Ashwani Kumar · Shinjiro Ogita
Yuan-Yeu Yau *Editors*

Biofuels: Greenhouse Gas Mitigation and Global Warming

Next Generation Biofuels and Role of Biotechnology

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Foreword

I am delighted to write this foreword to a timely and thorough book *Biofuels: Greenhouse Gas Mitigation and Global Warming, Next-Generation Biofuels and Role of Biotechnology* which is a topic of great interest to us all; it is edited by three international authorities in this area of research: Ashwani Kumar (of India), Shinjiro Ogita (of Japan) and Yuan-Yeu Yau (of USA).

In 1874, Jules Verne wrote, "Water will one day be employed as fuel, that hydrogen and oxygen that constitute it, used singly or together, will furnish an inexhaustible source of heat and light". While Monsieur Verne was likely not thinking about water in the context of photosynthesis and in growing biofuels, it is there that his prophecy seems closest to fruition. Today, biofuels are poised to make a substantial contribution in solving the global challenge of a sustainable zero-carbon energy source. With all of us working together, there is hope of surmounting the problems facing the implementation of biofuels on a global scale. It is in this context that this book holds importance in recounting progress and providing a roadmap for moving forward.

What is the big picture? Global population is increasing and with it global energy demand (including those related to industry as well as agriculture). The focus of this book is an attempt to address the problem of increasing energy demand by enhancing the production of renewable biofuels. Currently, this is largely being satisfied by burning fossil fuel, but the concomitant emission of greenhouse gases is of grave concern. Although the contribution of renewal solar and wind energy is steadily increasing, these do not address the need for high-energy-density liquid fuels. For the first time in human history, carbon dioxide (CO_2), the primary suspect of global warming, has reached a very high level of 400 ppm (parts per million) in our atmosphere, almost a twofold increase over pre-industrial (~1850) atmospheric CO_2 levels. The increase in atmospheric concentration of greenhouse gases (CO_2, methane (CH_4), nitrous oxide (N_2O) and ozone (O_3)) is leading to heating of the atmosphere by absorption of infrared radiation. The CO_2 level in 2012 was about 40% higher than it was in the nineteenth century. The 2015 Paris Climate Conference led to a universal agreement on climate, which aims at keeping the global warming below 2°C. The book, edited by Kumar, Ogita and Yau, based on extensive research in several countries, is dedicated to finding solutions to these daunting challenges.

Photosynthesis is the very basis of all life on Earth. It provides food, feed, fuel, oxygen, and much more, while playing a central role in the hydrological and

biogeochemical C and N cycles. Photosynthesis is inefficient under field conditions; it operates in nature with just 1% solar energy efficiency, but there is strong evidence that photosynthesis could be "engineered" to be much more efficient. Thus, today, not only biologists but also chemists, physicists, computer scientists and engineers are all involved in finding ways and means to enhance photosynthetic efficiency including biomimicry and fully artificial systems. On p. 21 of the January 2012 issue of *Scientific American*, David Biello talks about the hope of producing biofuels (through tweaking photosynthesis) that could make economic sense. Willem (Wim) J. Vermaas (of Arizona State University, Tempe, AZ, USA) and others are already engineering a cyanobacterium *Synechocystis* into a "milking cow", so to speak, and producing valuable products for human use. On the other hand, Anastasios (Tasso) Melis (of UC–Berkeley, USA) has succeeded in diverting photosynthesis to generate hydrogen gas from microalgae. Further, by manipulating the antenna size, his group has improved productivity of photosynthesis in mass algal cultures in bright sunlight. And, now, his lab is producing, through photosynthesis, isoprene (C_5H_8) hydrocarbons, used in medicine, from cyanobacteria and microalgae; this work serves as a case study for the generation of many types of biofuels and other useful bioproducts. And most recently, the labs of Krishna (Kris) Niyogi (UC–Berkeley) and Stephen (Steve) P. Long (University of Illinois) have engineered accelerated relaxation of photoprotection resulting in up to 20% increase in dry matter production of tobacco in replicated field trials. Increasing photosynthetic efficiency will enable greater production of biofuel feedstock on a land area basis without increasing water requirements. We have hopes for the future.

Photosynthesis is indeed being "tweaked" (so to say) for the benefit of all of us (see, e.g. Ü. Niinemets, J.A. Berry, S. von Caemmerer et al. (2017) New Phytologist 213: 43–47). It is essential for all scientists and engineers to have a clear idea of the basics of photosynthesis. I urge the readers to consult *Photosynthesis* by Eugene Rabinowitch and Govindjee (1969; John Wiley & Sons (available free at: http://www.life.illinois.edu/govindjee/g/Books.html)) and *Molecular Mechanisms of Photosynthesis* by Robert Blankenship (2014; Wiley & Blackwell). There are many approaches to tweak photosynthesis which involve, e.g. (a) *decreasing antenna size* (D.R. Ort, S.S. Merchant, J. Alric et al. (2015) Proceedings of the National Academy of Science, USA 112: 8529–8536; H. Kirst, S.T. Gabilly, K.K. Niyogi et al. (2017) Planta 245: 1009–1020; Z. Perrine; S. Negi, R.T. Sayre (2012) Algal Research 1: 134–142) and (b) *engineering organisms, or using existing organisms, to capture extreme far-red light* (R.E. Blankenship, D.M. Tiede, J. Barber et al. (2011) Science 332: 805–809; M. Chen and R. Blankenship (2011) Trends in Plant Science 16:427–431). As a corollary, we ask if black plants, which would capture all visible light, would be better; although we really don't have a clear answer, we suspect that it may produce unnecessary heat load in the system. Further, we may ask as to why chlorophyll *a* (Chl *a*) was the chosen pigment? It seems the answer lies in the redox properties of chlorophyll *a* molecules bound to specific protein environment (see L.O. Björn, G.C. Papageorgiou, R. Blankenship and Govindjee (2009) *Photosynthesis Research* 99: 85–98), but chlorophyll shares a common biosynthetic pathway with an ancient molecule, haem, and thus may be a legacy of its synthesis; however,

minor tweaking of the proteins might indeed lead to better efficiency – these are just thoughts to explore. Other approaches include (i) *improving ways to protect against damage by excess light* (J. Kromdijk, K. Glowacka, L. Leonelli, et al. (2016) Science 354: 857–861); (ii) *manipulating photorespiration, a wasteful process* (M. Betti, H. Bauwe, F.A. Busch, et al. (2016) Journal of Experimental Botany 67: 2977–2988); (iii) *transferring an efficient C-4 system into C-3 plants* (D. Kandoi, S. Mohanty, Govindjee et al. (2016) Photosynthesis Research 130: 47–72); and (iv) *introducing the algal pyrenoid into higher plants* (R.E. Sharwood (2017) New Phytologist 214: 496–499). Further, there is a bottleneck reaction at the plastoquinone level, in the Z-scheme of photosynthesis, which needs to be examined to explore its potential for improving photosynthesis (see Govindjee, D. Shevela and L.O. Björn (2017) Photosynthesis Research 133: 5–15, for the historical evolution of the scheme).

Thus, there is hope of increasing photosynthesis by a multitude of possibilities, the end result being more biomass in the form of food and/or feedstock bioenergy. This book focuses on the generation of biofuels, which is also very important towards solving global issues. *Biofuels: Greenhouse Gas Mitigation and Global Warming, Next-Generation Biofuels and Role of Biotechnology* is indeed a highly useful book, written by 41 contributors from nine countries (Brazil, Canada, China, India, Japan, Malaysia, Mexico, Portugal and USA): Ebin Abraham (Jaipur, India), Sunita Agarwal (Jaipur, India), Kinya Akashi (Tottori, Japan), Birgit Arnholdt-Schmidt (Évora, Portugal), Muhammad Asif (Edmonton, Canada), Saikat K. Basu (Lethbridge, Canada), Shikha Bhansali (Jaipur, India), William Cetzal-Ix (Campeche, México), José Hélio Costa (Fortaleza, Brazil), Mona Easterling (Broken Arrow, OK, USA), Randhir S. Gajraj (Mumbai, India), Arti Gupta (Jaipur, India), Nidhi Gupta (Meerut, India), Yasuyo Himuro (Ibaraki, Japan), Arvind Hirani (Winnipeg, Canada), Nasir Javed (Winnipeg, Canada), Anand D. Karve (Pune, India), Masatomo Kobayashi (Ibaraki, Japan), Taichi Koshiba (Kyoto, Japan), Amit Kotiya (Jaipur, India), Ashwani Kumar (Jaipur, India), Elisete Santos Macedo (Évora, Portugal), Nidhi V Maheshwari (Meerut, India), Gunasekaran Mohanapriya (Coimbatore, India), Kalaivani K. Nadarajah (Bangi, Selangor, Malaysia), Yoshihiko Nanasato (Ibaraki, Japan), Shinjiro Ogita (Hiroshima, Japan), Anand Patwardhan (Maryland, USA), Anand B. Rao (Mumbai, India), Shikha Roy (Jaipur, India), Masahiro Sakamoto (Kyoto, Japan), Ramalingam Sathish Kumar (Coimbatore, India), Meghendra Kumar Sharma (Jaipur, India), Gajendra P. Singh (Jaipur, India), Chigullapalli Sreenivas (Mumbai, India), Ding-Qin Tang (LinAn City, Zhejiang Province, China), Toru Taniguchi (Ibaraki, Japan), Toshiaki Umeazawa (Kyoto, Japan), Yuan-Yeu (known to us as Frank) Yau (Broken Arrow, OK, USA), Peiman Zandi (Beijing, China) and Ming-Bing Zhou (LinAn City, Zhejiang Province, China). Each and every contributor deserves my congratulations to have participated in producing such a wonderful and timely book on a major problem facing our world.

Chapters in this book will help increase our understanding of the large challenges, facing us all, and the role of global initiatives in bioenergy that are being undertaken to address these challenges while helping mitigate global warming.

I am delighted to note that this book will be a key resource for graduate students, teachers and even laypersons interested in biofuel production. I would like to end this *foreword* by reminding everyone that we should move forward on all fronts to solve the global problems and should not be afraid to make mistakes. As Jules Verne (in *A Journey to the Centre of the Earth*) said, *"Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth"*. Let there be light and let there be hope. Yes, this book gives us both.

I thank Ashwani Kumar, Shinjiro Ogita and Yuan-Yeu Yau for giving me the opportunity to say a few words based on my background and perspective. I am grateful to Lars Olof Björn, Robert (Bob) Blankenship, Johannes (Wanne) Kromdijk, Donald (Don) R. Ort, Tasso Melis, Richard (Dick) Sayre and Wim Vermaas for reading this *foreword* and for making suggestions before its publication. I wish this book a great success.

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Preface

Global energy demands are directly linked with growing population numbers and the resulting increase in industrial and agricultural demands. Currently, 88 per cent of energy demands are met by burning fossil fuel. CO_2 concentrations in our atmosphere have increased from 250 to 400 ppm within a short span of 200 years. This has resulted in an increase of global mean temperature, commonly termed as global warming. Even a slight increase in mean temperature over a period of time results in changing climate. Floods, cyclones, droughts and other natural calamities are occurring with increasing frequency and ferocity. This book is based on published research by leading field scientists, who are experts in this field; it focuses on key environmental changes and challenges towards the path of green growth and sustainable development. The negative impact of climate change is increasing in frequency and intensity. The impact on lives, economies, infrastructure and nature is real, in many cases irreversible and only destined to get worse. Much of the world, including North America and Europe, has witnessed a steep rise in temperature during the last 15 years. If we want to turn the tide, we must embrace clean energy solutions. With any great challenge comes great opportunity. Using renewable energy sources and natural resources efficiently in reducing the carbon intensity and improving people's environmental quality of life is the major aim of greenhouse gas reduction strategy. First and foremost, rapid and drastic emission reduction is urgently needed to keep global temperature rise below 1.5°. The chapters in this book are designed to help increase our understanding of the problem, its current status, global initiatives and possible measures that we can adopt to mitigate global warming. The benefit of biofuels can be appropriately managed through proper choice of plant, algal and bacterial species and production criteria for feedstock and fuel conversion technologies in any given geographic region. Examples include the use of algae or arid-tolerant plants as feedstocks, the use of light biomass or grasses and the use of plants that can tolerate and ameliorate poor or damaged soils, allowing the use of land not suitable for food or feed production.

Global concerns about and benefits of biofuels can be appropriately managed through proper choice of plant, algal and bacterial species. For the first time, cellulosic biofuels from lignocellulosic biomass are being manufactured and shipped commercially in the USA and in Europe. Algal biofuels are in the nascent stage of development and are derived from low-input and high-output production of suitable algal species. Green algae are being used to generate third-generation biofuels.

Fourth-generation biofuels are derived from the bioconversion of living organisms (microorganisms and plants) using biotechnological tools. Fourth-generation biofuel production incorporates carbon capture and storage (CCS) technology to create a carbon-negative hope for the future, which would exceed all carbon-neutral options currently available. Biotechnological approaches to improve quality and quantity of biomass have been amply demonstrated. Synthetic and semi-synthetic metabolic pathways for biofuel production have been covered in this book.

This book includes information concerning the four generations of biofuel currently in production, basic process procedures for each and feedstock used and considered, as well as benefits and risks within those categories. The Intergovernmental Panel on Climate Change (IPCC) reports stated that climate change is accelerating, that the changes are to a significant extent man-made and that the need to adopt counter-measures is urgent if we are to prevent a global climate crisis from arising in the near future and threatening the basis of human life. Considering the importance of this crisis and its possible solutions, the 2007 Nobel Peace Prize was awarded jointly to this panel (IPCC) and to Albert Arnold (Al) Gore Jr. "for their efforts to build up and disseminate greater knowledge about man-made climate change, and to lay the foundations for the measures that are needed to counteract such change" (http://www.nobelprize.org/nobel_prizes/peace/laureates/2007/).

This book presents contemporary biotechnology options and potential improvements, which will impact biofuel production. Readers will learn about the flexibility and broad-range utility of plant cell tissue culture for producing fertile energy plants. Energy crop breeding improves lignocellulosic biomass by the use of molecular tools such as transposons, DNA markers and specific mutants. Some of the chapters, in this book, contain expert discussion of novel molecular tools for the manipulation of metabolic pathways in microalgae and plant cell walls. Genetic engineering techniques can increase lipids, such as triacylglycerols (TAG), or decrease lignin, as needed.

In this book, references have been provided to update current research data from multiple, credible scientific journals. The most popular technology today, CRISPR/Cas9, is being explored for potential use in generating lignin-reduced transgenic plants for lignocellulosic biomass-based biofuel production.

We are thankful to Professor Govindjee, of the University of Illinois at Urbana-Champaign, for writing an excellent foreword for our book (see a brief write-up on him on p. xi). We heartily thank all of our co-authors and many others who have contributed to this book.

This book provides plentiful resources for biofuel researchers and is also designed to provide general information to graduate and advanced undergraduate students, teachers and laymen who are interested in biofuel production.

We dedicate our book to Professor Dr. Sven Schubert (Germany), AK's teacher, on his 60th birthday for his excellent contributions to plant science (see a brief write-up on him on p. xiii).

Jaipur, Rajasthan, India — Ashwani Kumar
Syoubara, Hiroshima, Japan — Shinjiro Ogita
Broken Arrow, Oklahoma, USA — Yuan-Yeu Yau
August 2017

About Govindjee: Mister Photosynthesis

The Editors and authors of this book are delighted to celebrate Govindjee's 85th birthday in 2017. Why did we invite him to write the *foreword* for our book? Our book deals with *biofuels,* which is totally dependent on photosynthesis. And, Govindjee has been entrenched in the basic research in the area of photosynthesis for 60 years! He has been rightfully called *Mr. Photosynthesis*; and he is an institution in himself. Since 1999, Govindjee has been professor emeritus of biochemistry, biophysics and plant biology at the University of Illinois at Urbana-Champaign (UIUC), Illinois, USA, after serving on the faculty of the UIUC for ~40 years. He learned his plant physiology from Shri Ranjan, who was a student of Felix Frost Blackmann (of Cambridge, UK). Then, Govindjee studied *photosynthesis* at the UIUC, under two giants in the field, Robert Emerson (a student of 1931 Nobel laureate Otto Warburg) and Eugene Rabinowitch (who had worked with James Franck, a 1926 Nobel laureate in physics), obtaining his Ph.D., in *biophysics* (with minors in physics and in chemistry), in 1960!

Govindjee is best known for his extensive research on excitation energy transfer, light emission (prompt and delayed fluorescence and thermoluminescence), primary photochemistry, and electron transfer in *photosystem II* (PS II, water-plastoquinone oxidoreductase). His research, with many others, includes the discovery of a short-wavelength form of chlorophyll *a* functioning in PS II; the two-light effect in Chl *a* fluorescence; and, with his wife, Rajni Govindjee, the two-light effect (Emerson enhancement) in $NADP^+$ reduction in chloroplasts. His major achievements, together with several others, include an understanding of the basic

relationship between Chl *a* fluorescence and photosynthetic reactions; a unique role of bicarbonate/carbonate on the electron acceptor side of PS II, particularly in the protonation events involving the Q_B binding region; the theory of thermoluminescence in plants; the first picosecond measurements on the primary photochemistry of PS II; and the use of fluorescence lifetime imaging microscopy (FLIM) of Chl *a* fluorescence in understanding photoprotection by plants against excess light. His current focus is on the *history of photosynthesis research* and in *photosynthesis education*. Professor Govindjee has received a large number of honors including fellow of the American Association for the Advancement of Science (AAAS); distinguished lecturer of the School of Life Sciences, UIUC; fellow and lifetime member of the National Academy of Sciences (India); past president of the American Society for Photobiology (1980–1981); Fulbright scholar (1956), Fulbright senior lecturer (1997), and Fulbright specialist (2012); honorary president of the 2004 International Photosynthesis Congress (Montréal, Canada); the first recipient of the Lifetime Achievement Award of the Rebeiz Foundation for Basic Biology (2006); recipient of the Communication Award of the International Society of Photosynthesis Research (2007); Lifetime Achievement Award of the College of Liberal Arts and Sciences, UIUC (2008). Further, Govindjee has been honored at his 75th and 80th birthdays by Special Issues of "Photosynthesis Research". At his 85th birthday, he is being honored by a Special Issue of "Photosynthetica".

Govindjee's unique teaching of the Z-scheme of photosynthesis, where students act as different intermediates, has been published in *Photosynthesis Research* by P. K. Mohapatra and N. R. Singh (2015; Volume 123, pp.105–114) and by S. Jaiswal, M. Bansal, S. Roy, A. Bharati and B. Padhi (2017; Volume 131, pp. 351–359).

Since 2007, each year, a *Govindjee and Rajni Govindjee Award* is given to graduate students, by the Department of Plant Biology (odd years) and by the Department of Biochemistry (even years), at the UIUC, to recognize excellence in biological sciences (see http://www.life.illinois.edu/govindjee/photooftheyear2017.html, and links).

For an interview of Govindjee by Donald R. Ort for the Annual Reviews, Inc., see https://www.youtube.com/watch?v=cOzuL0vxEi0. Further information on Govindjee is at https://en.wikipedia.org/wiki/Govindjee; for "Govindjee, the living legend", see https://www.linkedin.com/pulse/govindjee-living-legend-i-met-dr-ravi-sharma; and for "Govindjee and Rajni Govindjee – Confluence of Photosynthesis and Photobiology", see https://www.linkedin.com/pulse/govindjee-rajni-confluence-photosynthesis-dr-ravi-sharma. Finally, Govindjee's website at http://www.life.illinois.edu/govindjee is a gold mine of information on him and on *photosynthesis*.

Dedication to Professor Dr. Sven Schubert on his 60th Birthday for his Devotion to Plant Sciences

Professor Dr. Sven Schubert is the director of the renowned Institute of Plant Nutrition, Justus Liebig University, Giessen, Germany, which was originally founded in 1840 by Justus von Liebig (1803–1873), one of the greatest agricultural chemists of the world. Professor Schubert completed his Ph.D. on "the plasma membrane H^+-ATPase of plants" at the Faculty of Nutrition at the same university in 1985 and proceeded to Davis, California, USA, for studies on "the salt resistance of maize". He then completed his *habilitation* and became professor of plant nutrition at the University of Hohenheim in Stuttgart, Germany, at a young age of 35. In 1997, he was appointed professor and director of the Institute of Plant Nutrition at Giessen. A large number of students from different parts of the world such as China, Egypt, India, Pakistan, Romania, Russia, Turkey and the USA have been attracted to study, under his guidance, at the Institute of Plant Nutrition in Giessen. Salt resistance of plants, membrane biochemistry, physiological functions of magnesium, kernel development in maize, phosphorus efficiency and biochar (for carbon sequestration) are among the many areas of his research.

Professor Dr. Sven Schubert is well known for his broad perspective, clear vision, positive thinking and supportive attitude. He has developed maize hybrids that can tolerate high levels of soil salinity under extreme conditions. Thorough studies on various physiological, biochemical and biotechnological aspects have resulted in a

large number of top-ranking research publications from his own work and from doctoral theses guided by him. He has authored two textbooks on plant nutrition (*Pflanzenernährung*) and biochemistry (*Biochemie*), and his textbook on plant nutrition is running into its third edition.

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About the Editors

Dr. Ashwani Kumar obtained his M.Sc. in botany (gold medal was awarded for standing first in order of merit in M.Sc. (Botany) examination of University of Rajasthan, 1967) and Ph.D. (1971) at the University of Rajasthan. He was selected through IAS examination of Govt of India into IPS in 1972 but preferred to remain as University Professor by choice. He was awarded an Alexander von Humboldt fellowship in Germany, a JSPS visiting professorship in Japan, an INSA-DFG visiting professorship in Germany and a British Council visitorship in the UK. During his career, he held many important administrative and research positions like head of the Department of Botany, coordinator of SAP(1995–1998), director of Life Sciences (2001–2004), director of the Central Library and Information Science (2000–2005), member of the senate and research board and convener of the Board of Studies of Botany and COC Biotechnology. He worked in areas of plant pathology, physiology, renewable sources of energy, biofuel biotechnology and photosynthetic carbon fixation in C_3 and C_4 plants. He carried out research projects granted by UGC, USDA-ICAR, MNES, CSIR, DST and DBT of the Govt of India and visited countries such as the USA, Canada, Denmark, Italy, the Netherlands, UK, Japan, France, Sweden, Spain and Portugal for research. He has published around 181 papers in national and international journals. He is elected / nominated fellow of various societies including the Association of Biotechnology and Pharmacy (ABAP), Indian Botanical Society (IBS), Indian Phytopathological Society (IPS), Indian Society of Mycology and Plant Pathology (ISMPP) and Mendelian Society of India (MSI). He has guided the Ph.D. research work of 39 students and is consultant of a World Bank project sanctioned to SPRI-HPPI and Biodis Spain. He has also published a total of 16 books including *Plant Cell and Tissue Culture – A Tool in Biotechnology: Basics and Application* and edited a book *Working with ferns: issues and applications* with Helena Fernandez and Maria Angeles Revilla both by Springer. He also has 14 books mainly in the field of biotechnology to his credit. He was awarded a V. Puri Medal in 2008 for botany and a CEE award for excellence in teaching and research in 2015 (www.science20.com/profile/professor_ashwani_kumar).

Dr. Shinjiro Ogita has over two decades of experience in the field of plant biotechnology. In 1992, he started his research career as a master's student at the United Graduate School of Agriculture, Tokyo University of Agriculture and Technology

(TUAT), Japan, and in 1997, he received his Ph.D. in agriculture (subject: sciences of resources and environment). He is an expert in the field of cell and tissue culture and transformation technologies for higher plants. He has worked at the following institutes based on projects worked:

1. To elucidate embryogenic capacity of elite coniferous trees at the Laboratory of Cell Manipulation, Division of Bio-resources Technology, Forestry and Forest Products Research Institute, Ministry of Agriculture, Forestry and Fisheries, Japan (October 1997–September 2000).
2. To establish genetically modified decaffeinated coffee plants at the Laboratory of Plant Molecular Breeding, Research and Education Center for Genetic Information, Nara Institute of Science and Technology (NAIST), Japan (October 2000–March 2003).
3. To teach plant biotechnology, microbiology and molecular biology as assistant professor (2003–2006), lecturer (2006–2010) and associate professor (2010–2015) at the Laboratory of Plant and Cell Engineering, Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University (TPU), Japan (April 2003–March 2015). He presently works as a full professor at the Faculty of Life and Environmental Sciences, Department of Life Sciences, Prefectural University of Hiroshima (PUH), Japan (April 2015–).

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2016: 7/211 (Plant Sciences); 10/158 (Biotechnology & Applied Microbiology)) and *Plant Cell Reports*. He joined the Chinese Academy of Sciences as a professor in 2010 and then joined Northeastern State University of Oklahoma (USA) as a research scientist and nontenured professor in 2012. Dr. Yau is an active member of *ResearchGate* (https://www.researchgate.net). He mentors numerous students from all over the world on *ResearchGate*.

Global Warming, Climate Change and Greenhouse Gas Mitigation

1

Ashwani Kumar

"Continued emission of greenhouse gases will cause further warming and long-lasting changes in all components of the climate system, increasing the likelihood of severe, pervasive and irreversible impacts for people and ecosystems. Limiting climate change would require substantial and sustained reductions in greenhouse gas emissions which, together with adaptation, can limit climate change risks."

(Source IPCC 2014)

Abstract

In recent years fossil fuels, smog, carbon monoxide, particulates, free radicals and toxic chlorofluorocarbons and deforestations have increased significantly mainly due to anthropogenic activities. Urbanization is a global trend and is associated with increases in income, and higher urban incomes are correlated with higher consumption of energy and GHG emissions. This has resulted in increasing levels of greenhouse gases which absorb heat mainly infrared radiation, emitted from the Earth's surface. Increases in the atmospheric concentrations of these gases cause Earth to warm by trapping more of this heat. The CO_2 level in 2012 was about 40% higher than it was in the nineteenth century. During Conference of the Parties (COP21), at Paris Climate Conference, (2015) a legally binding and universal agreement on climate change was achieved, with the aim of keeping global warming below 2 °C. Substantial cuts in anthropogenic GHG emissions by mid-century through large-scale changes in energy systems and altered land use can be achieved. Biofuels, according to the IEA, could displace enough petroleum to avoid the equivalent of 2.1 Gigatons (Gt) of carbon dioxide emission each year if produced sustainably – about as much as net carbon dioxide absorbed by the oceans. Currently, second-generation cellulosic biofuels and

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third-generation algal biodiesels are prominent biological approaches to sequester and convert CO_2. The race is on to optimize the technology that can produce biofuels from lignocellulose sources, and it is expected that biotechnological advancement is expected to make this happen in the near future for the benefit of mankind. This review is supported by various publications from the United Nations, and citations are acknowledged.

Keywords
Biofuel • Climate change • Global warming • Greenhouse gases

1.1 Introduction

Climate change is any long-term significant change in average temperature, precipitation and wind patterns. Climate is driven by large-scale factors such as the level of radiation received from the Sun, the atmospheric composition, the landscape, the distance from the ocean, movement of currents in the ocean and other atmospheric processes such as the El Niño-Southern Oscillation. Many of these factors change gradually and do so in a more predictable, less chaotic manner than those factors affecting our daily weather. Hence, the climate can be projected further into the future with more confidence (IPCC 2014).

1.2 Greenhouse Gases (GHGs)

The Earth's temperature is maintained by reradiated infrared radiations by CO_2, CH_4, O_3, NO and NO_2 and slightly by water vapours. These gases are called greenhouse gases, and the effect is called greenhouse effect. This greenhouse effect is significant to maintain Earth's temperature by 33° C. If our environment would have had oxygen and nitrogen alone, the Earth would have been very cool (−15 to 17 °C). However, our atmosphere had carbon dioxide, methane, ozone and water vapours which help in keeping Earth warm and don't allow heat to dissipate to the atmosphere, and average temperature is 15 °C. CO_2 has risen by 40% in just the past 200 years, contributing to human alteration of the planet's energy budget that has so far warmed Earth by about 0.8 °C (1.4 °F).The three major non-CO_2 gases (or groups of gases) are provided – CH_4, N_2O and the group of fluorinated gases (F-gases), including CF_4, HFCs and SF_6 that also contribute to the GHG emissions.

The CO_2 is added to the atmosphere by burning fossil fuel. Besides this, CFCs and oxides of nitrogen and methane also exert greenhouse effect i.e. around 60% CO_2, 20% methane, 14% CFCs and 6% N_2O (making around 100%).

The increase in temperature due to increasing level of GHGs in the atmosphere is called global warming. This speed of warming is more than ten times than that at the end of an ice age, the fastest known natural sustained change on a global scale.

Annual anthropogenic GHG emissions have increased by 10 $GtCO_2eq$ between 2000 and 2010, with this increase directly coming from energy supply (47%), industry (30%), transport (11%) and building (3%) sectors (medium confidence). Accounting for indirect emissions raises the contributions of the buildings and industry sectors (high confidence). Since 2000, GHG emissions have been growing in all sectors, except AFOLU. Of the 49 (±4.5) $GtCO_2eq$ emissions in 2010, 35% (17 $GtCO_2eq$) of GHG emissions were released in the energy supply sector, 24% (12 $GtCO_2eq$, net emissions) in Agriculture Forestry and Other Land Use (AFOLU), 21% (10 $GtCO_2eq$) in industry, 14% (7.0 $GtCO_2eq$) in transport and 6.4% (3.2 $GtCO_2eq$) in buildings. When emissions from electricity and heat production are attributed to the sectors that use the final energy (i.e. indirect emissions), the shares of the industry and buildings sectors in global GHG emissions are increased to 31% and 19.7%, respectively (IPCC 2014).

1.3 Global Warming

In recent decades, changes in climate have caused impacts on natural and human systems on all continents and across the oceans. Impacts are due to observed climate change, irrespective of its cause, indicating the sensitivity of natural and human systems to changing climate. Continued emission of greenhouse gases will cause further warming and long-lasting changes in all components of the climate system, increasing the likelihood of severe, pervasive and irreversible impacts for people and ecosystems.

According to IPCC (2014) 5th assessment report (AR5) since the beginning of the industrial era, oceanic uptake of CO_2 has resulted in acidification of the ocean; the pH of ocean surface water has decreased by 0.1, corresponding to a 26% increase in acidity, measured as hydrogen ion concentration. Over the period 1992–2011, the Greenland and Antarctic ice sheets have been losing mass, likely at a larger rate over 2002–2011. Glaciers have continued to shrink almost worldwide. Northern Hemisphere spring snow cover has continued to decrease in extent. There is high confidence that permafrost temperatures have increased in most regions since the early 1980s in response to increased surface temperature and changing snow cover. The annual mean Arctic sea-ice extent decreased over the period 1979–2012, with a rate that was very likely in the range 3.5–4.1% per decade. Arctic sea-ice extent has decreased in every season and in every successive decade since 1979, with the most rapid decrease in decadal mean extent in summer. It is very likely that the annual mean Antarctic sea-ice extent increased in the range of 1.2–1.8% per decade between 1979 and 2012. However, there is high confidence that "there are strong regional differences in Antarctica, with extent increasing in some regions and decreasing in others" (IPCC 2014).

Reducing GHG is essential. GHG emission growth is expected to persist driven by growth in global population and economic activities if no action is taken. Baseline scenarios, those without additional mitigation, result in global mean surface temperature increases in 2100 from 3.7 to 4.8 °C compared to pre-industrial levels.

CO_2 emissions dominate GHG emissions from industry, but there are also substantial mitigation opportunities for non-CO_2 gases. CH_4, N_2O and fluorinated gases from industry accounted for emissions of 0.9 $GtCO_2eq$ in 2010. Key mitigation opportunities include, e.g., the reduction of hydrofluorocarbon emissions by process optimization and refrigerant recovery, recycling and substitution, although there are barriers.

1.3.1 Possible Causes

Globally, economic and population growth continue to be the most important drivers of increases in CO_2 emissions from fossil fuel combustion. The United Nations World Population Prospects 2015 revision puts the world population at 9.7 billion in 2050 and 11.2 billion in 2100. Most of this population growth is projected to take place in developing world and poorest nations. (United Nations, World Population Prospects, the 2015 revision). The contribution of population growth between 2000 and 2010 remained roughly identical to the previous three decades, while the contribution of economic growth has risen sharply. Between 2000 and 2010, both drivers outpaced emission reductions from improvements in energy intensity. Urbanization is a global trend and is associated with increases in income, and higher urban incomes are correlated with higher consumption of energy and GHG emissions. Increased use of coal relative to other energy sources has reversed the long-standing trend of gradual decarbonization of the world's energy supply (IPCC 2014). Globally, economic and population growth continue to be the most important drivers of increases in CO_2 emissions from fossil fuel combustion.

In addition to natural climatic variability, the anthropogenic climate change is taking place due to emissions of greenhouse gases which cause environmental damage to any given area. Both of these climatic factors are posing disaster risks in terms of droughts, floods, melting of glaciers, acidification of sea and rising sea levels. As of 2011, more than 52% of the global population lives in urban areas. In 2006, urban areas accounted for 67–76% of energy use and 71–76% of energy-related CO_2 emissions. CO_2 emissions from fossil fuel combustion and industrial processes contributed about 78% of the total GHG emission increase from 1970 to 2010, with a similar percentage contribution for the period 2000–2010.

Each of the last three decades has been successively warmer at the Earth's surface than any preceding decade since 1850. The period from 1983 to 2012 was likely the warmest 30-year period of the last 1400 years in the Northern Hemisphere, where such assessment is possible. The globally averaged combined land and ocean surface temperature data as calculated by a linear trend show a warming of 0.85° C [0.65–1.06° C], covering the period 1880–2012, when multiple independently produced datasets exist (IPCC 2014).

Total anthropogenic GHG emissions have continued to increase over 1970–2010 with larger absolute decadal increases towards the end of this period (IPCC 2014). Total anthropogenic GHG emissions were the highest in human history from 2000 to 2010 and reached 49 (±4.5) $GtCO_2eq$/year in 2010. Of the 49 (±4.5) $GtCO_2eq$/

year in total anthropogenic GHG emissions in 2010, CO_2 remains the major anthropogenic GHG accounting for 76% (38 ± 3.8 $GtCO_2eq$/year) of total anthropogenic GHG emissions in 2010. Sixteen percent (7.8 ± 1.6 $GtCO_2eq$/year) come from methane (CH_4), 6.2% (3.1 ± 1.9 $GtCO_2eq$/year) from nitrous oxide (N_2O) and 2.0% (1.0 ± 0.2 $GtCO_2eq$/year) from fluorinated gases (IPCC 2014) covered under the Kyoto Protocol (F-gases). (IPCC 2014: Annex II).

1.3.1.1 Ozone Pollution

Earth's atmosphere is divided into different strata:

Exosphere	150 km
Mesosphere	80 km
Stratosphere	50 km ozone layer
Troposphere	16 km

The ozone present in stratosphere is good ozone. This layer acts as ozone shield protecting the Earth biota from harmful effects of strong UV radiation. CFCs, CH_4 and N_2O cause destruction of the ozone and cause ozone depletion. The thinning of the ozone layer results in an increase in the UV-B radiations reaching Earth surface. Main greenhouse gases are CO_2, CH_4, CFCs and N_2O; in addition to this, SO_2, NO_2 and water vapours are also released from industries and agriculture which are also responsible for increase in the greenhouse effect. Although IPCC 2014 report does not consider them as greenhouse gases and they are not part of COP21 agreements on greenhouse gas reductions to be achieved by 2030 to zero emission levels, it is concluded that tropospheric ozone and industrial water vapours and agricultural activities' generated vapours could cause minor greenhouse effect also and may be considered as greenhouse gases in its widest sense in addition to core gases. The Global Warming Potential (GWP) for methane includes indirect effects of tropospheric ozone production and stratospheric water vapour production. However, tropospheric ozone triggered by car exhaust (smog) (25 ppb has risen to 34 ppb level in last 100 years) has also been considered a cause of greenhouse gas effect. Levels of this ozone may rise due to human activity. It is also formed by NO_2 under UV radiation effect. Minor amounts of ozone are also added to the atmosphere by electric discharges such as lightning flashes, by vertical flux of stratospheric ozone and by tropospheric storms.

1.3.1.2 Nitrous Oxide (N_2O)

Nitrous oxide is produced naturally in soils through the processes of nitrification and denitrification. Nitrous oxide is a gaseous intermediate in the reaction sequence of denitrification and a by-product of nitrification that leaks from microbial cells into the soil and ultimately into the atmosphere. N_2O emissions using human-induced net N additions to soils include synthetic or organic fertilizers, deposited manure, crop residues and sewage sludge, or mineralization of N in soil organic matter following drainage/management of organic soils or cultivation/land-use change on mineral soils.

1.4 United Nations Environment Programme (UNEP)

The United Nations Environment Programme (UNEP) established the *Intergovernmental Panel on Climate Change (IPCC)* and the *World Meteorological Organization (WMO)* in 1988 to provide the assessment of climate change and its potential environmental and socio-economic impacts. IPCC is open to all member countries of the United Nations (UN) and WMO, and currently 195 countries are members of the IPCC. IPCC is located at WMO headquarters in Geneva. According to IPCC report, the planet is warming rapidly, and the major contributor is the increase in heat trapping greenhouse gases (GHGs) from the combustion of fossil fuels and other industrial and agricultural processes.

The international political response to climate change began at the *Rio Earth Summit* in 1992, where the "Rio Convention" included the adoption of the *UN Framework Convention on Climate Change* (UNFCCC 2010). This convention set out a framework for action aimed at stabilizing atmospheric concentrations of greenhouse gases (GHGs) to avoid "dangerous anthropogenic interference with the climate system". The *UNFCCC* which entered into force on 21 March 1994 now has a near-universal membership of 195 parties.

It aims at policy linkages among regional, national and subnational climate policies which offer potential climate change mitigation and adaptation benefits. Various regional initiatives between the national and global scales are either being developed or implemented, but their impact on global mitigation has been limited to date.

Adaptation and mitigation are the two central approaches in the international climate change process. The agreements reached at the *UNFCCC* conference in Cancun in 2010 recognize that countries should take urgent action to limit the increase in global average temperature to less than 2 °C relative to pre-industrial levels. Scenarios reaching atmospheric concentration levels of about 450 ppm CO_2eq by 2100 (consistent with a likely chance to keep temperature change below 2 °C relative to pre-industrial levels) include substantial cuts in anthropogenic GHG emissions by mid-century through large-scale changes in energy systems and potentially land use. Global climate change has stimulated efforts to reduce CO_2 emissions (IPCC 2014).

1.4.1 Kyoto Protocol to Paris Agreement 2015

The Kyoto Protocol is an international agreement linked to the United Nations Framework Convention on Climate Change, which commits its Parties by setting internationally binding emission reduction targets. The Kyoto Protocol is seen as an important first step towards a truly global emission reduction regime that will *stabilize GHG emissions* and can provide the architecture for the future international agreement on climate change. The Kyoto Protocol was adopted in Kyoto, Japan, on 11 December 1997 and entered into force on 16 February 2005. The detailed rules for the implementation of the Protocol were adopted at COP7 in Marrakesh,

Morocco, in 2001, and are referred to as the "Marrakesh Accords". Its first commitment period started in 2008 and ended in 2012.

In Doha, Qatar, on 8 December 2012, the "Doha Amendment to the Kyoto Protocol" was adopted:

- New commitments for Parties to the Kyoto Protocol who agreed to take on commitments in a second commitment period from 1 January 2013 to 31 December 2020
- A revised list of greenhouse gases (GHG) to be reported on by Parties in the second commitment period

Under the Protocol, countries' actual emissions have to be monitored and precise records have to be kept of the trades carried out. Global efforts have concentrated on adaptation as well as mitigation.

1.4.1.1 Cancun Adaptation Framework

The Bali Action Plan, adopted at COP13 in Bali, December 2007, identified adaptation as one of the key building blocks required for a strengthened future response to climate change to enable the full, effective and sustained implementation of the Convention through long-term cooperative action. At the Cancun Climate Change Conference in December 2010, Parties established the Cancun Adaptation Framework (CAF) with the objective of enhancing action on adaptation, through international cooperation and coherent consideration of matters relating to adaptation under the Convention. At the COP18 in Doha, Qatar, Parties continued the implementation of the CAF and agreed on it (Source: http://unfccc.int/adaptation/items/7623.php).

1.4.2 The Intergovernmental Panel on Climate Change (IPCC)

The scientific evidence brought up by the first IPCC Assessment Report of 1990 underlined the importance of climate change as a challenge requiring international cooperation to tackle its consequences. After the first assessment report in 1990, the Second Assessment Report of IPCC (1995) (https://www.ipcc.ch/pdf/climate-changes-1995/ipcc-2nd-assessment/2nd-assessment-en.pdf) provided important material drawn on by negotiators in the run-up to adoption of the Kyoto Protocol in 1997. The Third Assessment Report came out in 2001 and the Fourth in 2007. The Fourth Assessment Report paid greater attention to the integration of climate change with sustainable development policies and relationships between mitigation and adaptation. At the end of 2007, the IPCC was awarded the Nobel Peace Prize. The Intergovernmental Panel on Climate Change and Albert Arnold (Al) Gore Jr. were awarded the Nobel Peace Prize "for their efforts to build up and disseminate greater knowledge about man-made climate change, and to lay the foundations for the measures that are needed to counteract such change". The Fifth Assessment Report (AR5)

was released in four parts between September 2013 and November 2014 (http://www.ipcc.ch/report/ar5/syr/).

Many governments have adopted a goal of avoiding global warming of more than 2 °C. This would require keeping concentrations of carbon dioxide below about 450 ppm. Because of the dynamics of absorption of carbon dioxide by oceans, soils and forests, this would require reducing emissions by 80% over the next half century at a rate of 3% per year. In addition, there is another 50 ppm CO_2eq of other GHGs including methane, nitrous oxide and industrial gases that need to be reduced by comparable amounts. The Sixth Assessment Report is expected to be finalized in 2022 in time for the global stocktake foreseen under the UNFCCC Paris Agreement.

However, in recent years, international climate policy has increasingly focused on *limiting temperature rise*, as opposed to achieving greenhouse gas concentration related objectives. The agreements reached at the United Nations Framework Convention on Climate Change conference in Cancun in 2010 recognize that countries should take urgent action to limit the increase in global average temperature to less than 2 °C relative to pre-industrial levels (United Nations Framework Convention on Climate Change report 2011 (http://unfcc.int/resource/docs/2010/cop16/eng/07a01.pdf.). If this is to be achieved, policymakers need robust information about the amounts of future greenhouse gas emissions that are consistent with such temperature limits. This, in turn, requires an understanding of both the technical and economic implications of reducing emissions and the processes that link emissions to temperature. The ultimate objective of the United Nations Framework Convention on Climate Change (UNFCCC 2011) (http://unfccc.int/meetings/durban_nov_2011/meeting/6245/php/view/reports.php) is to stabilize atmospheric concentrations of greenhouse gases at a level that will prevent dangerous interference with the climate system. According to the most stringent scenario of the IPCC, a long-term goal in line with the latest science would include a peak in emissions in the next 10–15 years and a decline of 50% over 2000 levels by 2050. This would stabilize emissions at around 450 ppm CO_2eq in the atmosphere and correspond to a 2–2.4 °C rise in temperatures. "As long as we keep emitting carbon dioxide, the climate will continue to warm. There is no way around a zero carbon economy sooner or later if we want to stay below 2 degrees," Target of 44 billion tons of carbon dioxide equivalent emissions ($GtCO_2$eq) by 2020 is difficult if not impossible to achieve. The world is currently at 48 $GtCO_2$eq/year. The United Nations Emissions Gap (2010) found that in 2020, emissions would still rise well beyond 50 $GtCO_2$eq (Rogelj et al. 2011). Collectively members of the G20 are on a likely track to meet their Cancun pledges for 2020, but these pledges do not deliver the necessary early emission reductions.

The UN Emission Gap report (2016) assessment shows that according to all available estimates, three of the G20 members – China, the EU-28 and India – are on track to meet their pledges without purchasing offsets. Three more – Brazil, Japan, and Russia – are on track according to most estimates.

1.5 Paris Agreement 2015

The Paris Agreement entered into force on 4 November 2016. The Paris Agreement builds upon the Convention and – for the first time – brings all nations into a common cause to undertake ambitious efforts to combat climate change and adapt to its effects, with enhanced support to assist developing countries to do so. The Paris Agreement's central aim is to strengthen the global response to the threat of climate change by keeping a *global temperature rise* this century well below 2° C above pre-industrial levels and to pursue efforts to limit the temperature increase even further to 1.5 °C (http://unfccc.int/files/essential_background/convention/application/pdf/english_paris_agreement.pdf).

The Paris Agreement shall be supervised by a body designated by the Conference of the Parties serving as the meeting of the Parties to this Agreement and shall aim:

(a) To promote the mitigation of greenhouse gas emissions while fostering sustainable development
(b) To incentivize and facilitate participation in the mitigation of greenhouse gas emissions by public and private entities authorized by a Party
(c) To contribute to the reduction of emission levels in the host Party, which will benefit from mitigation activities resulting in emission reductions that can also be used by another Party to fulfil its nationally determined contribution
(d) To deliver an overall mitigation in global emissions

However, only a limited number of studies have explored scenarios that are more likely than not to bring temperature change back to below 1.5 °C by 2100 relative to pre-industrial levels; these scenarios bring atmospheric concentrations to below 430 ppm CO_2eq by 2100. On 5 October 2016, the threshold for entry into force of the Paris Agreement was achieved. One hundred thirty-two Parties have ratified of 197 Parties to the Convention. The first session of the Conference of the Parties serving as the Meeting of the Parties to the Paris Agreement (CMA 1) took place in Marrakech, Morocco, from 15 to 18 November 2016. It is expected that the Paris Agreement on climate change will transform the global energy system for decades to come. Implementing current international pledges will only slow down the projected rise in energy-related carbon emissions from an average of 650 million tonnes per year since 2000 to around 150 million tonnes per year in 2040. On our present global trajectory, according to a recent Energy Information Administration forecast, global emissions of carbon dioxide will increase by 46% between 2010 and 2040, from 31 billion metric tons to 45 billion metric tons (US Department of Energy, May 2013) (Sheridan 2013).

1.5.1 Conference of the Parties 21 (COP21)

The main objective of the annual Conference of the Parties (COP) is to review the Convention's implementation. The first COP took place in Berlin in 1995, and significant meetings since then have included COP3 where the Kyoto Protocol was adopted, COP11 where the Montreal Action Plan was produced, COP15 in Copenhagen where an agreement to succeed Kyoto Protocol was unfortunately not realized and COP17 in Durban where the Green Climate Fund was created. In 2015 COP21, also known as the 2015 Paris Climate Conference, will, for the first time in over 20 years of UN negotiations, aim to achieve a legally binding and universal agreement on climate, with the aim of keeping global warming below 2 °C. On 5 October 2016, the threshold for entry into force of the Paris Agreement was achieved. The Paris Agreement came into force on 4 November 2016. WEO 2016 tracks progress with the implementation of the different pledges made at COP21 and judges what they mean for long-term energy trends. Based on this assessment, it examines and presents policy options to bridge the gap and reach climate objectives.

1.6 Reducing Greenhouse Gases (GHGs)

Annual anthropogenic GHG emissions have increased by 10 $GtCO_2eq$ between 2000 and 2010, with this increase directly coming from energy supply (47%), industry (30%), transport (11%) and building (3%) sectors. From the present level of 400-ppm CO_2eq, the chances are that it may overshoot to 500-550 ppm by the year 2100. It is imperative to keep it around 430-ppm CO_2eq by 2100.

Reducing emissions of non-CO_2 agents can be an important element of mitigation strategies. All current GHG emissions and other forcing agents affect the rate and magnitude of climate change over the next few decades, although long-term warming is mainly driven by CO_2 emissions. Emissions of non-CO_2 forcers are often expressed as "CO_2-equivalent emissions". Depending on the level of overshoot, overshoot scenarios typically rely on the availability and widespread deployment of *bioenergy with carbon dioxide capture and storage* (BECCS) and afforestation in the second half of the century.

1.6.1 Mitigation

Mitigation is a human intervention to reduce the sources or enhance the sinks of greenhouse gases. Mitigation, together with adaptation to climate change, contributes to the overall objectives of the United Nations Framework Convention on Climate Change (UNFCCC).

1.6.2 Energy

Most notably, about 1.3 billion people worldwide do not have access to electricity, and about three billion are dependent on traditional solid fuels for cooking and heating with severe adverse effects on health, ecosystems and development. Providing access to modern energy services is an important sustainable development objective (IPCC 2014). According to the World Energy Outlook (2008), current energy supplies are unsustainable from environmental, economic and societal standpoints. In addition, it is projected that world energy demands will continue to expand by 45% from 2008 to 2030, an average rate of increase in 1.6%/year. In 2007, the Intergovernmental Panel on Climate Change (IPCC 2007, 2008) released its Fourth Assessment Report confirming that climate change is accelerating, and if current trends continue, energy-related emissions of carbon dioxide (CO_2) and other greenhouse gases will rise inexorably, pushing up average global temperature by as much as 6 °C in the long term. Preventing catastrophic and irreversible damage to the global climate ultimately requires a major decarbonization drive.

The latest *World Energy Outlook* (WEO 2016) offers the most comprehensive analysis of what this transformation of the energy sector might look like, thanks to its energy projections to 2040. Renewable energy is the central pillar of the low-carbon energy transition, as well as the critical role for energy efficiency. Post-Paris world redefines the idea of *energy security*, particularly in the power sector of oil, natural gas and coal. WEO 2016 also outlines a course that would limit the rise in global temperature to below 2 °C and also plots possible pathways for meeting even more ambitious goals (source www.worldenergyoutlook.org).

1.6.3 Energy Production

A transition away from the use of traditional biomass and the more efficient combustion of solid fuels reduce air pollutant emissions, such as sulphur dioxide (SO_2), nitrogen oxides (NOx), carbon monoxide (CO) and black carbon (BC), and thus yield large health benefits (IPCC 2014). According to data, provided by the International Energy Agency (http://www.iea.org), the recently consumed primary energy sources are mainly represented by oil (36.0%), coal (27.4%) and natural gas (23.0%) that add up a total of 86.4% of the energy (fossil and non fossil) consumed in the world. The non-fossil energy sources include hydropower plants (6.3%), nuclear power (8.5%) and other sources of energy (geothermal, solar, tidal, wind, wood and waste burning) with a sum of 0.9%. However, over the last 5 years, a trend has been observed towards reduction of the consumption of traditional energy sources, with a simultaneous increase in the use of nontraditional sources (up to 25% in 2015), including solar energy, wind, biomass use, etc. (Sarsekeyev et al. 2015) (International Energy Agency (IEA): http://www.iea.org/publications/freepublications/publication/kwes.pdf_).

1.7 Role of Renewable Energy Sources

Recent environmental concerns on fossil fuels, smog, carbon monoxide, particulates, free radicals and toxic chlorofluorocarbons have shifted the concern on the alternative fuel usage. The second-generation fuels include gasification, pyrolysis, torrefaction and other thermochemical routes. Methane and carbon dioxide are the products of second-generation fuel which can range from 60 to 70% and 30%, respectively. For the process, biomass and municipal waste can be used thereby making this process cost effective. Nitration of methane can be done for production of nitromethane. Gasification of coal converts it into sugars which can directly be used as fuel.

The European Union (EU) sets out a strategy to double the share of renewable energy in gross domestic energy use in the EU by 2010 (from the recent 6–12%, with some 85% of the renewables being bioenergy). The EC's green paper in November 2000, "Towards a European Strategy for the Security of Energy Supply", introduced the objective of substituting 20% of traditional fuels by alternative fuels in the road transport sector by 2020.

1.7.1 Biomass as Potential Resources

Biomass can be defined as the collection of all organic matter composing biological organisms, but the main components utilized for biofuel production are sugars (starch, simple sugars and lignocelluloses) and lipids (Hill et al. 2006; Roy and Kumar 2013). Carbohydrate content of biomass can be converted to alcohols (ethanol, butanol), fatty acid esters (biodiesel) and long-chain and cyclic hydrocarbons (gasoline equivalents and jet fuels), using both well-known fermentation pathways and the newly developed methods of synthetic biology (see www.synberc.org/content/articles/what-synthetic-biology for a description). Caspeta and Nielsen (2013) suggested that driver for biofuel production is also the opportunity to reduce GHG emissions. To evaluate energy and environmental feasibilities of producing advanced biofuels, they calculated the net energy balance (NEB) and greenhouse gas (GHG) emissions resulting from different technologies. Their results show that NEBs are higher for microbial biodiesel production than for ethanol production using equivalent raw material and that this is associated with larger reduction of GHG emissions.

Biodiesel is typically made by chemically reacting lipids (e.g. vegetable oil, soybean oil, animal fat) with an alcohol-producing fatty acid esters. Biodiesel has a higher *cetane* number than petroleum diesel because of its oxygen content. The higher the *cetane* number, the more efficient the fuel – the engine starts more easily, runs better and burns cleaner. Global biodiesel market is estimated to reach 37 billion gallons by 2016 (Muniraj et al. 2015). Countries like Thailand are aiming for a 10% renewable mix in the next 5 years and India 20% by 2020. Sweden has stated that it aims to become 100% energy independent by 2020; most of this

independence will come through its own nuclear power, but renewable fuels will likely make up the balance (Kumar 2013).

Biodiesel has many environmentally beneficial properties. There are several advantages of using biofuels: biodiesel burns up to 75% cleaner than petroleum diesel fuel. Biodiesel reduces unburned hydrocarbons (93% less), carbon monoxide (50% less) and particulate matter (30% less) in exhaust fumes, as well as cancer-causing PAH (80% less) and nitrated PAH compounds (90% less) (US Environmental Protection Agency), and sulphur dioxide emissions are eliminated (biodiesel contains no Sulphur). Biodiesel is plant-based and using it adds no extra CO_2 greenhouse gas to the atmosphere. Nitrogen oxide (NO_2) emissions may increase or decrease with biodiesel but can be reduced to well below petro-diesel fuel levels. Biodiesel exhaust is not offensive and doesn't cause eye irritation (Kumar 2013).

Biofuels offer one of the best alternative options as they have much lower life cycle GHG emissions compared to fossil fuels (Source: http://unfccc.int/ghg_data/items/3825.php). These are liquid fuels derived from renewable biological sources (Kumar 1994, 1998, 2001, 2008, 2011) (See also Chaps. 4, 12, 13, and 14 this volume). One of the directives of the European Union (2009/28/CE) imposes a quota of 10% for biofuels on all traffic fuel until 2020 (Xavier et al. 2010). The most common renewable fuel is ethanol, which is produced from direct fermentation of sugars (e.g. from sucrose of sugarcane or sugar beet) or polysaccharides (e.g. starch from corn and wheat grains) (Mussatto et al. 2010). Cellulosic biomass also has great potential to contribute to the demand for liquid fuel (Himmel and Bayer 2009; Wilson 2009). There has also been recent interest in conversion of gaseous feedstocks such as CO_2 and H_2 (as off-gases, as syngas or as producer gas from biomass gasification) to products such as ethanol, butyrate and acetate (Wilkins and Atiyeh 2011).

Third-generation biofuels usually refer to microalgal triacylglycerols (TAGs), which are extracted from cells and used for biodiesel production (Chisti 2007; Pribyl et al. 2014). Algal biofuels are in the nascent stage of development and are derived from low-input/high-output production organisms such as algal biomass (Sarsekeyeva et al. 2015) (see also Chap. 17 this volume). Fourth-generation biofuels are derived from the bioconversion of living organisms (microorganisms and plants) using biotechnological tools (Rutz and Janseen 2007; FAO 2008) (Gopinathan and Sudhakaran 2009) (see also Chap. 24 this volume). Thus, fourth-generation biofuels combine the properties of third-generation biofuels with an advantage of genetic optimization of their producers (Al-Thani and Potts 2012; Nozzi et al. 2013).

1.8 Conclusion

A world of rapid economic growth and rapid introductions of new and more efficient technologies would require policies supporting technology development, diffusion and transfer, as well as finance for responses to climate change, which can complement and enhance the effectiveness of initiatives that directly promote

adaptation and mitigation. Globally, economic and population growth continue to be the most important drivers of increases in CO_2 emissions from fossil fuel combustion. Explicit consideration of interactions among water, food, energy and biological carbon sequestration plays an important role in supporting effective decisions for climate-resilient pathways. The International Energy Agency (IEA) published WEO 2016 and suggested that the entry into force of the Paris Agreement has raised hopes and expectations of more concerted global efforts to tackle climate change, but how will the various country climate pledges made in Paris really affect the efficiency and carbon footprint of the energy sector? Will market dynamics change for oil, natural gas and coal – or might the slump in prices for some fuels be here to stay? How can governments address the impact of local pollution, often energy-related, on air quality?

IEA suggested that the 2 °C pathway is very tough and the road to 1.5 °C goes through uncharted territory. The challenges to achieve the 450-ppm scenario are immense, requiring a major reallocation of investment capital going to the energy sector. By 2040, the share going to fossil fuels drops towards one-third. In addition, $35 trillion is needed for improvements in energy efficiency. The 450 ppm scenario puts the energy sector on course to reach a point, before the end of this century, when all residual emissions from fuel combustion are either captured and stored, or offset by technologies that remove carbon from the atmosphere. The transformation required for a reasonable chance of remaining within the temperature goal of 1.5 °C is stark. It would require net-zero emissions at some point between 2040 and 2060 (even if negative emission technologies can be deployed at scale), thus requiring radical near-term reductions in energy sector CO_2 emissions, employing every known technological, societal and regulatory decarbonization option.

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Historical Development of Biofuels

2

Sunita Agarwal and Ashwani Kumar

Abstract

Biofuel, also called as agrofuel, is derived from biomass and may exist in solid, liquid, or gaseous form. The use of waste biomass to generate energy may reduce waste management problems like pollution, greenhouse gas emissions, and the use of limited amount of fossil fuels. Unlike fossil fuels, biofuels are a renewable energy source. Because they are derived from crops that can be harvested annually, or in the case of algae monthly, biofuels are theoretically unlimited. There is a potential for bioenergy obtained from waste to decrease the speed of global warming. Biofuels have emerged as an ideal choice to meet these requirements. Huge investments in research and subsidies for production are being done in most of the developed countries. The choice of raw material for biofuel production ranges from molasses for bioethanol and nonedible oil for biodiesel depending on nations. Global initiatives started in 1970s with pioneer work by Melvin Calvin in the United States, D.O. Hall in King's College London, Stewart in CSIRO, Australia, and Prof. Sato and Yamada were some leading groups to undertake biofuel work. Melvin Calvin inspired us (AK and team) to take work on laticifers in Rajasthan. We were among the pioneers to start work on biofuels in Rajasthan with support from Department of Non Conventional Energy Sources (DNES) Govt of India in early 1980's. Initial researches were confined to discover candidate crop species, the agrotechnological zones in which they might be grown and predicted yields. Cost of production of feedstock was assessed in

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relation to cost of alternative crops such as wheat and grain sorghum in southern and eastern Australia. At present, governments have initiated the use of alternative sources of energy for ensuring energy security, generating employment, and mitigating CO_2 emissions. Attempts were also made to produce energy from waste. One waste management strategy is anaerobic digestion. In anaerobic digestion, any sort of biomass can be utilized. This waste breaks down and gives rise to methane. This methane is harvested and burned to create heat and power to run some automobiles and vehicles.

Keywords
Biofuel • Waste management • Ethanol production • Microalgae • Fossil fuels

2.1 Introduction

2.1.1 Definition

Biofuels can be defined as liquid fuels produced from biomass for either transport or burning purposes. They can be produced from agricultural and forest products and the biodegradable portion of industrial and municipal waste. The use of biomass to generate energy reduces problems like pollution, greenhouse gas emissions, and the use of limited amount of fossil fuels. Unlike fossil fuels, biofuels are a renewable energy source. Because they are derived from crops that can be harvested annually, or in the case of algae monthly, biofuels are theoretically unlimited. Plant oils were the original choice of Otto Diesel in his early engine, and Henry Ford preferred grain ethanol to power his early vehicles. Solar energy, accumulated under earth in the form of fossil fuels since the inception of life, accounts for more than 97% of the world consumption of energy of which the share of oil is about 39%. The biomass accounts for 43% of the total energy supply in the developing countries as compared to only 1% in the developed countries (Hall 1982).

Liquid fuel is a very precious resource used abundantly and somehow indiscriminately by modern man. The primary source of liquid fuel is currently crude oil which is becoming harder and more expensive to recover as conventional reserves are depleted and as foreign suppliers increase the price for their declining reserves. The drastic jump in oil prices between 1973 and 1979 started the agitation that made many governments to adopt policies to develop alternative energy sources.

2.2 History of Biofuels

2.2.1 Biofuels Milestones

B.C.E. (Before Common Era)

4000 Sumerians discover the process of fermentation.
Tenth century Assyrians use biogas for heating bathing water.

C.E. (Common Era)

Seventeenth century Helmont observes that organic matter emits flammable gases.
1808 Davy discovers methane as the end product of anaerobic digestion.
Mid-1800s Transesterification of plant oils is used to distill glycerin during soap production.
1858–1864 French biologist Antoine Bechamp experiments with fermentation and concludes that ferments are living organisms.
1864 French chemist Louis Pasteur describes the process of fermentation scientifically.
1876 First successful internal combustion engine is produced.
1880s First successful internal combustion engine using producer gas is produced.
1892–1893 Rudolf Diesel files a patent for a "Working Method and Design for Combustion Engines ... a new efficient, thermal engine."
1895 Biogas is used to fuel street lamps in Exeter, Great Britain.
1897 First diesel engine suitable for practical use operates at an efficiency of 75%.
1908 Henry Ford's Model T is designed to run on ethanol.
1920s–1930s Attempts to promote ethanol as motor fuel are made. Anaerobic bacteria responsible for methane production are identified.
1940s First US ethanol plant opens.
1939–1945 Extensive use of biogas to replace gasoline occurs.
1979 Commercial alcohol-blended fuels are marketed.
1984 The number of ethanol plants peaks at 163 in the United States, producing over 2.2 billion liters of ethanol during the year.
1988 Ethanol is used for the first time as an oxygenate to lower pollution caused by burning gasoline.
1990 Ethanol plants begin to switch from coal to natural gas and to adopt other cost-reducing technologies. Ethanol plants are subsidized by the US government to support farmers. Gasohol becomes commonly available in the US Midwest.
1997–2002 Three million US cars and light trucks that could run on E85, a blend of 85% ethanol and 15% gasoline. However only limited gas stations sell this fuel. Concerns about climate change cause leading alternative energies such as biofuel, solar, and wind to expand by 20–30% yearly.
2003 California becomes the first state to start replacing the oxygenate MTBE with ethanol. Several other states start switching soon afterward. California consumes 3.4 billion liters of ethanol a year—about a third of all ethanol produced in the United States.

2004 Crude oil prices rise by 80%, gasoline prices rise by 30% in the United States, and diesel fuel prices rise by almost 50%. The US ethanol industry makes 225,000 barrels per day in August, an all-time record. Oil companies invest heavily in alcohol fuel.

2005 E85 sells for less than gasoline on average in the United States (see also chapter 12 this volume). More than 4 million flexible-fuel vehicles (vehicles that run on E85 and gasoline) exist in the United States. About 400 filling stations that sell E85 fuel exist in the United States, mostly in the Midwest. Gasoline prices rise as ethanol prices stay the same, due to rapidly growing ethanol supply and federal tax subsidies for ethanol.

2006–2017 Indy Racing League switches to a 10% ethanol and 90% methanol fuel mixture (Meher et al. 2006). In 2017 most of the gas stations in United States sell gasoline containing upto 10 percent ethanol (Personal observation for Alabama and Georgia by AK).

2.2.2 Initial Efforts

First efforts to cultivate hydrocarbon-producing plants for fuel production were made by Italians in Ethiopia and French in Morocco. Later on, Calvin and his collaborators have revived the idea again and have advocated the study of petro-crops as a possible feedstock for petroleum-like materials. Presently, the largest fuel program is in Brazil where the government currently spends a considerable amount on subsidizing the production of alcohol, mostly from biomass of sugarcane. Production was estimated to increase so much that around 11–14 million cars will use alcohol (with gasoline) by the year 2000. With the wide majority of atmospheric scientist now agreeing that global warming is already well underway, there are now more strident calls to replace crude oil as our liquid fuel source in order to reduce the buildup of greenhouse gases in the environment. Thus an additional emphasis is being placed on the development, production, and the use of alternative fuel considered being friendlier to the environment than fossil fuel. Generally, bio-sourced fuels are termed biofuel examples of which are biomethanol, bioethanol, biobutanol, biomethane, biohydrogen, biodiesel, etc. (Houghton et al. 2001). However, numerous popular articles and scientific papers have cautioned against the global drive toward a biofuel economy generally, highlighting the potential impacts on food security (Shaik and Kumar 2014).Therefore, initial efforts were concentrated on biofuels from arid and semiarid conditions using laticifers.

2.3 Biomass as Potential Resources

Biomass resources are potentially the world's largest and sustainable energy source, a renewable resource comprising 220 billion oven dry tonnes (about 4500 EJ) of annual primary production. The annual bioenergy potential is about 2900 EJ though only 270 EJ could be considered available on sustainable basis and at competitive

prices. Most major energy scenarios recognize bioenergy as an important component in the future world's energy. Projections indicate the biomass energy use to the range of 85–215 EJ in 2025 compared to current global energy use of about 400 EJ of which 55 EJ are derived from biomass (Hall and Rosillo-Calle 1998).

Melvin Calvin initiated work on *Euphorbia* spp during 1970's onwards which was followed by several groups world wide. Early technical and political discussion of biofuels—and more broadly bioenergy—focused on solutions to some of the key energy challenges facing many developed countries, i.e., on ways to improve the security of energy supply in an environmentally sound way (Kumar 2013).

Systematic search for plants with hydrocarbon contents has been made sporadically in the past. Initial studies on latex-bearing plants were confined to the rubber-yielding plants. However, during the Second World War, considerable interest was generated for alternative energy sources for fuel and rubber (Hall 1982). Buchanan et al. (1978) surveyed 100 plant species from Illinois for natural rubber as well as oil content and developed selection criteria for identifying potential plant species. Species were rated in four categories on the basis of their uses such as fiber, protein, oil, and rubber production. Oil and rubber contents were determined by extraction of dried plant material with various solvents. In this survey, 14 species were identified, which were judged to have good potential as hydrocarbon and rubber-producing crops according to the criteria of Buchanan et al. (1978). The most promising species belonged to Euphorbiaceae, Asclepiadaceae, and Compositae. In the US Department of Agriculture (USDA), researchers screened 6500 species of wild plants as oil-producing plant species (Stewart et al. 1982). McLaughlin and Hoffman (1982) conducted a survey of over 400 samples of plants from the Southern United States. The plant collection encompassed considerable taxonomic diversity; 195 species belonging to 107 genera and 35 families were examined for hydrocarbon or chemical feedstock. Ten species were identified by the Arizona selection criteria as having high potential for further development: *Pedilanthus macrocarpus* (Euphorbiaceae); *Asclepias albicans*, *A. subulata*, and *A. erosa* (Asclepiadaceae); *Amsonia grandiflora* and *A. kearnevama* (Apocynaceae); and *Chrysothamnus paniculatus*, *C. nauseous*, *Grindelia camporum*, and *Xanthocephalum gymnospermoides* (Compositae). Two species in the family Asciepiadaceae, *Calotropis procera* and *Asclepias syriaca*, have been investigated as potential sources of hydrocarbon-like materials. *C. procera* has been reported as hydrocarbon-yielding crop by several workers (Erdman and Erdman 1981; Williams et al. 2006). Bhatia and Srivastava (1983) screened 386 indigenous laticiferous plants belonging to families Euphorbiaceae, Asclepiadaceae, Apocynaceae, Urticaceae (Moraceae), Convolvulaceae, and Sapotaceae and resulted in the selection of 16 potential plants for further studies. Our lab carried out pioneer work on hydrocarbon-yielding plants in the early 1980s onward in collaboration with National Botanical Research Institute, Lucknow and Indian Institute of Petroleum, Dehradun with support from Department of Non Convetional Energy sources which was later raised to the level of Ministry of Non Conventinal Energy Sources Government of India (Kumar 1984; see Chap. 12, 13, 14 of this volume).

During the 1970s, work on biofuel was taken up due to high price of fossil fuels, and the aim was replacement of fossil fuels. It is noteworthy to say that Melvin Calvin initiated the work on biofuels in Arizona state with several laticifers in the early 1970s (Calvin 1979a, b; Hall and Calle 1998). Different countries like the United States, Australia, and Japan initiated work on hydrocarbon-yielding plants. During a visit to California University, the author (Ashwani Kumar) was invited to visit the laboratory of Nobel Laureate Prof. Melvin Calvin, and he advised him to work on laticiferous plants in Rajasthan as it has broadly similar climatic conditions as that of Arizona desert. The work on *Euphorbia lathyris* (Garg and Kumar 1987; Kumar 2001) was commenced in India successfully which was later extended to other hydrocarbon-yielding crops like *Euphorbia antisyphilitica* (Johari et al. 1990; Johari and Kumar 1992; Kumar 1995). DNES also initiated all India-coordinated project with the National Botanical Research Institute Lucknow and Indian Institute of Petroleum Dehradun, a Council of Scientific and Industrial Research-funded institution (Bhatia et al. 1983).

In vitro micropropagation methods for mass multiplication of *E. lathyris* (Kumar and Joshi 1982), *Euphorbia antisyphilitica* (Johari and Kumar 1992), and *Pedilanthus tithymaloides* var. *Green* (Rani and Kumar 1994) were developed successfully.

Subsequently, Kumar (1996) presented a model system for biofuel cultivation in semiarid and arid regions. Agrotechnology for the laticifers was developed at the University of Rajasthan for different laticiferous plants (Kumar 2011, 2013). The plants included are *Euphorbia lathyris* (Garg and Kumar 2012, 2013), *Calotropis procera* (Kumar et al. 2002), and *Euphorbia antisyphilitica* (Johari and Kumar 2013, 2016).

In India, 68.5% of the energy used in households is from the firewood, and 64.2% of it is collected from natural sources. The shortfall in fuel production is likely to rise to 137 million tonnes in 2000 A.D. from the present 84 million tonnes (Vimal 1986). Source-wise, energy consumption in the household sector in the rural areas is as follows: noncommercial sources like fuel wood (68.5%) and animal dung (8.3%); commercial energy sources like oil (16.9%), coal (2.3%), and electricity (0.6%); and others (3.4%) (Vimal and Tyagi 1984).The indiscriminate falling of trees has reduced the forest cover to 23% against 33% during the last decade (Murty 1985). The annual production of dry dung is of the order of 350 million tonnes from about 240 million cattle which is capable of generating 70 billion cubic meters of gas annually in biogas processing plants. However, most of the dry dung is used for burning (Murty 1985).Tropical forests in the world are estimated to be vanishing at an annual rate of about 7 million hectares, while the corresponding rate for woodlands in the semiarid zones is 4 million hectares. Kumar and Roy (1996) presented model for wood energy plants cultivation in Rajasthan.

The best solar-converting machine available today is the green plant which can produce fuel and material on renewable basis (Szego and Kemp 1973; Calvin 1976, 1977, 1979a, b, 1980, 1983a, b, 1985; Calvin et al. 1981, 1982; Buchanan et al.

1978; Vergara and Pimental 1978; Weisz and Marshall 1979; Bagby et al. 1980; Hall 1980; Johnson and Hinman 1980; Coffey and Halloran 1981; Tideman and Hawker 1981; Khoshoo 1982; McLaughling et al. 1983; Stewart et al. 1982; Bhatia and Srivastava 1983; Hoffman 1983; Nemethy 1984; Vimal and Tyagi 1984).

Subsequently, work on *Jatropha* has been carried out at our Energy Plantation Demonstration Project Centre, University of Rajasthan, Jaipur supported by the Department of Biotechnology, Government of India (see also chapter 12 this volume). However, the use of *Jatropha* for oil and biodiesel produced discussions on food vs fuel. Arguments were made that even if nonedible oil-yielding crops were raised on wastelands, the ecological balance was disturbed in the long run. Recently, in a meeting of the German Plant Nutrition Society, issue was raised of nitrogen imbalance in soil and groundwater. These controversies paved way for the next generation biofuels. Currently, cellulosic biofuels and algal biodiesels are prominent biological approaches to sequester and convert CO_2. However, another biofuel feedstock, lignocelluloses—the most abundant biological material on earth—is being explored. Lignocelluloses is everywhere—wheat straw, corn husks, prairie grass, discarded rice hulls, or trees. The race is on to optimize the technology that can produce biofuels from lignocellulose sources more efficiently—and biotech companies are in the running. Second- and third-generation biofuels require altering host material by metabolic engineering for entire product and developing new enzyme systems. Industrial application of biofuel inclusive of related bioproducts of commercial value from fourth-generation products is being adapted on a large scale. Carbon captured in cellulosic biofuels and algal biodiesels are prominent biological approaches to sequester and convert CO_2. Lipid productivity of many algae greatly exceeds that of the best cellulosic ethanol production. Another approach is direct conversion of CO_2 to fuels or chemicals. Biofuels will reduce greenhouse gas emissions, promote energy independence, and encourage rural development.

The discovery that mechanical energy could be extracted from the wind and from the kinetic energy of falling water was made independently in many parts of the world over the past millennia. The use of various water wheels to convert the gravitational energy of falling water into shaft energy was used for many tasks including grinding of grain and formed an initial basis for the textile industry at the start of the industrial revolution before steam and electricity proved more effective. Modern hydroelectric systems were developed only a little over a century ago with the harnessing first of natural falls such as Niagara Falls on the US-Canadian border and Three Gorges Dam in China. In the past two decades, modern wind turbine electric generators as large as 6 Gwatts of power have been developed and deployed. Wind turbines now provide more than 1% of global electricity, and wind is the fastest-growing energy supply sector. Solar energy has always been used directly for heat and light, and this principle of "passive solar gain" is now being used in the design of new buildings.

2.4 Uses of Biofuels

1. Ethanol is used in fuel for gasoline-based engines. Ethanol is mixed upto 10 percent of gasoline and is sold as E10.
2. Gasoline cars use up to 85% Ethanol (E85) in flex-fuel vehicles.
3. Biodiesel is used in normal diesel-burning vehicles. It's mixed up to 20% (B20) for regular diesel.
4. Modified engines use upto 100% (B100).
5. Ethanol is produced from corn in USA and sugarcane in Brazil and distillation purifies the alcohol to 100%.
6. Lastly, ethanol is mixed with gasoline in low-enough quantities that the corrosive nature of ethanol doesn't damage a car's fuel system components.
7. Similar to ethanol, biodiesel is processed from various crops. In some cases, it's even possible to convert waste vegetable oil from restaurants to burn safely in a diesel car.
8. Biodiesel is used as a means of recycling oils, which are relatively easy to convert to regular diesel fuel. After it has been refined, biodiesel works in regular diesel engines as well as diesel fuel. Experts are studying means of turning plant matter into ethanol, which is used in a small but significant number of cars.
9. Biofuels release carbon dioxide, but the carbon dioxide release is offset by the carbon dioxide absorbed by plants during photosynthesis. Because of this, biofuels are considered carbon neutral.
10. Nearly 30% of all energy consumed in the United States is used in transportation. To put this into perspective, residential and commercial uses combined only account for 10%. This means that humans in industrial nations use, on an average, three times more energy to get around than they use to cook their food and heat their homes. This number does not include electricity generation, which accounts for 40% of all energy used. The solution, at least for now, appears to be algal-based biofuels, which are still years if not decades away from commercialization (see also chapter 17 this volume). The idea is simple. Algae have lipid, and lipid can be converted to a number of fuels including diesel, ethanol, butanol, and methanol. Because algae absorb CO_2 to make lipid, the net impact on the environment should be very small. Additionally, biofuels are biodegradable, so if they do spill, less harm is done compared to when fossil fuels spill.
11. The generation of electricity is the single largest use of fuel in the world. In 2008, the world produced about 20,261 TWh of electricity. About 41% of that energy came from coal, another 21% came from natural gas, and the rest was covered by hydro, nuclear, and oil at 16%, 13%, and 5% respectively. Of the fuel burned, only 39% went into producing energy, and the rest was lost as heat.

Only 3% of the heat was then used for cogeneration. Of the 20,261 TWh produced, 16,430 TWh were delivered to consumers, and the rest was used by the plants themselves. Biofuels may provide at least a partial answer. Cogeneration plants often use methane derived from landfills, and there is vigorous interest in the use of syngas in many agricultural areas. Like any biofuel, the balance of the equation lies in carbon generation. For syngas made from the agricultural waste, the net impact is lower than if the waste were allowed to decompose on its own. This is because natural decomposition in oxygen-rich environments produces nitrogen dioxide, which is over 300 times more potent of a greenhouse gas than carbon dioxide, as well as methane, which is over 20 times more potent. The same benefits exist for methane harvested from landfills.

12. The major use of natural gas from fossil fuels is heat, though a good deal of it also goes to energy. In the United States, a boom in hydraulic fracturing (called fracking) has led to a huge surge in the production of natural gas from shale (a fossil fuel) and to the prediction that this will soon become the predominant form of energy, perhaps as soon as 2040. Of course, natural gas need not come from fossilized plant material; it can also be produced from recently grown plant material. However, the majority of biofuel used in heating is solid. Wood is both an aesthetic and a practical method of heating, and homes may use wood burning stoves as supplements to other heating systems like natural gas or electricity. Wood gasification boilers can reach efficiencies as high as 91% (Clark 2007).

2.5 Biomass as Feedstock

Biomass derived from trees, agroforest residues, grasses, plants, aquatic plants, and crops is versatile and important renewable feedstock for chemical industry. Through the process of photosynthesis, plants convert carbon dioxide and water into primary and secondary metabolites. Both of these are industrially important chemicals. Primary metabolites are carbohydrate (simple sugar, cellulose, hemicelluloses, starch, etc.) and lignin called lignocellulose present in high volume in biomass. The lignocellulosic biomass can be converted into biofuels. The secondary metabolites are high-value biochemical such as gums, resins, rubber, waxes terpenes, terpenoids, steroids, triglyceride, tannin, plant acids, alkaloids, etc. and are present in low volume in the plants (Clark 2007; Naik et al. 2010). The secondary metabolites can be utilized for production of high-value chemicals such as food flavors, feeds, pharmaceuticals, cosmeceuticals, nutraceutical, etc., using integrated processing technique.

Enhancement of biomass utilization requires tremendous effort to develop new biomass systems in which production, conversion, and utilization of bio-based products are carried out efficiently in near harmony with nature (Osamu and Carl 1989). Huang et al. (2008) reviewed separation methods and technologies related to lignocellulosic biorefineries for production of ethanol and other products. Clark and

Buldarni (2006) reported the use of green chemical technologies to transform low-value waste biomass to green chemicals such as waxes, ethanol, etc. However, Chew and Bhatia (2008) have reported on different types of catalysts and their role in the catalytic processes for production of biofuels in a typical palm oil based refinery in Malaysia.

2.6 Biodiesel

The functionality of biodiesel as a possible and likely candidate to replace fossil fuels as primary energy source for machineries and vehicular flow remains a driving force for scientists to keep researching into the world of biodiesel. Since vegetable oils which are the major feedstock for biodiesel are widely grown and used for food and animal feed, hence there is the current debate "food or fuel?" Nonetheless, lengthy list of academia has worked on surplus and yet inedible oil as major feedstock for biodiesel (Kumar and Sharma 2005). Kumar (2013) discussed the importance of nonedible oil *Jatropha curcas* in biodiesel production. Shah and Gupta (2007) produced biodiesel from *Jatropha* by enzyme in a solvent-free system. The Central Salt and Marine Chemicals Research Institute Bhavanagar, Gujrat, has played a leading role in developing agrotechnology for *Jatropha* biodiesel production including genetic improvements (see also chapter 12 this volume). Attempts have also been made to produce biodiesel from nonedible sources like used frying oil, greases, tallow, and lard (Alcantara et al. 2000; Canakci and Gerpen 2001; Dorado et al. 2002). Soap has also been presented as a suitable feedstock for biodiesel production (Arjun et al. 2008). Nevertheless, numerous references revealed the importance of biodiesel especially with respect to the future energy needs (Demirbas and Balat 2006; Marchetti and Errazu 2008; Mohibbe Azam et al. 2005; Sarin et al. 2007; Zhang et al. 2003).

2.7 Biorefinery System

The biorefinery system is based on biomass as processing input (feedstock) for production of multiple bio-based products. The basic concept of the biorefinery system is to produce biofuel and platform of chemicals from biomass. The development of comparable biorefineries—however not in the sense of a direct copy of petroleum refinery—is necessary to produce a broad variety of bio-based products in an efficient construction set system. The product palette of a biorefinery includes not only the products produced in the petroleum refinery but also in particular products that are not accessible in petroleum refineries (Kamm and Kamm 2004): (1) lignocellulosic feedstock biorefinery, including lignocellulosic feedstock (LCF) pretreatment and effective separation in lignin, cellulose, and hemicellulose; (2) further development of thermal, chemical, and mechanical processes; (3) development of biological processes; and (4) combination of substantial conversions, such as biotechnological and chemical processes (Kamm et al. 2006).

2.8 Conversion Processes

2.8.1 First Generation

2.8.1.1 Transesterification

This reaction is used to produce biodiesel or vegetable oil-based fatty acid methyl esters. The product of the reaction is glycerol—a high-value coproduct and biodiesel (Kulkarni et al. 2006; Meher et al. 2006). Transesterification is a reversible reaction and proceeds essentially by mixing the reactant in which the catalyst is a liquid acid or liquid base (called homogeneous catalysis); however, in the cases of high free fatty acids, this process fails, hence solid catalyst is recommended. This is because the solid catalysts can simultaneously catalyze the transesterification of triglycerides and free fatty acids present in biomass to methyl esters (Kulkarni et al. 2006).

2.8.1.2 Fuel Ethanol Overview

Ethanol is a biofuel produced from sugar and starch as raw materials by fermentation. In a large number of countries, ethanol attained a predominant position among biofuels as a blending agent with petrol because of its oxygenation properties, energy balance, environmentally friendly nature, possible employment benefits in the rural sector, and contribution to energy security at the national level (Faaji et al. 2008). Global production of fuel ethanol increased by 18% over 2006 to 46 billion liters in 2007, marking the sixth consecutive year of double-digit growth (Worldwatch Institute 2009). The United States became the leading fuel ethanol producer in 2007, producing over 24.5 billion liters and jumping ahead of long-standing leader Brazil. Brazil and the United States accounted for 95% of all ethanol production in 2007. Several important political, technological, and federal policies and incentives led to both countries becoming world leaders in the use of bioethanol. Other countries implementing fuel ethanol programs are Australia, Canada, China, Colombia, the Dominican Republic, France, Germany, India, Jamaica, Malawi, Poland, South Africa, Spain, Sweden, Thailand, and Zambia (Faaji et al. 2008).

2.8.1.3 Ethanol Conversion Process

A wide variety of carbohydrates containing raw materials have been used for production of ethanol by fermentation process. The fermentation process refers to the metabolic conversion of organic substrate by the activity of enzymes secreted by microorganisms. There are two basic kinds of fermentation, (1) aerobic and (2) anaerobic, depending upon oxygen needed in the process or not. There are many microorganisms capable of providing fermentative changes to both sugars and starches.

2.8.2 Conversion technologies

There are two basic routes for conversion of biomass to liquid biofuels viz. thermochemical processing and biochemical processing.

- Biochemical—in which enzymes and other microorganisms are used to convert cellulose and hemicellulose components of the feedstock to sugars prior to their fermentation to produce ethanol.
- Thermochemical—(also known as biomass to liquids) where pyrolysis/gasification technologies produce a synthesis gas ($CO+H_2$) from which a wide range of long carbon chain biofuels, such as synthetic diesel, aviation fuel, or ethanol, can be reformed, on the basis of the Fischer–Tropsch conversion. The clear advantage of thermochemical processing is that it can essentially convert all the organic components of the biomass compared with biochemical processing that focuses mostly on the polysaccharides (Gomez et al. 2008). Thermochemical conversion is thus direct combustion, gasification, thermochemical liquefaction, and pyrolysis (USDOE 2002). The biological process of energy conversion of biomass into other fuels includes anaerobic digestion, alcoholic fermentation, and photobiological hydrogen production (Grant 2009).

2.8.3 Next-Generation Biofuels

The second-generation biofuel derived from lignocellulosic agriculture and forest residue addresses some of the problems such as strain on food markets, contribution to water shortage, and destruction of the forest cover that are posed by the first-generation biofuels. However, there is a concern over the competing land use or required land-use changes (Brennan and Owende 2010). In the latest generation of biofuels—the third generation—scientists are looking toward microscopic organisms. These biofuels are derived from microbes and microalgae and are considered to be a viable alternative energy resource that is devoid of drawbacks associated with first- and second-generation biofuels. There have been researches that show microalgae and some species of microbes can be used as substrate to produce biodiesel, as they can synthesize and store a large amount of fatty acids in their biomass (Xiong et al. 2008). Zhu et al. (2008) have worked on production of microbial biofuels from waste molasses and have reported that lipids produced in microbial biomass can be utilized for biodiesel production.

Currently, a few companies and some research groups are working on algae cultivation. In April 2006, the start-up company Solix Biofuels was set up in Fort Collins, Colorado, to develop a microalgae reactor technology that could be used in conjunction with existing power stations, running the carbon dioxide in closed cycles. In June of the same year, oil company PetroSun started a wholly owned subsidiary called Algae Biofuels to operate in the United States and Australia to investigate the production of biodiesel, ethanol, methanol, methane, and hydrogen

from microalgae (Brennan and Owende 2010). However further researches are going in such areas (see also chapter 17 and 23 this volume). For production on a commercial scale, there are a large number of issues that are to be tackled such as the following:

- Most efficient algal strain
- Cultivation of selected strain at best growing rates
- Designing the metabolic pathways and engineering those reactions that control lipid synthesis to produce algal cells having the desired lipid content
- Designing an efficient and economical method to recover oil from algal cells (Xiong et al. 2008)

2.8.4 Bioethanol from Lignocellulosic Biomass

The ethanol that is produced from a renewable biomass is called bioethanol; its use is both environmental friendly and renewable (Johnston 2008). It can be used directly in modified spark engines or can be blended with petrol. Ethanol also improves fuel combustion in vehicles, hence reducing emissions. In comparison with petrol, ethanol contains only a trace amount of sulfur, so mixing ethanol with petrol helps reduce the sulfur content of fuel, simultaneously lowering the emission of sulfur oxide that is the major component of acid rain (FAO 2008; Woods 2008).

Ethanol available in the biofuel market is mainly produced from starch (FAO 2008; Woods 2008). Sugar and starch are fermented to alcohol. This is, in fact, the least complex method used for producing ethanol (Verma et al. 2000; Singh et al. 1995).

With plant biomass, it is a different story altogether. Plant biomass consists of cellulose microfibers embedded in hemicelluloses, pectin, and lignin. The amount of each component varies among different plant species and parts. The following steps are involved in production of ethanol (see also chapter 7 and 15 this volume).

- Pretreatment of substrates.
- Saccharification process to release the fermentable sugars from polysaccharides and fermentation of released sugars.
- Finally, distillation step to separate ethanol. Pretreatment is designed to facilitate in the separation of cellulose, hemicellulose, and lignin so that complex carbohydrate molecules constituting the cellulose and hemicellulose can be broken down by enzyme-catalyzed hydrolysis into their constituent simple sugars.

The complex structure of cellulose makes it difficult to depolymerize into simple sugars, but once the polymer structure has been broken down, the sugar molecules are fermented to ethanol using fermentative microorganisms (Barron et al. 1995).

Hemicellulose consists of five-carbon sugars, which although are easily broken down into its constituent sugars such as xylose and pentose, the fermentation

process is much more difficult and requires efficient microorganisms that are able to ferment five-carbon sugars to ethanol.

Lignin consists of phenols and, for practical purposes, is not fermentable, although it can be recovered and utilized as a fuel, providing process heat and electricity for the alcohol (ethanol, butanol) production facility.

The hydrolysis is usually catalyzed by cellulase enzymes, and the fermentation is carried out by yeast or bacteria. The factors that affect the hydrolysis of cellulose include porosity, that is, an accessible surface area of the waste materials, cellulose fiber crystallinfity, and lignin and hemicellulose content (Sun and Cheng 2002). The presence of lignin and hemicellulose makes the access of cellulase enzymes to cellulose difficult. The lignin and hemicellulose removal, reduction of cellulose crystallinity, and increase of porosity in pretreatment processes can significantly improve the hydrolysis. The cellulose crystallinity can be reduced by a combination of chipping, grinding, and milling (Morjanoff and Gray 1987). Steam explosion is the most commonly used method for pretreatment of plant biomass (Sun and Cheng 2002).

Lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of H_2O_2 (Schurz and Ghose 1977). Microorganisms such as brown, white, and soft rot fungi are used in biological pretreatment processes to degrade lignin and hemicellulose (Bhoominathan and Reddy 1992). Brown rots mainly attack cellulose, whereas white and soft rots attack both cellulose and lignin. The white rot fungus *Phanerochaete chrysosporium* produces lignin-degrading enzymes, lignin peroxidases, and manganese-dependent peroxidases, during secondary metabolism in response to carbon or nitrogen limitation (Blanchette 1991). Other enzymes including polyphenol oxidases, laccases, H_2O_2-producing enzymes, and quinine-reducing enzymes can also degrade lignin (Azhar et al. 1981). The advantages of biological pretreatment include low-energy requirement and mild environmental conditions, but the hydrolysis rate is very low (Morjanoff and Gray 1987).

Furfural is an important inhibitor of ethanol production from hemicellulose hydrolysate (Ranatunga et al. 1997) even at low concentrations (Zaldivar et al. 1999; Boopathy and Daniels 1991). Various bacteria and yeast have been reported to partially transform furfural to either furfural alcohol or furoic acid, or a combination of both (Gutierrez et al. 2002; Wang et al. 1994; Beguin and Aubert 1994).

A few microbial species such as *Neurospora*, *Monilia*, *Paecilomyces*, and *Fusarium* have been reported to hold the ability to ferment cellulose directly to ethanol by simultaneous saccharification and fermentation (Lynd et al. 2005). Consolidated bioprocessing featuring cellulase production, cellulose hydrolysis, and fermentation in one step is an alternative approach with outstanding potential (Banat et al. 1998a, b).

The recombinant strain of *Escherichia coli* with the genes from *Zymomonas mobilis* for the conversion of pyruvate into ethanol has been reported by Dien et al. (2001). A key challenge to commercializing production of fuels and chemicals from cellulosic biomass is higher processing costs (Wyman 1999; Banat et al. 1998a, b). Biological conversion opens such low-cost production path as it has the potential to achieve a higher yield, and the modern tools of biotechnology can improve key process steps.

A range of residual substrates such as sugarcane bagasse, sugarcane molasses (Farrell et al. 1998; Sheoran et al. 1998; Gough et al. 1997; McMillan 1994), and starch (Aggarwal et al. 2001) have been found suitable for the bioconversion of available carbohydrates in these substrates to produce ethanol. A variety of mesophilic and thermophilic microorganisms were employed to optimize the fermentation process (Singh et al. 1998; Wati et al. 1996; Yadav et al. 1996; Banat et al. 1998a, b, 2000; Abdel-Fattah et al. 2000; Huang et al. 2009), which could be practically viable in different climatic conditions, particularly to reduce the cost of temperature maintenance in large fermenters operating in warmer countries in summer months (Sheoran et al. 1998; Abdel-Fattah et al. 2000; Huang et al. 2009).

Sukumaran et al. (2009) have reported on bioethanol production from the saccharification of wheat bran, a lignocellulosic waste. The cost of cellulose degrading enzymes is a major factor in the enzymatic saccharification of agricultural biomass, which contains lignin. Production cost of cellulases and hence ultimately the cost of ethanol production may be brought down by multifaceted approaches. One important approach is the use of cheaper lignocellulosic substrates for the biosynthesis of the enzyme, and second strategy is the use of cost-efficient fermentation process such as solid state or solid substrate fermentation at much cheaper cost.

Although bioethanol production has been greatly improved by development of new technologies, there are still challenges that need further improvements in the developed technology to bring forward to commercial scale. These challenges include maintaining a stable performance of the genetically engineered microorganisms and developing more efficient pretreatment technologies for the lignocellulosic biomass and integrating the optimal components into economic ethanol production systems (Chakraborty et al. 2012).

2.8.5 Perspectives on First- and Second-Generation Biofuels

Metrics that can be useful for understanding and evaluating first- and second-generation biofuel systems include land-use efficiency, net life cycle energy balance, net life cycle greenhouse gas balance, and economics (Larson 2006).

2.8.5.1 Land-Use Efficiency for Providing Transportation Services

Land is ultimately the limiting resource for biofuels production. There is a wide variation in the total amount of biomass that can be produced on a unit area of land, depending on species chosen, soil and climate conditions, and agronomic treatments.

Second-generation fuels, can provide an improvement of 50 % or more in land-use efficiency over sugar-based first-generation fuels (Larson 2006).

2.8.5.2 Greenhouse Gas Emissions

The effectiveness with which greenhouse gas emissions (GHGs, including CO_2, CH_4, and others) (see also chapter 1 this volume) can be avoided using biofuels is related to the amount and carbon intensity of the fossil fuel inputs needed to produce the biofuel, as well as to which fossil fuel is substituted by use of the biofuel.

However, typically some fossil fuel is consumed in the course of producing or converting the biomass or delivering the biofuels to the point of use, resulting in net positive GHG emissions on a life cycle basis. These emissions will offset to some degree the emissions that are avoided when the biofuel is used in place of a fossil fuel (Larson 2006). There is a rich literature on GHG life cycle analyses (LCAs) of biofuels (Larson 2006). Most published LCAs have been undertaken in a European or North American context, with an excellent study of Brazilian sugarcane ethanol being an exception (Macedo et al. 2004). There is considerable context-specific variability and uncertainty around input parameter values in LCA analysis, which may explain the wide-ranging results from different studies for the similar biofuel and biomass source. The estimated range in reductions of GHG emissions per vehicle kilometer (v-km) for rape methyl ester (RME) compared to conventional diesel fuel (for which RME can substitute) is 16% on the low end and 63 % on the high end—a range of a factor of four. The range in reduction indicated for SME (soy methyl ester) is 45–75%. The range for ethanol from sugar beets is somewhat smaller (but complicated by three alternative sets of assumptions about how GHG emission credits are assigned to the residual pulp coproduct of ethanol production). Ethanol from wheat shows anywhere from a 38% GHG emissions benefit to a 10 % penalty relative to gasoline. Understanding such diversity in LCA results require examining details of each analysis, including analytical boundaries, numerical input assumptions, and calculation methodologies. However, even without delving into that level of detail, it is possible to draw a few firm conclusions (Larson 2006).

Future improvements in cellulosic ethanol production are expected to eliminate the conversion efficiency advantage currently enjoyed by corn ethanol: yields from lignocellulose of 340 L/ton are projected for 2010 (Sheehan et al. 2004) and 437 L/ton for 2030 (Greene 2004). Technology for production of Fischer–Tropsch fuels from lignocellulose (which could be commercially ready in the 2010/2015 timeframe) can yield some 280 L of diesel equivalent (Larson et al. 2006), which corresponds to 471 L of ethanol equivalent.

Proposals have also been made for thermochemically coprocessing coal and biomass to make carbon-neutral liquid fuels by capturing and storing some CO_2 produced during the conversion process (Williams et al. 2006).

Finally, it is worth noting that biomass can be converted into heat or electricity as well as into liquid fuel. GHG emissions per unit land area that are avoided in this way may be greater than when making liquid fuel. However, for electricity or heat production, a variety of renewable resources are available (hydro, solar, geothermal, wind, etc.). Biomass is the only renewable source of carbon, for producing carbon-bearing liquid fuels (Wescott 2007).

2.9 Economics

With the exception of ethanol from sugarcane in Brazil, production costs of essentially all first-generation biofuels in all countries are subsidized. In most countries, including Brazil, demand is driven by regulatory mandates (Marrison and Larson 1995). The Brazilian ethanol industry has evolved since its inception in the 1970s to

be able to produce ethanol that is competitive with gasoline at oil prices much lower than today's levels. In contrast, even the most efficient producers of ethanol (outside Brazil) are not able to compete without subsidy unless oil prices are above the $50–$70 per barrel price range (Assis et al. 2007). The relatively high cost of the edible crops used as feedstocks for first-generation biofuels accounts for the high production costs outside of Brazil. In some cases, as with corn in the United States, prices for the feedstocks have increased dramatically recently because of the rapidly growing demand for feedstocks for biofuels production (Wyman et al. 1993). The US Department of Agriculture expects corn prices to continue rising through the end of this decade and acreage devoted to corn planting to reach unprecedented levels. Such market impacts highlight sharply the food vs. fuel issue associated with first-generation biofuels (United Nation Conference 2008).

2.10 Recent Advances in Biofuel Production

2.10.1 Biofuel from Microbes

Recent advances have shown that some microbial species such as yeast, fungi, and microalgae can be used as potential sources for biodiesel as they can biosynthesize and store large amounts of fatty acids in their biomass (Xiong et al. 2008). Therefore, these organisms can be used as a promising strain for microbial oil production. Zhu et al. (2008) have worked on production of microbial biofuel from waste molasses and have reported that lipids produced in microbial biomass can be utilized for biodiesel production.

2.10.2 Biofuel from Algae

Algae are recognized as one of the oldest life forms and are present in all existing earth ecosystems, representing a large variety of species living in a wide range of environmental conditions (Mata et al. 2010). Under natural growth conditions, phototrophic algae absorb sunlight and assimilate carbon dioxide from the air and nutrients from aquatic habitats (Brennan and Owende 2010).

2.11 Market Scenario of Biofuel in India

Biofuels are going to play an extremely important role in meeting India's energy needs (see also chapter 9 this volume). The country's energy demand is expected to grow at an annual rate of 4.8% over the next couple of decades (Planning Commission). Most of the energy requirements are currently satisfied by fossil fuels—coal, petroleum-based products, and natural gas. Domestic production of crude oil can only fulfill 25–30% of national consumption (Thurmond 2008). In fact, the crude oil imports are expected to a total of 147 million tonnes in

2006–2007. With the ever-escalating crude oil prices, if one assumes a price of $57/barrel ($420/tonne), the estimated crude oil import bill for 2006–2007 would be $61.74 billion, about 10% of the country's gross domestic produce (GDP).

Ethanol, currently produced in India by the fermentation of sugarcane molasses, is an excellent biofuel and can be blended with petrol. Likewise, biodiesel which can be manufactured by the transesterification of vegetable oil can be blended with diesel to reduce the consumption of diesel. Ethanol and biodiesel are gaining acceptance worldwide as good substitutes for oil in the transportation sector. Brazil uses pure ethanol in about 20% of their vehicles and a 22–26% ethanol–petrol blend in the rest of their vehicles. The United States and Australia use 10% ethanol blend. With a normal production rate of 1900 million L a year, India is the world's fourth largest producer of ethanol after Brazil, the United States, and China. Beginning 1st January 2003, the GOI mandated the use of a 5% ethanol blend in petrol sold in nine sugarcane-producing states. The development of biofuels is at a nascent stage in India but is being actively pursued to reduce dependence on oil imports. Favorable government policies and initiatives are driving the ethanol and biodiesel production in India. Indian oil majors and big corporate houses are foraying into the biofuel industry to cash on the untapped potential (UNCTAD 2005).

2.11.1 Current Scenario

India is one of the world's leading producers of sugarcane and sugar. Sugar molasses, a by-product of the sugar industry, is used for production of most of the rectified spirits (alcohol) produced in India including ethanol for fuel. Due to the cyclical nature of sugarcane and sugar production, sugarcane farmers and the sugar industry experience periodic market gluts of sugarcane, sugar, and molasses production which reflects in prices and farm incomes.

The Government of India (GOI) has been focusing on encouraging sugarcane juice/sugar molasses usage for ethanol production to bring stability in farm incomes. The commercial production and marketing of ethanol-blended gasoline started in January 2003, the first phase of the ethanol-blended petrol (EBP) program that mandated blending of 5% ethanol in gasoline in nine states and four union territories (UT). With a strong resurgence in sugarcane/sugar production in 2006–2007, the GOI announced the second phase of the EBP program in September 2006 that mandated 5% blending of ethanol with petrol (gasoline) subject to commercial viability in 20 states and 8 UT.

The GOI had initially planned to launch the third stage of the EBP from 1 October 2008 wherein (i) the ethanol blend ratio was to be raised from 5% to 10% and (ii) 5% blending was to be made mandatory across the country in all states. However, due to the short supply of sugarcane and sugar molasses in 2008–2009 and forecast short supplies in 2009–2010, the government has deferred the proposed implementation of the third phase of the EBP.

The GOI has developed an ambitious national biodiesel mission to meet 20% of the country's diesel requirements by 2011–2012. Since the demand for edible

vegetable oil exceeds supply, the government decided to use nonedible oil from *Jatropha curcas* oilseeds as biodiesel feedstock (Planning Commission 2003).

The current manufacturing cost of ethanol and biodiesel in India is about Rs. 27/L ($0.56/L), roughly the same as petrol and diesel. This puts biofuels in a favorable position for meeting India's energy needs, especially as the cost of petroleum is expected to continue its upward trend. In addition to provide energy security and a decreased dependence on oil imports, biofuels offer several significant benefits such as reduced emission of pollutants and greenhouse gases and increased employment in the agricultural sector.

It was clearly projected that the country has already shown the capability of producing ethanol for 10% blending in 2006–2007 through sugarcane alone. Another peculiarity of ethanol production in India is by and large based on by-product utilization (molasses). The area of sugarcane cultivation has come down in the recent past due to stagnation of sugarcane prices which led to shortfall in ethanol production in 2008–2009. It happened due to dichotomy between sugar and ethanol production and market price. Unless the government initiates sugarcane production stabilizing measures or petroleum companies agree to link ethanol prices with raw material prices, the EBP will be successful only during excess sugar production seasons. The implementation of EBP will not be successful in the near future (Murali and Hari 2011).

2.12 Biofuels in India

2.12.1 Commercial Initiatives for Biofuels

Large number of small and medium private enterprises have invested in plantations as well as commercial production of biodiesel; however, the market for biodiesel has not yet emerged on a commercial scale. In October 2005, the Ministry of Petroleum and Natural Gas initiated a biodiesel purchase policy with effect from January 2006. According to the policy, oil marketing companies are to purchase biodiesel in 20 purchase centers in 12 states (DIE 2008). As per the government notification, biodiesel has been completely exempted from the excise duty. The Global Exchange for Social Investment (GEXSI) (2008) has conducted a detailed survey of status of Indian *Jatropha* plantations. They report that *Jatropha* plantations in India fall into one of three types of ownership: private, public, and public–private partnerships with 31%, 31%, and 38%, respectively. The total area under plantation is estimated to be of 497,881 ha of which 84,000 ha is in Chhattisgarh, 33,000 ha is in Rajasthan, 20,277 ha is in Tamil Nadu, 16,715 ha is in Andhra Pradesh, 350 acres is in Uttaranchal, and 328 ha is in Haryana. Most of these crops are grown in nonirrigated land, and 60% are planted in wastelands. It is also projected that India will have 1,179,760 ha of crop in 2010 and 1,861,833 ha in 2015. German development institute (GTZ) confirmed that the biodiesel sector in India is different from elsewhere in the world (DIE 2008). Biodiesel production is restricted to nonedible oil plants and not related to the price increase of edible oils. The focus

is on non-intensive agricultural lands minimizing the competition between fuel and food. Biodiesel activity in rural areas can improve the food security as it provides additional income opportunities to the rural poor. The report also indicated that the biodiesel program addresses the five important development challenges such as energy supply, reduction of carbon dioxide emission, rural employment, rural energy securities, and protection of natural resources (Gopinathan and Sudhakaran 2009).

2.12.2 State Initiatives for Biofuels

Many states have initiated biodiesel programs based on the central policy directives or their own. Two hundred districts in 19 potential states have been identified on the basis of availability of wasteland, rural poverty ratio, below poverty line census, and agroclimatic conditions suitable for *Jatropha* cultivation over a period of 3 years. Each district is planned to be treated as a block, and under each block, a 15,000 ha *Jatropha* plantation is planned to be undertaken through farmers (DIE 2008; GOI 2003). However some bottlencks have to be overcome (see also chapter 12 this volume).

2.12.3 Government Policies for Biofuel Plantation in Rajasthan (India)

In exercise of the powers conferred by subsection (2) of section 22 of the Rajasthan Imposition of Ceiling on Agricultural Holding Act, 1973, the state government being of the opinion that it is necessary to do so for the public purpose hereby exempt the wastelands to be allotted for biofuel plantation and biofuel industrial and processing unit in the state from the operation of the said act. In exercise of the powers conferred by subsection (2) section 261 of the Rajasthan Land Revenue Act, 1956 (Rajasthan Act No. 15 of 1956) read with section 101 and 102 of the said Act, the state government hereby makes the following rules, namely, Waste Land Allotment rules (2007).

2.12.3.1 Short Title Extent and Commencement

1. These rules may be called the Rajasthan Land Revenue (allotment of waste land for biofuel plantation and biofuel-based industrial and processing unit) Rules, 2007.
2. They shall extend to the whole of the state of Rajasthan.
3. They shall come into force at once.
4. Words and expressions, not defined in these rules but defined in the Act, shall wherever used in these rules be construed to have the same meanings as assigned to them in the Act.

2.12.3.2 Purpose and Eligibility of Land Allotment

1. Land for biofuel plantation and for biofuel-based industry and processing unit under these rules may be allotted to:
   (a) Self-help group of BPL families
   (b) Village forest security and management committee
   (c) Gram panchayats
   (d) Agriculture cooperative societies
   (e) Societies
   (f) Government undertakings
   (g) Companies
2. Maximum of 30% of total wasteland available in a district may be allotted to the government undertakings and companies, and preference will be given to those government undertakings and companies which undertake to plant Ratanjot, Karanj, and other similar biofuel plants and to establish processing units, refineries, composite units, value addition of such biofuel plants and processing, and nursery for high-quality plants and seeds including research and development and to employ at least 50% of unskilled labor from local areas.
3. The remaining land shall be allotted to the other categories of sub-rule (1), and preference will be given to self-help groups of BPL families among other categories.

2.12.3.3 Terms and Conditions of Allotment of Land

The allotment of land under these rules shall be made on the following conditions:

(a) Land allotted under these rules shall be used only for the purpose for which it is allotted. However, the allottees may utilize 2% of the allotted area or 10 ha of land whichever is less for storage of raw material, storage of finished goods, labor quarters, and factory shed.
(b) The allottee shall have to utilize 50% of the land for plantation within 2 years from the date on which possession was handed over, and the balance shall have to be utilized for plantation within the next 1 year; otherwise, the allotment shall deemed to have been canceled automatically.
(c) The allottee shall be liable to make payment of all taxes, which may be leviable under the appropriate laws.
(d) The allottee shall abide by all the terms and conditions of these rules and other applicable laws as amended from time to time.
(e) The allottee shall give preference to the local residents of the area in employment.
(f) The allottee shall use the allotted land himself and shall not transfer/sublease the land.
(g) It shall be compulsory to adopt micro-irrigation management system as per latest technology.
(h) The allottee shall not make any construction of permanent nature without obtaining prior approval of the allotting authority.

(i) Allottees other than companies shall sell the produce to the company situated in that zone at the minimum support price as fixed by the biofuel authority.
(j) The company shall purchase the produce from the other allottees situated in the zone at the minimum support price fixed by the biofuel authority (Waste Land Allotment rules 2007).

2.13 Discussion

The biofuel debate has moved beyond discussion of energy security, less reliance on fossil fuels, and the production of more environmentally responsible energy sources. It is now a much broader debate, and within that debate, the central questions are about what is deemed to be responsible land use. Thus, bioenergy was considered as a promising option alongside other renewable energy sources, such as solar, wind energy, and hydropower (International Energy Agency (IDEA) http://www.iea.org/subjectqueries/index.asp). The arguments presented for biofuels highlighted the benefits of reducing dependence on fossil fuels and the countries producing them and therefore in this context the development and use of bioenergy.

However, biofuel production has itself become one of the major issues in an increasingly fierce debate over climate change and global food security.

Bioenergy, particularly biofuels, sustainability is a key aspect of the future energy development. The use of rapeseed biodiesel represents a good opportunity for the achievement of the European goals in terms of GHG emission reductions, considering a saving of emissions, measured in carbon dioxide (CO_2) equivalents, of 56 % with respect to conventional diesel (Finco 2012). However, this result does not take into account the negative environmental impacts caused by land-use changes (direct and indirect), which would lead to GHG emissions, in turn resulting in a decrease in the percentage of the estimated saving (Finco 2012). The land-use changes and intensification of cultivation following the increased demand for biofuels may cause new GHG emissions and affect the biodiversity, the soil quality, and the natural resources (Finco 2012; Perimenis et al. 2011). Moreover, Howard et al. (2009) affirmed that the first-generation biofuels are inefficient both in terms of economy and the environment.

Subsidizing biofuels and bioenergy with the aim of reducing GHG emissions is a less effective and costlier way of achieving this goal than many other more cost-effective solutions, such as improving the energy efficiency and conservation or encouraging more effective renewable energy options where feasible. The structure of the existing support (sum of biofuel subsidies and farm payments) will not only continue to be significant but is likely to rise over time (Steenblik 2007). Moreover, taxpayer costs of biofuel and renewable energy policies in general are very high, especially relative to their benefit, which can easily be negative (de Gorter and Just 2010).

The feedstocks are cultivated as intensive monocultures (as is the case for many important food crops), where the conversion of extensive agricultural systems and natural habitats such as grasslands into intensive monocultures is one of the major

threats to biodiversity; nonnative feedstocks are also potentially invasive and may have negative impacts on ecosystems; ecosystem services such as soil regeneration, carbon sequestration, natural chemical cycles, pollination, and protection against flood may be affected (Haines-Young and Potschin 2010).

2.14 Conclusions

The consideration of any process for the biotransformation in the production of biofuels needs critical evaluation. Despite so many obvious advantages, still no large-scale production facilities have been established using biotransformation of lignocellulosic materials. This is one of the main issues to focus in future development for biofuel production. Bioethanol is currently being produced from sugarcane and starch-containing substrates. Although there are similarities in between the lignocellulosic and the starch process, the technical and economical challenges in bioconversion of lignocellulosic substrates are large. The conflict between food and fuel needs further study to find a common pathway to avoid it. This includes biofuel production that utilizes wastes, is flexible in its feedstock requirements, and/or uses technology or process synergy, hybrids, or feedstock fractionation to ensure affordable renewable energy access that does not compromise, but rather compliments, the food production.

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Perspective of Biofuel Production from Different Sources

3

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Abstract

Biofuel is an emerging agri-industry that has the potential to change the agricultural economics of both developed, as well as developing, and underdeveloped nations in the not so distant future. Biofuels are regularly replacing fossil fuels to generate power, heat, and chemicals. The extensive use of biofuels in the future for energy generation is of great interest since it has the potential to reduce the concentration of greenhouse gases in the atmosphere and could serve as an important step toward establishing energy independence and open new

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employment opportunities globally. It is therefore important to understand the different sources of biofuel productions for long-term and sustainable use depending upon the nature and energy dynamics of the regional demands for biofuel. This review investigates the sustainability of biofuel production from different sources and their role in energy generation.

Keywords
Biofuels • Biomass • Environment • Feedstock • Fossil fuels • Renewable resources

3.1 Introduction

Nearly more than one-third of total global commercial energy used belongs to worldwide agricultural residuals and also the forest product industry (Demirbas 2009a). Today, bioenergy contributes about 10% of the global energy demand (International Energy Agency (IEA) 2014). Thus, the bioenergy resources are divided into two main groups:

- Dedicated energy crops plantations
- Organic food municipal residuals and waste materials

These biomass items can be converted into modern energy transporters, i.e., methanol, ethanol, electricity, or hydrogen (Azar et al. 2003). Fuel type choice in the forwarding sector greatly depends on the accessibility of biomass resources.

With the increase of population and expansion of cities, the need for fuel and energy has increased annually. Due to rapid reduction of natural energy resources like fossil fuels, the studies for finding potential alternative energy sources have started long back all across the globe. The availability of large amount of oily wastes in nutritional industries and factories, waste cooking oil in restaurants, the existence of large amount of cellulose wastes from lump sugar production and rice factories, and the possibility of economic use for them in producing clean fuel have encouraged researchers to produce new and alternative energy sources from these waste products. Using biotechnology to produce different enzymes from different microorganisms, instead of chemical catalysts, is one innovative approach in modern biofuel production (Hama et al. 2013). Biofuels include bio-hydrogen, methanol, DME (dimethyl ether), biodiesel, ethanol, and bio-oil (Hamelinck and Faaij 2006).

H_2 (hydrogen) can be obtained by using plant material or agricultural by-products through two techniques (Ardehali 2006; Demirbas 2009b): (a) partial oxidation process (gasification) followed by reformation of synthesis gas (syngas) and (b) fast pyrolysis after reformation of carbohydrate existing in the bio-oil. In either process, the shift reaction of gas-water for converting gas into H_2 is applied. In the process

of gas making with high-pressure water-steam flow, biomass is converted into a mixture of combustive gases with little oxidation of biomass in high temperature ranging from 1073.2 to 1173.2 K (Goyal et al. 2007). Fischer-Tropsch synthesis (FTS) process is also used for producing chemical materials, gasoline, and diesel fuel. They are often hydrocarbons with linear chain and so they produce high-quality diesel fuel. On the other hand, pure syngases are used in this process that cause products free from N (nitrogen) and S (sulfur) (Ardehali 2006). Through hydrolysis, carbohydrates such as cellulose, hemicellulose, etc. can be transformed into sugar and thereby used as energy fuel resource downstream.

Fermentation is an energy-releasing, microbial reaction that causes anaerobic decomposition of carbohydrates (sugars and starches) into organic compounds such as alcohol. Hydrolysis breaks down the hydrogen bands in carbohydrates into sugar compounds with 5- and 6-carbon, and finally these sugars are converted to bioethanol (Ardehali 2006; Demirbas 2009b). Methanol ($2H_2 + CO \rightarrow CH_3OH$) is produced by syngas or biogas. Transformation of coal, biomass, and natural gas into H_2 is less expensive and much more energy efficient than their reformation to methanol (Azar et al. 2003). Since the process is too expensive, hence, the utilization of biomass wastes like old wood or biological wastes (as alternative feedstock) is currently of considerable interest among researchers (Ardehali 2006).

Dimethyl ether (DME) can also be considered as an alternative for fossil fuels. It can be compared to ordinary fuels like methane, ethanol, methanol, biofuels, and hydrogen and Fischer-Tropsch (FT) fuels. DME is commercially manufactured in two steps in which syngas (obtained usually from steam-methane reforming) is converted to methanol first and then to DME after dehydration (Semelsberger et al. 2006). This review investigates the sustainability of biofuel production from different sources and their role in energy generation.

3.2 Potential of Biofuels Application

The superiority of using oil fuels remained until the Organization of the Petroleum Exporting Countries (OPEC) announced the oil crisis in 1970. Since then countries started looking for viable and alternative energy resources in the transportation section. Although it will possibly take quite a long time for biofuels to replace the current fossil fuel-based oil economy, they have demonstrated great potential in several regions of the globe as an alternative fuel resources for the future (Loppacher and Ker 2005; Lichts 2013). According to the envisioned roadmap for fuel production by 2050, 27% of the global transport fuel is devoted to biofuels in the future (Fig. 4.1). It has now been projected that the lion's share of biofuel use will increase until 2050 (International Energy Agency (IEA) 2011). Renewable resources are spreading around the world more consistently than fossil or even nuclear fuels, and the amount of energy from these sources is three times as much as the current world consumption (Demirbas 2009b). The biodiesel production trend in European Union has been presented in Fig. 4.2.

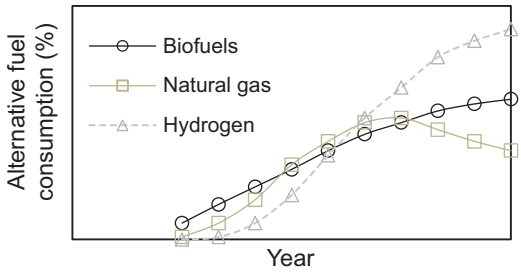

Fig. 4.1 Showing the futuristic view of the percentage of alternative fuels used according to total fuel consumption in vehicles in the word (Adeeb 2004; Çeper 2012)

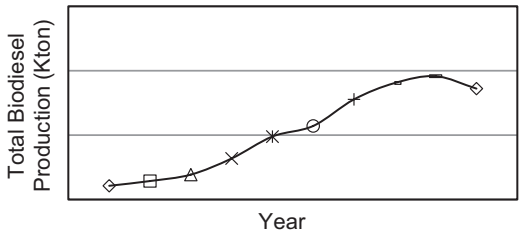

Fig. 4.2 Biodiesel production in the European Union (2002–2011) (European Biodiesel Board (EBB) 2014)

3.3 Bio-Oil

Bio-oil fuels (in liquid or gaseous forms) are commonly produced bio- or thermo-chemically, from different biomass feedstock, including agricultural crops, forest and agricultural by-products, and urban solid wastes (Demirbas 2007). It has been projected that such biofuel sources have the potential to replace conventional fuel sources in the future either totally or partly in the not so distant future (European Commission (EC) 2004). When the organic compounds from biomass sources such as animal wastes, sewage sludge, and industrial effluents are broken down via anaerobic digestion into a mixture of carbon dioxide (CO_2) and methane (CH_4), it is known as biogas production (Demirbas 2004a). Important properties of such fuel have been reported to be cheap, clean, versatile, and environment friendly (Kapdi et al. 2005). Breaking down complex molecules into smaller molecules with the aid of thermal energy is defined as cracking or pyrolysis. The H_2 molecules can be generated from wood-type biomass (Demirbas 2009a). Moreover, H_2 can also be produced via thermal process generated by gasification or pyrolysis. The major gaseous products of biomass are (a) H_2+CO_2+CO+gaseous and liquid hydrocarbons by pyrolysis, (b) H_2+CO_2+CO by catalytic steam reforming, and (c) gaseous and liquid hydrocarbons by FTS over H_2+CO complex (Babu and Chaurasia 2004). The products of interest in association with conventional and fast pyrolysis of biomass are high charcoal content and tar at low temperature (Di Blasi 2008; Bruchmüller et al. 2012) or gas at high temperature (Bridgwater 2003; Demirbas 2009b), respectively.

3.4 Biomethanol

Methanol which is also known as wood alcohol can also be used like conventional motor fuels. It was used in the past mostly in automobiles when inexpensive gasoline is not easily available. Its application as motor fuel drew attentions in the period of oil crises in the 1970s for being low cost and easily available. In recent years, an increased demand has been seen in the direct application of methanol in the Otto-powering engines or in vehicles containing fuel cells (Chmielniak and Sciazko 2003). Biomethanol production facilities comprise of (a) catalytic synthesis of $CO+H_2$, (b) liquid distribution from wood pyrolysis, (c) biomass gasification and gaseous products, and (d) syngas from coal and biomass (Demirbas 2007). Presently, the sustainable approaches in making methanol are practically no more stable and economic than other fuels (Demirbas 2009b). Methanol is manufactured by biogas or syngas (H_2/CO = 2:1 ratio) process and aptly supplies the required fuels in engines possessing internal combustion (Demirbas 2007). However, the trend is of cost-bearing process; hence, nowadays, exclusively waste biomass is used for such production (Vasudevan et al. 2005).

Biomass is a renewable resource which in turn demonstrates a possibly interminable feedstock supply basis for biomethanol synthesis. The bio-syngas compositions, required for methanol production, include H_2, carbon monoxide (CO), CO_2, CH_4, and ethene (C_2H_4). Renewable methanol can compete economically with natural gas feedstock yearlong, being easily available and cheaper in prices. The synthesis of methanol with natural gas feed is in progress by means of available technologies and needs to be improved and scaled up efficiently and economically in the future (Demirbas 2009a). Supercritical water gasification (SCWG) is a practical trend in which the ratio of biomass conversion to gas under high moisture content (100%) and the H_2 volumetric relativity (50%) is high (Demirbas 2004b; Matsumura and Minowa 2004). In terms of environmental benefits, the trend appears to be more efficient with respect to fuel reforming from fossil sources. Thermochemical conversion of biomass materials is supposed to be a considered technique having momentous, economic, great-scale, and unceasing H_2 renewability properties (Demirbas 2005, 2009b). The concomitant generation of biomethanol, gained via hydrogenation of CO_2 (generated within sugar juice fermentation) in line with bioethanol production, looks economically efficient in places with high availability of hydroelectricity at very low cost and where excess amount of lignocellulosic residues is cheaply and easily available.

3.5 Bioethanol

Hemicellulose and cellulose compounds or carbohydrates, in herbal substances, have the ability to generate sugars via hydrolysis. After the conversion of hydrocarbons to sugars, they are next converted to alcohol (i.e., ethanol) in an anaerobic biological process (fermentation) caused usually by the action of yeast. The efficiency of such conversion processes is probably related to the range of pretreatment

items: chemical and mechanical parameters. In addition, type of wood, method of pulping, and use of recycled products like paper and pulp could also affect availability of cellulase enzymes from the cellulose substrates (Adeeb 2004; Demirbas 2006a). The presence of hemicelluloses or noncellulosic substances (i.e., xylanase, arabanes, mannanase, and galactans) in the plant membrane appears in short-chain molecules rather than long-chain ones (i.e., cellulose) and is thereby readily exposed to thermal decomposition.

Bioethanol is an additive or substitute fuel derived from different feedstock resources like *Beta vulgaris* L., *Triticum* spp., and *Zea mays* L., agricultural by-products (e.g., straw) and wood-based materials. Homemade oil wastes have the potential to serve as base material for low-cost bioethanol production too. The highest and lowest record for ethanol production among different continents, up to now, belongs to America (77 billion liters per year, 90% of global output) and Oceania (0.2 billion liters per year), respectively (Renewable Fuels Associations (RFA) 2014). Bioethanol can also be generated by extended types of carbohydrates. Sucrose fermentation is chemically processed using yeast (*Saccharomyces cerevisiae* Meyen ex E.C. Hansen). Ethanol production from sucrose is often facilitated by the action of two catalyzed enzymes; invertase and zymase in a two-stage reaction (Du et al. 2004; Demirbas 2006a).

The corresponding enzyme for D-glucose production from starch is glucoamylase. D-Glucose is then fermented, distilled, and dehydrated to finally form dewatered bioethanol. Globally available, dominant feedstock in starch-to-bioethanol industry belongs to corn (*Zea mays* L.) by more than 60% starch. Lignocellulosic materials possessing carbohydrates are confronted by a series of reactions by which bioethanol is manufactured from their carbohydrate base such as removal of polymer lignin from plant structures that deals with delignification process, steam-exploded biomass (lignocellulosic substances), pre-hydrolysis of wood material with dilute sulfuric acid, enzymatic hydrolysis, and fermentation (Kim and Dale 2005). Through the hydrolysis process over the cellulose/hemicellulose fractions, the hydrogen bonds in such carbohydrates are broken down into their corresponding sugar parts: pentoses and hexoses (Kim and Dale 2005).

3.6 Biodiesel

The idea that biodiesels can be produced from herbal oils was first proposed by prominent researchers like Bala (2005) and Demirbas (2009a). Initially, biodiesels were too expensive compared to the conventional fossil fuels. However, uncertainties in fossil fuel availability and recent events of price hikes have rejuvenated the interest in biodiesel, which is a low-pollutant, renewable fuel compared to conventional diesels (Ghadge and Raheman 2006). Biodiesel or fatty acid methyl esters (FAMEs) are mono-alkyl esters produced by a reaction between renewable feedstocks such as herbal oils, animal fats, and algae with an alcohol like ethanol or methanol with active participation of an acid/base catalyst (Canakci and Sanli 2008; Demirbas 2009b). In other words, herbals oils have the potential to be considered as

a fuel in diesel sector after being transesterified (Demirbas 2007). Soybean oil, alkaline catalyst, and methanol are currently the main sources of biodiesel production (Demirbas 2009b). One of many resources for producing clean-burning fuel is edible herbal oil obtained from oily seeds like corn, soybean, sunflower, etc. (Meher et al. 2006). The main obstacle in the commercial production of biodiesel is related to its high manufacturing cost from crude herbal oils. Hence, waste cooking oil (WCO) has become an encouraging alternative resource in the biodiesel sector. WCO is reasonably low-priced than crude herbal oils from sunflower, soybean, or canola and is usually discarded or used as animal feed (Kulkarni and Dalai 2006).

Biodiesel is generated through four primary pathways: direct mixing of herbal oils and diesel (direct-blending); stable and isotropic liquid blending of water, oil, and surfactant (microemulsions); pyrolysis (thermal cracking); and reaction of one-unit triglyceride (oil) with an alcohol promoted by a catalyst (TP, transesterification process) (Demirbas 2009b). Among all these approaches, TP is considered as the best alternative due to its low cost and is well-known for conversion of fats or oils into FAMEs by the action of lipase or acid/base catalysts (Bisen et al. 2010). Methyl ester yielded by TP is highly affected by important variables like reaction temperature and the alcohol/herbal oil molar ratio. To avoid saponification, the TP process requires two prerequisite factors: free fatty acid (FFA) content in lower levels and higher-quality herbal oil (Yu et al. 2013). Usually, saponification causes some difficulties in glycerol (by-products) separation and low biodiesel conversion. Until now there are little studies reported on non-catalytic TP under supercritical methanol (SCM) procedure (Demirbas 2003, 2009b). Newly developed process of biodiesel production includes a non-catalytic SCM method. The advantages of SCM method in comparison to catalytic processes exposed by barometric pressure are that the former is non-catalytic, is simpler to use in the purification of products, requires shorter time for reaction, uses less energy, and is more eco-friendly (Demirbas 2003, 2009a).

3.7 Bio-Hydrogen

It is produced from a mixture of natural constituents like lignin, cellulose, and hemicellulose and several other accessory contents from extracts representing different biomass materials. Such constituents can be pyrolyzed at various amounts and by fundamentally different pathways and processes. They can also be converted to energy by two mechanisms—biological and thermochemical. In the biomass conversion process, the carbon becomes less reactive forming a stable chemical structure followed by an increase in the activation energy (Demirbas 2009b). Pyrolysis from biomass gasification usually happens at higher temperatures and yields a mixture of different gases containing 6–6.5% H_2 (Bridgwater 2003). The production of H_2 from biomass sources can take place with two thermochemical pathways (Demirbas 2009c).

3.7.1 Steam-Reforming Trajectory

In this process, commonly regarded as syngas, hydrocarbons in the feedstock undergo reaction with steam to finally produce H_2 and CO. The process includes various solid waste materials such as paper mill sludge, refuse-derived fuel, black liquor, sewage sludge, waste oil, cooked oil, municipal organic waste, as well as agricultural wastes. Bulk H_2 production from steam reforming of natural gas is a frequently used approach and sometimes defined as steam CH_4 reforming. It is important to note that multiple catalytic reaction phases need to be applied in the process of producing H_2 from waste-originated carbonaceous solid materials. This can be achieved for generating high-purity H_2 in the long run by following the reforming procedure with two important WSG reaction steps, including purification of CO and subsequent removal of CO_2 (Demirbas 2007, 2009b).

3.7.1.1 Stepwise Steam Reforming of CH_4

So far producing a CO-free hydrogen through stepwise steam reforming of CH_4 at different process conditions has been once investigated (Demirbas 2009c). Stepwise steam reforming of CH_4 comprises two consecutive steps: (a) the CH_4 degradation to CO-free hydrogen and surface carbon and (b) steam gasification of surface carbon.

3.7.1.2 Supercritical Water Gasification (SCWG)

The superiority of SCWG technique to the other biomass thermochemical gasification (i.e., steam gasification, air gasification) is mainly for its higher efficiency of gasification at lower temperatures and for direct dealing with wet biomass without drying. Nonetheless, the present expense for H_2 production from steam-CH_4 reforming is much less than production by SCWG of wet biomass (Demirbas 2007). Gasification of biomass feedstock situated in supercritical water and exposed to a set of pressure and temperature at different times of reaction, thereby generating a mixture of different gases (CO, CO_2, H_2, CH_4), and to some extent, C_2H_6 (Ethane) and C_2H_4 (Demirbas 2004b, 2009c).

3.7.1.3 Supercritical Fluid Extraction

Supercritical fluid extraction (SCFE), steam gasification, and slow pyrolysis at different temperatures are major methods in the yield of H_2. The critical temperature for pure water, applied in the SCFE, is set at 647.7 K. It is understood that at lower temperatures, the yield of H_2 from SCFE is remarkably high (~50%). In addition, pyrolysis and steam gasification were performed using moderate and high temperatures, respectively (Demirbas 2006b).

3.7.2 Production of Bio-Syngas Fuels by Fischer-Tropsch Synthesis (FTS)

The synthesis of hydrocarbons, in their liquid forms based on coal-made syngas via FTS approach, has recently generated attention for converting coal-to-liquid and/or gas-to-liquid fuels (Demirbas 2009c). Continuing worries about enhanced global energy demands by putting pressure on oil supplies have brought about the application of FTS in making transportation fuels and other chemicals via synthesis (Demirbas 2009b). As a prominent strategy for biofuel production from biomass resources, the synthesis of hydrocarbons from biologically derived syngas has been reported (Demirbas 2006b), in a way that it can possibly yield carbon neutral, liquid, and transportation fuels (Pöhlmann and Jess 2015). The predominant constituents of bio-syngas are CO_2, CO, CH_4, and H_2. To promote efficiency of carbon sources, biomass syngas steam reforming with supplementary natural gas feedstock can prove to be quite useful (Demirbas 2009c).

3.7.2.1 Catalyst Function in FTS Process

The most important parameters determining the diversity of products are composition of the feed gas, temperature, nature of catalyst used, pressure, and promoters (Jahangiri et al. 2014). Of all the available catalysts, the iron-based ones are more attractive due to highly activated FTS and for the low-cost WGS reactivity (Wu et al. 2004). This feature can easily compensate the H_2 deficiency in the syngas process from coal gasification via newly released technology (Wu et al. 2004). Large amounts of LAOs are recommended to be produced in a fluidized bed FT reactors situated at high temperature with iron-based catalyst (Demirbas 2009b; Hu et al. 2012). The activity of the iron-based catalyst and the selectivity of products in the FTS method are influenced by Al_2O_3/SiO_2 ratio (Demirbas 2009b; Jahangiri et al. 2014).

3.7.2.2 FTS Products

Several FT products, such as gasoline, diesel fuel, and chemicals, are mostly linear hydrocarbons; thus, their quality (e.g., diesel fuels) is high (Demirbas 2009b). All the products from FTS using purified syngas do not contain any N/S (Hu et al. 2012) and are also low in aromatics. Hence, they have attracted much attention for being more environment friendly against those products with crude oil basis (Demirbas 2009b; Pöhlmann and Jess 2015). Syngas conversion, syngas generation, and hydroprocessing are three processing steps that directly correspond to gas-to-liquid (GTL) technology on the basis of FTS (Demirbas 2009b). At present, the FT process is commercially aimed at producing linear alpha olefins (LAOs) and liquefied petroleum gas (LPG) like gasoline, diesel, and kerosene (Jahangiri et al. 2014).

3.8 Conclusion

Biofuels constitute an important field of research that holds great promises for the future. Biofuel opportunities are being steadily hunted and investigated all across the planet in the research laboratories of developed as well as developing and underdeveloped countries based on the local natural resources and technology alternatives available. Although biofuels have the potential to transform into a completely independent alternative fuel source, however, as discussed in this review, they have their own unique challenges too to overcome. Like any other developing field of research, this realm also suffers from the occasional excitements and frustrations, moments of utter joy and cruel failures in terms of success and achievements, and scientific and technological triumphs. The past decade has seen a grand momentum with respect to biofuel research and related technology developments. We anticipate that the next two decades of biofuel research are going to be even more exciting but challenging and daunting at the same time.

Several new alternative pathways and reactions are expected to be explored, and new approaches, instrumentation, and technological surges are also expected. The future holds great promises for the biofuel research and technology development. However, we must all reasonably expect that since the success of biofuel is also closely tied with the economic developments and economic fluctuations in the not so distant future, we should be prepared to face some major glitches and obstacles regarding the successful commercialization and adoption of biofuel technology and production across the globe. The future success of biofuels turning into a viable alternative replacing fossil fuels will be surely dictated by our compulsions to protect our environment, governed by the existing economic parameters and directed by the accessibility to cheaper and affordable global biofuel technology.

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Potential Biomass for Biofuels from Wastelands

4

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Abstract

Biomass production for biofuels in wasteland offers an attractive proposition. India is situated between 8°4″ and 37°6″ N latitude and 68°7″ and 97°25″ E longitude. The total land area of India is approximately 329 mha, out of which 150 mha is uncultivable and around 90 mha is characterized as wasteland. Wastelands in India are scattered all over the country as in the Himalayas, the Western Ghats, the Eastern Ghats, hilly areas, and desert of Rajasthan. The broad subdivision of wastelands is categorized as degraded forest, overgrazed revenue wasteland, ravines, hilly slopes, eroded valleys, drought-stricken pastures, over-irrigated "usar" and "khar" soils (saline and alkaline), wind- and water-eroded lands, and waterlogged marshlands. Crop production in such types of soils is not economical at all. Even with productive soils, our crop yields are dependent on erratic and very poor seasonal monsoons. With the increasing population pressure there is an urgent need to trace some potential sources of biomass for sustainable utilization. During the present investigation, attempts were made to categorize some potential bioenergy sources in different habitats of arid and semiarid regions of Rajasthan. Our studies present alternative sources of energy to reduce the pressure on forest biomass for sustainable utilization.

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Keywords
Wasteland • Arid and semiarid • Biomass • Sustainable development • Rajasthan

4.1 Introduction

There is an urgent need to develop bioenergy resources in Rajasthan (Pal and Sharma 2001). Some of the plant species are listed in Tables 4.1, 4.2, 4.3, and 4.4 from the point of view of their suitability for different regions (Kotia and Kumar 2001a, b, c, d). Biodiversity characterization has been carried out in different parts of the world (Nilsson 1997). Biodiversity of Rajasthan and its bioenergy potential have been characterized by Roy and Kumar (1995) and Kumar et al. (1995). However, studies on characterization of plants appearing in wasteland during different stages of vegetation development are lacking (Kotia and Kumar 2000). The present investigations were undertaken with an objective to characterize plant species colonizing nonsaline wastelands.

The arid region of India lies between 24° and 29° N latitude and 70° and 76° E longitude and covers an area of 317,090 km^2 spread over the seven states (Rajasthan, Gujarat, Haryana, Maharashtra, Karnataka, Andhra Pradesh, and portions of Jammu and Kashmir) of the Indian Union. Wastelands in India are scattered all over the country in the Himalayas, the Western Ghats, the Eastern Ghats, hilly areas, and desert of Rajasthan. This indicates that each state has large areas of wasteland which have been categorized as saline and alkaline and wind- and water-eroded lands, and almost 60% of this lies in Rajasthan (Bhumbla and Khare 1984; Gupta and Kothari 1987; Gonzales 1996).

The Rajasthan state is located between 23°3′ and 30°12′ N latitude and 69°30′ and 78°17′ E longitude. Rajasthan is the largest state in India with a total land area of about 324,239 km^2, out of which about 196,150 km^2 is arid and the rest is semi-arid. A major part of Rajasthan in western area is desert and this area is broadly categorized as sandy plains and sand dunes. These sandy plains and sand dunes occupy an area of approximately 120,983 km^2. Forest covers only about 37,638 km^2, i.e., 11% of the total area. This forest includes roughly 7% of depleted and denuded forests, while the remaining area is fairly well stacked.

The Aravalli hills running over a length of 680 km divide the state into two parts, northwestern and southeastern occupying 60% and 40% area of the state, respectively. The southeastern part is comparatively productive and around 20% of the area is under forest cover. However, around 4.5 lakh ha is under ravines, which poses a continuous threat to the adjoining fields. As compared to the all India average value of 0.14 ha per capita forest area, Rajasthan has one of the lowest values (0.01 ha). In comparison to the world's average annual increment of forest of 2.1 m^3 per ha, India's average is only 0.5 m^3 per ha. The productivity of land in Rajasthan is low, even in comparison to that of the adjoining states of Gujarat, Madhya Pradesh, and Uttar Pradesh. The soils of the desert plains are loamy sand to loam, and the eastern part has alluvial soil which supports the forests and agricultural crop. The average annual rainfall in the state is 525–675 mm, and the annual precipitation in different tracts of Rajasthan varies from 13 to 1766 mm. Out of the total area, forests cover only about 37,638 km^2 and are rich in biodiversity. Rajasthan is rich in biodiversity which has a great economic value.

Table 4.1 Showing tree species of Rajasthan for biomass and other economic values

S. no	Plant species	Local name	Family	Economic value
1	*Acacia catechu* Willd.	Khair	Mimosaceae	Dye, fodder
2	*Acacia leucophloea* (Roxb.) Willd.	Rounj	Mimosaceae	Fiber, gum, fuelwood, fodder
3	*Acacia nilotica* (L.) Del.	Babul	Mimosaceae	Fiber, gum, fuelwood, fodder
4	*Acacia senegal* (L.) Willd.	Kumta	Mimosaceae	Gum, fuelwood, fodder
5	*Acacia tortilis* (Forsk.) Hayne.	Kikar	Mimosaceae	Gum, fuelwood, fodder
6	*Aegle marmelos* (L.) Correa	Beel	Rutaceae	Edible, medicinal
7	*Ailanthus excelsa* Roxb.	Ardu	Simaroubaceae	Fuelwood, fodder
8	*Albizia lebbeck* (L.) Willd.	Siras	Mimosaceae	Gum, fuelwood, fodder
9	*Anogeissus latifolia* Wall.	Dhawra	Combretaceae	Gum, fuelwood, fodder, medicinal
10	*Anogeissus pendula* Edgew. (Plate 4.3b)	Dhokra/dhok	Combretaceae	Gum, fuelwood, fodder
11	*Azadirachta indica* A. Juss.	Neem	Meliaceae	Medicinal, nonedible oil
12	*Balanites aegyptiaca* (L.) Delile.	Hingot	Simaroubaceae	Fuelwood, nonedible oil, medicinal
13	*Bauhinia purpurea* Linn.	Kachnar	Caesalpiniaceae	Fuelwood, fodder medicinal
14	*Bauhinia racemosa* Lamk.	Seta/jhinjha	Caesalpiniaceae	Fuelwood, fodder medicinal
15	*Bauhinia variegate* Linn.	Kachnar	Caesalpiniaceae	Fuelwood, fodder, medicinal
16	*Bombax ceiba* Linn.	Semal	Bombacaceae	Fuelwood, fodder, medicinal
17	*Borassus flabellifer* Linn.	Tad	Arecaceae	Seed (edible)
18	*Boswellia serrata* Roxb.	Salar	Burseraceae	Fuelwood, medicinal, gum resins
19	*Buchanania latifolia* Roxb.	Chironji	Anacardiaceae	Seed (edible)
20	*Butea monosperma* (Lamk.) Taub.	Dakh	Fabaceae	Fuelwood, fodder, medicinal
21	*Capparis decidua* (Forsk.) Edgew. (Plate 4.5d)	Kair	Capparaceae	Fruits (edible), fuelwood, fodder, medicinal
22	*Cassia fistula* Linn.	Amaltas	Caesalpiniaceae	Medicinal
23	*Cassia siamea* Lam.	–	Caesalpiniaceae	Fuelwood, fodder
24	*Cochlospermum religiosum* (L.) Alston	Ganiara	Cochlospermaceae	Fiber, oil
25	*Cordia dichotoma* Farsk.	Gonda	Ehretiaceae	Medicinal
26	*Cordia gharaf* (Farsk.) Her. & Asch.	Gondi	Ehretiaceae	Medicinal

(continued)

Table 4.1 (continued)

S. no	Plant species	Local name	Family	Economic value
27	*Dalbergia sissoo* Roxb.	Shisham	Fabaceae	Timber, fodder
28	*Dichrostachys cineraria* (L.) Wt. & Arn.	Goya-khair	Mimosaceae	Fuelwood, fodder, medicinal
29	*Diospyros melanoxylon* Roxb. (Plate 4.3d)	Timru	Ebenaceae	Biri leaves, fruits (edible)
30	*Diospyros montana* Roxb.	Chikon	Ebenaceae	Fuelwood, fodder
31	*Diospyros tomentosa* Roxb.	Tendu	Ebenaceae	Fuelwood
32	*Emblica officinalis* Gaertn.	Awala	Euphorbiaceae	Fruits (edible)
33	*Erythrina suberosa* Roxb.	Gadha palas	Fabaceae	Medicinal
34	*Ficus benghalensis* Linn.	Vat/bad	Moraceae	Medicinal
35	*Ficus religiosa* Linn.	Pipal	Moraceae	Lakh worm hosts
36	*Garuga pinnata* Roxb.	Karpata	Burseraceae	Medicinal
37	*Holarrhena antidysenterica* (L.) Wall. ex A. DC. (Plate 4.3c)	Indra jav	Apocynacae	Medicinal
38	*Holoptelea integrifolia* (Roxb.) Planch.	Churel/bandarrotti	Ulmaceae	Fuelwood, seed use as fodder
39	*Jatropha curcas* Linn.	Ratanjot	Euphorbiaceae	Medicinal, dye, nonedible oil
40	*Kigelia pinnata* DC.	Jhad fanush	Bignoniaceae	Plantation
41	*Lannea coromandelica* (Houtt.) Merril. (Plate 4.2b)	Godal	Anacardiaceae	Dye, timber, tannin
42	*Leucaena leucocephala* (Lamk.) de Wit.	Subabul	Mimosaceae	Fuelwood, fodder
43	*Madhuca indica* Gmel. (Plate 4.2a)	Mahua	Sapotaceae	Fruit edible, nonedible oil
44	*Mallotus philippinensis* Muell. Arg.	Siduri/rohini	Euphorbiaceae	Fuelwood, fodder
45	*Mangifera indica* Linn.	Aam	Anacardiaceae	Fruits (edible)
46	*Maytenus emarginata* (Willd.) Ding Hou	Kaker	Celastraceae	Fuelwood
47	*Miliusa tomentosa* (Roxb.) J. Sinclair	Umb	Annonaceae	Timber
48	*Mimusops elengi* Linn.	Maulsiri	Sapotaceae	Medicinal, nonedible oil
49	*Morinda tinctoria* Roxb.	Aal	Rubiaceae	Medicinal
50	*Moringa oleifera* Lam.	Sainjana	Caesalpiniaceae	Fruits (edible)

51	*Morus alba* Linn.	Satut	Moraceae	Fruits (edible), fuelwood, fodder
52	*Nyctanthes arbor-tristis* Linn.	Harsinghar	Oleaceae	Essential oil
53	*Ougeinia oojeinensis* (Roxb.) Hochreut	Sandan	Fabaceae	Poisonous plant
54	*Phoenix sylvestre* Roxb.	Khajur	Arecaceae	Fruits (edible)
55	*Pithecellobium dulce* (Roxb.) Benth. (Plate 4.2d)	Jangaljalbi	Mimosaceae	Fruits, timber fodder, fuelwood
56	*Prosopis cineraria* (L.) Druce (Plate 4.5b)	Khejari	Mimosaceae	Fruits (edible), timber fodder, fuelwood
57	*Prosopis juliflora* (Swartz.) DC. (Plate 4.5c)	Vilayati babul	Mimosaceae	Fuelwood
58	*Pterocarpus marsupium* Roxb.	Bijasal	Fabaceae	Medicinal
59	*Ricinus communis* Linn.	Arandi	Euphorbiaceae	Medicinal, nonedible oil
60	*Salvadora persica* Linn. (Plate 4.4a)	Kharajal	Salvadoraceae	Nonedible oil
61	*Salvadora oleoides* Decne.	Pilu	Salvadoraceae	Nonedible oil
62	*Santalum album* Linn.	Chandan	Santalaceae	Extraction and distillation
63	*Sapindus emarginatus* Vahl.	Aritha	Sapindaceae	Soap substitutes, nonedible oil
64	*Soymida febrifuga* A. Juss.	Rohan	Meliaceae	Medicinal
65	*Sterculia urens* Roxb.	Katria	Sterculiaceae	Oil, medicinal
66	*Tamarix aphylla* (L.) Karst.	Farash	Tamaricaceae	Medicinal
67	*Tecomella undulata* (Sm.) Seemann. (Plate 4.3a)	Roheda	Bignoniaceae	Timber
68	*Terminalia alata* Heyne	Sadad	Combretaceae	Medicinal, fodder
69	*Terminalia arjuna* (*R*oxb.) Wight. & Ara. (Plate 4.2c)	Arjun	Combretaceae	Medicinal, silkworm host
70	*Terminalia bellirica* Roxb.	Baheda	Combretaceae	Medicinal
71	*Wrightia tinctoria* R.Br.	Khirni/dudhi	Apocynaceae	Medicinal
72	*Ziziphus mauritiana* Lamk.	Bor	Rhamnaceae	Fruits (edible)
73	*Ziziphus glabrata* Santapau	Ghatbor	Rhamnaceae	Fruits (edible), fuelwood

Table 4.2 Shrub species of Rajasthan for biomass and other economic values

S. no	Plant species	Local name	Family	Economic value
1	*Abutilon indicum* (Linn.) Sweet.	Kanghi	Malvaceae	Fiber, fodder
2	*Acacia jacquemontii* Benth.	Baonli	Mimosaceae	Medicinal
3	*Aerva tomentosa* (Burm.f.) Juss.	Bui	Amaranthaceae	Medicinal
4	*Agave americana* Linn.	Rambans	Agavaceae	Medicinal
5	*Calotropis procera* (Ait.) R.Br. (Plate 4.4d)	Aak	Asclepiadaceae	Biomass
6	*Carissa carandas* Linn. (Plate 4.4b)	Apocynaceae	Karaunda	Fruits (edible)
7	*Cassia auriculata* Linn.	Anwal	Caesalpiniaceae	Medicinal
8	*Commiphara wightii* (Arn.) Bhandari.	Gugal	Burseraceae	Medicinal
9	*Crotalaria burhia* Buch. Ham.	Senia	Fabaceae	Fodder
10	*Crotalaria medicaginea* Lamk.	Jangali gass	Fabaceae	Fodder
11	*Grewia tenax* (Forsk.) Fiori.	Gangan	Tiliaceae	Medicinal, fodder
12	*Haloxylon recurvum* (Moq.) Bunge. ex Boiss.	Lana	Chenopodiaceae	Fiber, fodder, medicinal
13	*Helicteres isora* Linn.	Morорphali	Sterculiaceae	Medicinal
14	*Hibiscus ovalifolius* Vahl.		Malvaceae	Fiber, fodder
15	*Lantana indica* Roxb.	Lanten	Verbenaceae	Fuel
16	*Lawsonia inermis* Linn.	Mehandi	Lythraceae	Dye, medicinal
17	*Leptadenia pyrotechnica (*Forsk.*)* Decne. (Plate 4.4c)	Khimp	Asclepiadaceae	Medicinal, fruits (edible)
18	*Pandanus tectorius* Sol. ex Park.	Kewra	Pandanaceae	Medicinal
19	*Punica granatum* Linn.	Anar	Punicaceae	Fruit (edible)
20	*Rhus mysurensis* Heyne	Dansara	Anacardiaceae	Fruits (edible)
21	*Salsola baryosoma* (R.et S.) Dandy	Lunki	Chenopodiaceae	Fiber, fodder, medicinal
22	*Sericostoma pauciflorum* Stocks.	–	Boraginaceae	Fuel biomass
23	*Sida cordifolia* Linn	Bala	Malvaceae	Fuel biomass, medicinal
24	*Suaeda maritima* (Linn.) Dumort	Lana	Chenopodiaceae	Fiber, fodder, fuel, medicinal
25	*Verbesina encelioides* (Cav.) Benth. & Hook.	–	Asteraceae	Fuel biomass
26	*Waltheria indica* Linn	–	Sterculiaceae	Fuel biomass
27	*Withania somnifera* (Linn.) Dunal.	Ashwagandha	Solanaceae	Medicinal
28	*Xanthium strumarium* Linn.	Aadha-shishi	Asteraceae	Medicinal
29	*Ziziphus nummularia* (Burm.f.) Wt.et Arn. (Plate 4.6c)	Jhadi-Bor	Rhamnaceae	Fruits (edible)

Table 4.3 Climber/twiner/runner species of Rajasthan for biomass and other economic values

S. no	Plant species	Local name	Family	Economic value
1	*Celastrus paniculata* Willd.	Malkanghani	Celastraceae	Medicinal
2	*Cocculus pendulus* (Forst.) Diels.	Jaljamni	Menispermacea	Medicinal
3	*Tinospora cordifolia* (Willd.) Miers.	Giloy	Menispermacea	Medicinal
4	*Blastania fimbristipula* (Fensl.) Kotschy. et Peyr.	Bel	Cucurbitaceae	Medicinal
5	*Abrus precatorius* Linn.	Rati	Fabaceae	Medicinal
6	*Citrullus colocynthis* (L.) Schrad. (Plate 4.6b)	Tumba	Cucurbitaceae	Medicinal, nonedible oil
7	*Cucumis callosus* (Rottl.) Cogn.	Kachari	Cucurbitaceae	Medicinal
8	*Mukia maderaspatana* (L.) M. Roem.	–	Cucurbitaceae	Medicinal
9	*Pergularia daemia* (Forsk.) Chiov.	–	Asclepiadaceae	Medicinal
10	*Ipomoea eriocarpa* R.Br.	–	Convolvulaceae	Medicinal
11	*Ipomoea pes-tigridis* Linn.	–	Convolvulaceae	Medicinal

Rajasthan covers largest area of wasteland in India. It is mainly spread in Districts of Bikaner, Barmer, Churu, Sri Ganganagar, Jaisalmer, Jodhpur, and Nagaur. While only 4% of wasteland areas are covered by Ajmer, Jaipur, Jalore, Jhunjhunu, Sikar, and Pali, these wastelands are categorized as barren hill ridge or rock outcrop, gullied or ravenous, sandy area, salt-affected, and undulating upland with or without scrub (Gupta and Kothari 1987). Due to lack of rain, this wasteland is not used for cultivation.

4.2 Material and Methods

During the present investigation, different wastelands in various districts of Rajasthan state were surveyed, viz., Ajmer, Barmer, Bhilwara, Bikaner, Churu, Jodhpur, Jaisalmer, Jhunjhunu, Nagaur, Dausa, Sikar, Udaipur and Jaipur. The plant species were examined and collected from these sites and attempts were also made to examine their primary and secondary colonizers. Identification of these plant species was done using standard monographs and their local flora (Sharma 1976; Bhandari 1978; Shetty and Singh 1987). The plant specimens have been deposited in the herbarium of the Botany Department, University of Rajasthan, Jaipur.

4.3 Observation

Plant species have been collected from different habitats of the Rajasthan state in different seasons. Beside the biomass and wasteland colonizer these plants species have economic value also. The recorded plant species were categorized as tree,

Table 4.4 Herbaceous species of Rajasthan for biomass and other economic values

S. no.	Plant species	Habit	Family	Other uses
1	*Acanthospermum hispidum* DC.	Annual	Asteraceae	Biomass
2	*Achyranthes aspera* Linn.	Perennial	Amaranthaceae	Medicinal
3	*Alysicarpus monilifer* DC.	Annual	Fabaceae	Fodder
4	*Alysicarpus vaginalis*	Ephemerals	Fabaceae	Fodder
5	*Amaranthus caudatus* Linn.	Annual	Amaranthaceae	Edible, fodder
6	*Amaranthus spinosus* Linn.	Ephemerals	Amaranthaceae	Edible, fodder
7	*Anagallis arvensis* Linn.	Annual	Anagalaceae	Biomass
8	*Anisomeles indica* (L.) Ktze.	Perennial	Lamiaceae	Biomass
9	*Argemone mexicana* Linn.	Annual	Papaveraceae	Nonedible oil
10	*Arnebia hispidissima* (Lehm.) DC.	Annual	Boraginaceae	Medicinal
11	*Artemisia scoparia* Waldst. et Kit.	Ephemerals	Asteraceae	Medicinal
12	*Bidens biternata* (Lour.) Merr. & Sherff.	Ephemerals	Asteraceae	Fodder
13	*Blainvillea acmella* (Linn.) Philipson.	Ephemerals	Asteraceae	Fodder
14	*Boerhavia diffusa* Linn.	Perennial	Nyctaginaceae	Medicinal
15	*Borreria articularis* (L.) F.N. Will.	Ephemerals	Rubiaceae	Fodder
16	*Cassia angustifolia* Vahl. (Plate 4.6d)	Perennial	Caesalpiniaceae	Medicinal
17	*Cassia occidentalis* Linn.	Perennial	Caesalpiniaceae	Medicinal
18	*Cassia tora* Linn.	Annual	Caesalpiniaceae	Biomass
19	*Catharanthus roseus* (Linn.) Don.	Perennial	Apocynaceae	Medicinal
20	*Chenopodium album* Linn.	Annual	Chenopodiaceae	Edible, fodder
21	*Chenopodium murale* Linn.	Annual	Chenopodiaceae	Edible, fodder
22	*Cleome gynandra* Linn.	Ephemerals	Capparaceae	Nonedible oil
23	*Cleome viscosa* Linn.	Ephemerals	Capparaceae	Nonedible oil
24	*Commelina benghalensis* Linn.	Ephemerals	Commelinaceae	Fodder
25	*Commelina forskalaei* Vahl.	Ephemerals	Commelinaceae	Fodder
26	*Convolvulus microphyllous* Sieb. ex Spreng.	Annual	Convolvulaceae	Medicinal
27	*Corchorus aestuans* Linn.	Ephemerals	Tiliaceae	Fodder
28	*Corchorus tridens* Linn.	Ephemerals	Tiliaceae	Fodder
29	*Croton bonplandianum* Baill.	Annual	Euphorbiaceae	Biomass
30	*Datura innoxia* Mill.	Annual	Solanaceae	Medicinal
31	*Digera muricata* (L.) Mart.	Annual	Amaranthaceae	Fodder
32	*E. antisyphilitica* Zuce.	Annual	Euphorbiaceae	Biomass

(continued)

Table 4.4 (continued)

S. no.	Plant species	Habit	Family	Other uses
33	*Echinops echinatus* Roxb.	Perennial	Asteraceae	Medicinal
34	*Euphorbia hirta* Linn.	Annual	Euphorbiaceae	Biomass
35	*Euphorbia prostrata* Ait.	Ephemerals	Euphorbiaceae	
36	*Evolvulus alsinoides* Linn.	Ephemerals	Convolvulaceae	Medicinal
37	*Fagonia cretica* Linn.	Annual	Zygophyllaceae	Medicinal
38	*Farsetia hamiltonii* Royle.	Annual	Bracicaceae	
39	*Fumaria indica* (Haussk.) Pugsley.	Annual	Fumariaceae	Medicinal
40	*Gisekia pharnaceoides* Linn.	Ephemerals	Aizoaceae	Biomass
41	*Gnaphalium indicum* Linn.	Annual	Asteraceae	Fodder
42	*Gomphrena celosioides* Mart.	Annual	Amaranthaceae	Fodder
43	*Heliotropium ellipticum* Ledeb.	Annual	Boraginaceae	Biomass
44	*Heliotropium marifolium* Retz.	Annual	Boraginaceae	Biomass
45	*Heliotropium subulatum* Hochst. ex DC.	Annual	Boraginaceae	Biomass
46	*Indigofera astragalina* DC.	Ephemerals	Fabaceae	Biomass
47	*Indigofera cordifolia* Heyne ex Roth.	Ephemerals	Fabaceae	Fodder
48	*Indigofera linifolia* (L.) Retz.	Annual	Fabaceae	Fodder
49	*Indigofera linnaei* Ali.	Annual	Fabaceae	Fodder
50	*Indigofera sessiliflora* DC.	Annual	Fabaceae	Fodder
51	*Launaea procumbens* (Roxb.) Ramayya. et Rajagopal.	Annual	Asteraceae	Medicinal
52	*Launaea resedifolia* (L.) Druce	Perennial	Asteraceae	Biomass
53	*Lepidagathis trinervis* Wall. ex Nees.	Perennial	Acanthaceae	Medicinal
54	*Leucas aspera* (Willd.) Spreng.	Ephemerals	Lamiaceae	Medicinal
55	*Martynia annua* Linn.	Ephemerals	Pedaliaceae	Medicinal
56	*Medicago laciniata* (L.) Mill.	Annual	Fabaceae	Fodder
57	*Melilotus indica* All.	Annual	Fabaceae	Fodder
58	*Mollugo cerviana* (L.) Ser.	Ephemerals	Aizoaceae	Biomass
59	*Mollugo nudicaulis* Lamk.	Ephemerals	Aizoaceae	Biomass
60	*Ocimum canum* Sims.	Perennial	Lamiaceae	Medicinal
61	*Oligochaeta ramosa* (Roxb.) Wagenitz.	Perennial	Asteraceae	Medicinal
62	*Parthenium hysterophorus* Linn.	Annual	Asteraceae	Biomass
63	*Pedalium murex* Linn.	Ephemerals	Pedaliaceae	Medicinal
64	*Peristrophe bicalyculata* (Retz.) Nees.	Ephemerals	Acanthaceae	Medicinal
65	*Phyllanthus asperulatus* Hutch.	Ephemerals	Euphorbiaceae	Medicinal
66	*Physalis minima* Linn.	Annual	Solanaceae	Medicinal
67	*Polycarpaea corymbosa* (L.) Lamk.	Ephemerals	Polycarpaceae	Fodder
68	*Polygala erioptera* DC.	Ephemerals	Polygalaceae	Fodder

(continued)

Table 4.4 (continued)

S. no.	Plant species	Habit	Family	Other uses
69	*Polygala irregularis* Boiss.	Ephemerals	Polygalaceae	Fodder
70	*Portulaca oleracea* Linn.	Annual	Portulacaceae	Fodder
71	*Portulaca* sis *suffruticosa* Wt.	Annual	Portulacaceae	Fodder
72	*Pulicaria angustifolia* DC.	Annual	Asteraceae	Biomass
73	*Pulicaria crispa* Sch.-Bip.	Perennial	Asteraceae	Biomass
74	*Pupalia lappacea* (L.) Juss.	Ephemerals	Amaranthaceae	Biomass
75	*Rostellularia procumbens* (L.) Nees.	Ephemerals	Acanthaceae	Biomass
76	*Rumex dentatus* Linn.	Annual	Amaranthaceae	Biomass
77	*Sesamum indicum* Linn.	Ephemerals	Pedaliaceae	Nonedible oil
78	*Sida ovata* Forst. f.	Ephemerals	Malvaceae	Medicinal
79	*Sisymbrium irio* Linn.	Annual	Bracicaceae	Medicinal
80	*Solanum nigrum* Linn.	Annual	Solanaceae	Medicinal
81	*Solanum surattense* Burm.	Perennial	Solanaceae	Medicinal
82	*Sonchus asper* (L.) Gars	Annual	Asteraceae	Medicinal
83	*Tephrosia hamiltonii* Drumm. (Plate 4.6a)	Perennial	Fabaceae	Fodder
84	*Tephrosia strigosa* (Dalz.) Sant.	Ephemerals	Fabaceae	Fodder
85	*Trianthema portulacastrum* Linn.	Ephemerals	Aizoaceae	Biomass
86	*Trianthema triquetra* Rottl. ex Willd.	Ephemerals	Aizoaceae	Biomass
87	*Tribulus terrestris* Linn.	Ephemerals	Zygophyllaceae	Medicinal
88	*Trichodesma indicum* R.Br.	Perennial	Boraginaceae	Biomass
89	*Tridax procumbens* Linn.	Annual	Asteraceae	Medicinal
90	*Trigonella polycerata* Linn.	Annual	Fabaceae	Fodder
91	*Triumfetta pentandra* A. Rich.	Ephemerals	Tiliaceae	Fodder
92	*Vernonia cinerea* (L.) Less.	Ephemerals	Asteraceae	Medicinal
93	*Zaleya govindia* (Buch-Ham.) N.C. Nair.	Ephemerals	Aizoaceae	Fodder
94	*Typha elephantina* Roxb.	Perennial	Typhaceae	Biomass
95	*Bulbostylis barbata* (Rottb.) Kunth.	Annual	Poaceae	Fodder
96	*Vetiveria zizanioides* (L.) Nash.	Perennial	Poaceae	Essential oil
97	*Cymbopogon martini* (Roxb.) Wats.	Perennial	Poaceae	Essential oil
98	*Cyperus arenarius* Retz.	Annual	Cyperaceae	Biomass
99	*Cyperus bulbosus* Vahl.	Annual	Cyperaceae	Biomass
100	*Cyperus triceps* (Rottb.) Endl.	Annual	Cyperaceae	Biomass
101	*Aristida funiculata* Trin. et Rupr.	Annual	Poaceae	Fodder
102	*Cenchrus biflorus* Roxb.	Annual	Poaceae	Fodder
103	*Cenchrus ciliaris* Linn.	Annual	Poaceae	Fodder

(continued)

Table 4.4 (continued)

S. no.	Plant species	Habit	Family	Other uses
104	*Cenchrus pennisetiformis* Hochst. et Steud.	Annual	Poaceae	Fodder
105	*Chloris virgata* Sw.	Annual	Poaceae	Fodder
106	*Dactyloctenium sindicum* Boiss.	Annual	Poaceae	Fodder
107	*Eragrostis ciliaris* (L.) R.Br.	Annual	Poaceae	Fodder
108	*Eragrostis pilosa* (L.) P. Beauv.	Annual	Poaceae	Fodder
109	*Eragrostis tremula* Hochst. ex Steud.	Annual	Poaceae	Fodder
110	*Saccharum bengalense* Retz.	Annual	Poaceae	Fodder
111	*Cynodon dactylon* (L.) Pers.	Perennial	Poaceae	Fodder
112	*Perotis indica* (L.) O. Ktze.	Annual	Poaceae	Fodder
113	*Brachiaria reptans* (L.) Gardener. et Hubb.	Annual	Poaceae	Fodder
114	*Brachiaria ramosa* (L.) Stapf.	Annual	Poaceae	Fodder
115	*Digitaria adscendens* (HBK) Henr.	Annual	Poaceae	Fodder
116	*Lasiurus sindicus* Henr. (Plate 4.5a)	Annual	Poaceae	Fodder

shrub, climber, herb and grasses. Their economic importance is listed in Tables 4.1, 4.2, 4.3, and 4.4. Vast areas of desert in the western region are characterized by different plant associations and different formations, viz., *Leptadenia pyrotechnica* (Forsk.) Decne., *Calotropis procera* (Ait.) R.Br., and *Salvadora persica* Linn. (Plate 4.1a) and *Crotalaria burhia* Buch.-Ham. and *Aerva tomentosa* (Burm.f.) Juss. (Plate 4.1b).

4.3.1 Trees

Acacia catechu Willd.; *Acacia senegal* (Linn.) Willd.; *Azadirachta indica* A. Juss.; *Anogeissus pendula* Edgew. (Plate 4.3b); *Anogeissus latifolia* Wall.; *Ailanthus excelsa* Roxb.; *Aegle marmelos* (L.) Correa.; *Acacia tortilis* (Forsk.) Hayne.; *Acacia nilotica* (Linn.) Willd. ex Del.; *Acacia leucophloea* (Roxb.) Willd.; *Butea monosperma* (Lamk.) Taub.; *Buchanania latifolia* Roxb.; *Boswellia serrata* Roxb.; *Bombax ceiba* Linn.; *Bauhinia racemosa* Lamk.; *Balanites aegyptiaca* (Linn.) Delile.; *Balanites aegyptiaca* (L.) Delile.; *Capparis decidua* (Forsk.) Edgew. (Plate 4.5d); *Cordia oblique* Willd.; *Cordia gharf* (Farsk.) Her. & Asch.; *Cassia fistula* Linn.; *Cassia auriculata* Linn; *Diospyros tomentosa* Roxb.; *Diospyros montana* Roxb.; *Diospyros melanoxylon* Roxb. (Plate 4.3d); *Dichrostachys cinerea* (Linn.) Wight. et Arn.; *Erythrina suberosa* Roxb.; *Emblica officinalis* Gaertn.; *Ficus religiosa* Linn.; *Ficus religiosa* Linn.; *Ficus benghalensis* Linn.; *Garuga pinnata* Roxb.; *Leucaena leucocephala* (Lam.) de Wit.; *Lannea coromandelica* (Houtt.)

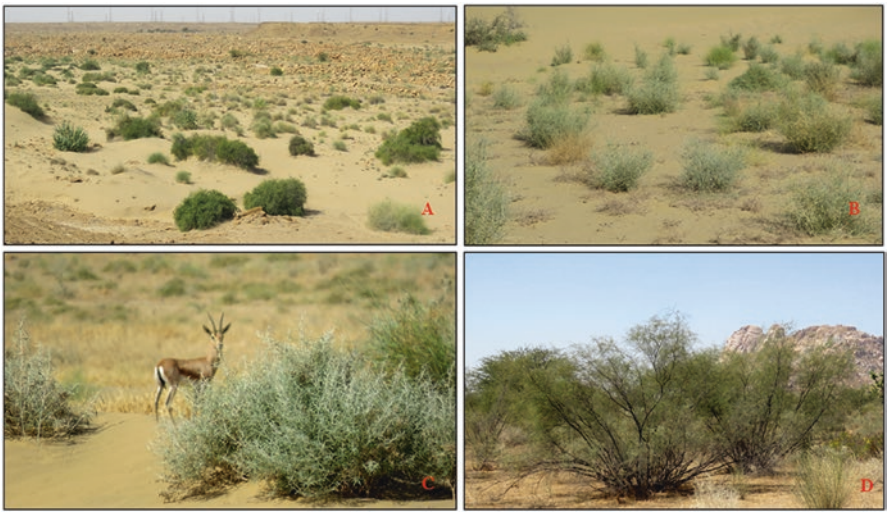

Plate 4.1 Figure **a**: Showing a scrub of *Leptadenia pyrotechnica* (Forsk.) Decne., *Calotropis procera* (Ait.) R.Br., *Salvadora persica* Linn.; Figure **b**: showing a scrub of *Crotalaria burhia* Buch. Ham. and *Aerva tomentosa* (Burm. f.) Juss. Figure **c**: *Aerva tomentosa* (Burm. f.) Juss. Figure **d**: *Acacia jacquemontii* Benth

Plate 4.2 Figure **a**: *Madhuca indica* Gmel.; Figure **b**: *Lannea coromandelica* (Houtt.) Merril; Figure **c**: *Terminalia arjuna* (Roxb.) Wight. & Ara. Figure **d**: *Pithecellobium dulce* Benth

Merril. (Plate 4.2b); *Jatropha curcas* Linn.; *Holarrhena antidysenterica* (L.) Wall. ex A. DC. (Plate 4.3c); *Holoptelaea integrifolia* Planch.; *Helicteres isora* Linn.; *Morus alba* Linn.; *Moringa oleifera* Lam.; *Morinda tinctoria* Roxb.; *Mimusops elengi* Linn.; *Miliusa tomentosa* (Roxb.) J. Sinclair; *Maytenus emarginata* (Willd.) Ding Hou; *Mangifera indica* Linn.; *Madhuca indica* Gmel. (Plate 4.2a); *Nyctanthes*

Plate 4.3 (**a**) *Tecomella undulata* (Sm.) Seem.; (**b**) *Anogeissus pendula* Edgew. (**c**) *Holarrhena antidysenterica* (L.) Wall. ex A. DC. (**d**) *Diospryos melanoxylon* Roxb

arbor-tristis Linn.; *Pterocarpus marsupium* Roxb.; *Prosopis juliflora* (Swartz.) DC. (Plate 4.5c); *Prosopis cineraria* (Linn.) Druce (Plate 4.5b); *Pongamia pinnata* (L.) Pierre.; *Pithecellobium dulce* Benth. (Plate 4.2d); *Phoenix sylvestris* (Linn.) Roxb.; *Phoenix sylvestris* Roxb.; *Sterculia urens* Roxb.; *Sapindus emarginatus* Vahl.; *Santalum album* Linn.; *Salvadora persica* Linn. (Plate 4.4a); *Salvadora oleoides* Decne.; *Ricinus communis* Linn.; *Wrightia tinctoria* R.Br.; *Terminalia bellirica* Roxb.; *Terminalia arjuna* (*R*oxb.) Wight. & Ara. (Plate 4.2c); *Terminalia alata*

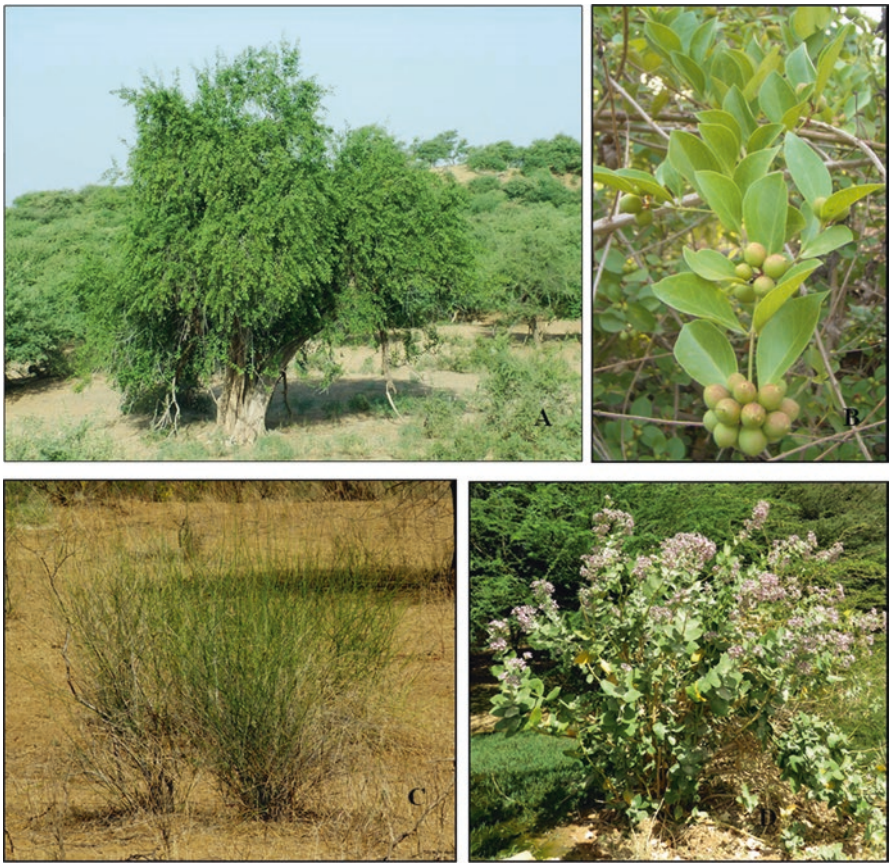

Plate 4.4 (**a**) *Salvadora persica* Linn.; (**b**) *Carissa carandas* Linn.; (**c**) *Leptadenia pyrotechnica (*Forsk.*)* Decne.; (**d**) *Calotropis procera* (Ait.) R. Br

Heyne; *Tecomella undulata* (Sm.) Seemann (Plate 4.3a); *Tamarix aphylla* (L.) Karst.; *Ziziphus glaberrima* Santapau; *Ziziphus mauritiana* Lamk.

4.3.2 Shrubs

Abutilon indicum (Linn.) Sweet.; *Acacia jacquemontii* Benth. (Plate 4.1d); *Aerva tomentosa* (Burm.f.) Juss. (Plate 4.1b and c); *Calotropis procera* (Ait.) R.Br. (Plates 4.1a and 4.4d); *Carissa carandas* Linn. (Plate 4.4b); *Commiphara wightii* (Arn.) Bhandari.; *Crotalaria burhia* Buch.-Ham. ex Benth.; *Crotalaria medicaginea* Lamk.; *Grewia tenax* (Forsk.) Fiori.; *Hibiscus ovalifolius* Vahl.; *Lantana indica* Roxb.; *Lawsonia inermis* Linn.; *Leptadenia pyrotechnica* (Forsk.) Decne. (Plate 4.4c); *Mallotus philippinensis* Muell. Arg.; *Punica granatum* Linn.; *Rhus mysurensis* Heyne; *Salsola baryosoma* (R.et S.) Dandy.; *Sericostoma pauciflorum* Stocks.; *Sida cordifolia* Linn.; *Suaeda maritima* (Linn.) Dumort.; *Verbesina encelioides* (Cav.) Benth. &

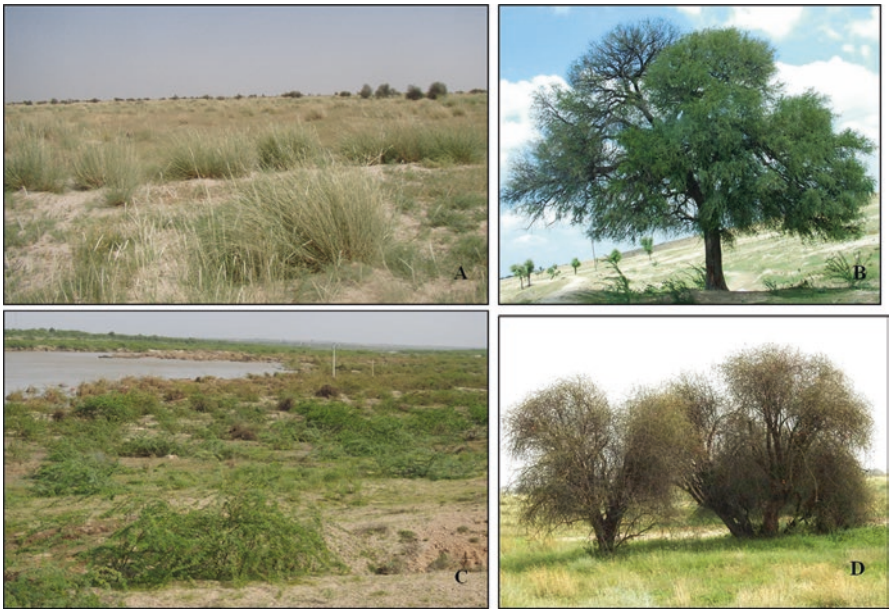

Plate 4.5 (**a**) Showing the grassland of *Lasiurus sindicus* Henr.; (**b**) *Prosopis cineraria* (L.) Druce.; (**c**) Showing a scrub of *Prosopis juliflora* (Swartz.) DC.; (**d**) *Capparis deciduas* (Forsk.) Edgew

Hook.; *Waltheria indica* Linn.; *Withania somnifera* (Linn.) Dunal.; *Xanthium strumarium* Linn.; *Ziziphus nummularia* (Burm.f.) Wt. and Arn. (Plate 4.6c).

4.3.3 Climbers and Twiners

Blastania fimbristipula (Fensl.) Kotschy et Peyr.; *Celastrus paniculatus* Willd.; *Citrullus colocynthis* (Linn.) Schrad. (Plate 4.6b); *Cocculus pendulus* (Forst.) Diels.; *Cucumis callosus* (Rottl.) Cogn.; *Ipomoea eriocarpa* R.Br.; *Ipomoea pestigridis* Linn.; *Mukia maderaspatana* (Linn.) M. Roem.; *Pergularia daemia* (Forsk.) Chiov.; *Tinospora cordifolia* (Willd.) Miers.

4.3.4 The Ground Cover

The flora of herbaceous plant species in Rajasthan comprises of more than 70% of herbaceous plants among all the flowering plants. These plant species or grasses are categorized according to their life span.

4.3.4.1 Perennial Herbs

Achyranthes aspera Linn.; *Amaranthus caudatus* Linn.; *Boerhavia diffusa* Linn.; *Borreria articularis* (Linn.) F.N. Will.; *Echinops echinatus* Roxb.; *Catharanthus roseus* (Linn.) Don.; *Convolvulus microphyllous* Sieb. ex Spreng.; *Croton*

Plate 4.6 (**a**) *Tephrosia hamiltonii* Drumm.; (**b**) *Citrullus colocynthis* (L.) Schrad.; (**c**) *Ziziphus nummularia* (Burm.f.) Wt. and Arn.; (**d**) *Cassia angustifolia* Vahl

bonplandianum Baill.; *Datura metal* Linn.; *Euphorbia hirta* Linn.; *Farsetia hamiltonii* Royle.; *Indigofera linnaei* Ali.; *Launaea procumbens* (Roxb.) Ramayya et Rajagopal; *Launaea resedifolia* (Linn.) Druce; *Lepidagathis trinervis* Wall. ex Nees.; *Oligochaeta ramosa* (Roxb.) Wagenitz; *Pulicaria crispa* Sch. Bip.; *Pupalia lappacea* (Linn.) Juss.; *Solanum nigrum* Linn.; *Solanum surattense* Burm.; *Tephrosia hamiltonii* Drumm. (Plate 4.6a); *Tephrosia purpurea* (Linn.) Pers.; *Trianthema portulacastrum* Linn.; *Zaleya govindia* (Buch-Ham. ex G. Don) N.C. Nair.

4.3.4.2 Annual Herbs

Acanthospermum hispidum DC.; *Alysicarpus monilifer* DC.; *Anagallis arvensis* Linn.; *Argemone mexicana* Linn.; *Arnebia hispidissima* (Lehm.) DC.; *Artemisia scoparia* Waldst et Kit.; *Cassia angustifolia* Vahl. (Plate 4.6d); *Chenopodium album* Linn.; *Chenopodium murale* Linn.; *Datura innoxia* Mill.; *Leucas aspera* (Willd.) Spreng.; *Fagonia cretica* Linn.; *Fumaria indica* (Haussk.) Pugsley.; *Gnaphalium indicum* Linn.; *Gomphrena celosioides* Mart.; *Heliotropium ellipticum* Ledeb.; *Heliotropium marifolium* Retz.; *Heliotropium subulatum* Hochst. ex DC.; *Indigofera cordifolia* Heyne; *Indigofera hochstetteri* Baker.; *Medicago laciniata* (Linn.) Mill., *Melilotus indica* All.; *Ocimum canum* Sims.; *Phyllanthus asperulatus* Hutch.; *Portulaca oleracea* Linn.; *Portulaca suffruticosa* Wt.; *Pulicaria angustifolia* DC.; *Sisymbrium irio* Linn.; *Sonchus asper* (Linn.) Gars.; *Tephrosia strigosa* (Dalz.) Sant.; *Trianthema triquetra* Rottl. ex Willd.; *Trigonella. polycerata* Linn.; *Vernonia cinerea* (Linn.) Less.

4.3.4.3 Ephemerals

Alysicarpus vaginalis (Linn.) DC.; *Amaranthus spinosus* Linn.; *Anisomeles indica* (Linn.) Ktze.; *Bidens biternata* (Lour.) Merr. & Sherff.; *Blainvillea acmella* (Linn.) Philipson.; *Cassia occidentalis* Linn.; *Cassia tora* Linn.; *Cleome gynandra* Linn.; *Cleome viscose* Linn.; *Commelina benghalensis* Linn.; *Commelina forskalaei* Vahl.; *Digera muricata* (Linn.) Mart.; *Euphorbia prostrata* Ait.; *Evolvulus alsinoides* Linn.; *Gisekia pharnaceoides* Linn.; *Indigofera astragalina* DC.; *Indigofera linifolia* (Linn.) Retz.; *Indigofera sessiliflora* DC.; *Martynia annua* Linn.; *Mollugo cerviana* (Linn.) Ser.; *Mollugo nudicaulis* Lamk.; *Pedalium murex* Linn.; *Peristrophe bicalyculata* (Retz.) Nees.; *Physalis minima* Linn.; *Polycarpaea corymbosa* (Linn.) Lamk.; *Polygala erioptera* DC.; *Polygala irregularis* Boiss.; *Rostellularia procumbens* (Linn.) Ness.; *Sesamum indicum* Linn.; *Sida ovata* Forst.; *Corchorus tridens* Linn.; *Tribulus terrestris* Linn.; *Trichodesma indicum* R. Br.; *Triumfetta pentandra* A. Rich.

4.3.4.4 Grasses

Aristida funiculata Trin. et Rupr.; *Brachiaria ramosa* (Linn.) Stapf.; *Brachiaria reptans* (Linn.) Gardener et Hubb.; *Bulbostylis barbata* (Rottb.) Kunth.; *Cenchrus biflorus* Roxb.; *Cenchrus ciliaris* Linn.; *Cenchrus pennisetiformis* Hochst. et Steud.; *Chloris virgata* Sw.; *Cyperus arenarius* Retz.; *Cyperus bulbosus* Vahl.; *Cyperus triceps* (Rottb.) Endl.; *Dactyloctenium sindicum* Boiss.; *Eragrostis ciliaris* (Linn.) R.Br.; *Eragrostis pilosa* (Linn.) P. Beauv.; *Eragrostis tremula* Hochst. ex Steud.; *Lasiurus sindicus* Henr. (Plate 4.5a); *Saccharum bengalense* Retz.; *Typha elephantina* Roxb.; *Vetiveria zizanioides* (L.) Nash.

4.3.5 Biomass for the Wasteland

The growing demand for fuelwood as a result of rapid population growth has made it increasingly difficult for many people in this region to meet their basic energy need. Most of the users of fuelwood lack access to alternative fuels. By the year 2020, a situation of acute scarcity and fuelwood deficit could affect 2400 million people in the world, over 1400 million of them in the Asian and Pacific regions, unless adequate corrective action is taken. Studies sponsored by the Energy Survey of Indian Committee (1965) have revealed that the estimated consumption of firewood in urban and rural areas was 0.41 and 0.58 kg/head/day, respectively, which is around 150 kg and 212 kg/capita/annum. The gap between availability and projected demand for fuel resources is increasing day by day (Vimal 1986; Kale 2002).

Around 200 plants species were selected for biomass production in their natural habitat, and the following plant species were suitable for soil biomass production. These plants included *Echinops echinatus* Roxb.; *Verbesina encelioides* (Cav.) Benth. & Hook.; *Calotropis procerag* (Ait) R.Br.; *Leptadenia pyrotechnica* (Forsk.) Decne.; *Sericostoma pauciflorum* Stocks.; *Amaranthus spinosus* Linn.; *Withania somnifera* (L.) Dunal.; *Lepidagathis trinervis* Wall. ex Nees.; *Lantana indica* Roxb.; *Aerva tomentosa* (Burm.) Juss.; *Croton bonplandianum* Baill.; *Abutilon indicum*

(L.) Sweet.; *Acacia jacquemontii* Benth.; *Crotalaria burhia* Buch.-Ham. ex Benth.; *Saccharum bengalense* Retz.; and *Artemisia scoparia* Waldst. et Kit. Although these plant species grow in wasteland they have great potential as colonizers and maintain their ecosystem.

The biomass production changes with seasons. The Rajasthan state is categorized as arid and semiarid region. Several plant species appear in the region during their respective growth periods. Major plant species are small herbs which grow during rainy season. They are the first colonizers which have important uses (Woodard and Prine 1993; Morgana et al. 1994, Houerou and Houerou 2000). As mentioned above some shrubs and perennial herbs are significant for biomass production and can be used as an alternative source for bioenergy (Woodard and Prine 1993; Pedreira et al. 1999; Vazquez-de-Aldana et al. 2000).

The following are the different colonizers that appear in these wastelands:

4.3.6 The Early Colonizers

Some of the early colonizers include small ephemerals *P. erioptera*, *P. corymbosa*, *G. phamacioides*, *M. cerviana*, *S. ovals*, *C. tridens*, *T. pentandra*, *I .sessiliflora*, and *I. linnaei* Ali. These plant species are the initial colonizers and are not suitable as biomass resources because their yield potential is very low. These early colonizers provide helpful association for any subsequent plants to come in the succession like *A. scoparia*, *F. hamiltonii*, *T. purpurea*, *C. colocynthis*, *B. diffuse*, and other herbs. Among the shrub species which come in the next season include *L. pyrotechnica*, *C. procera*, *S. cordifolia*, *C. burhia*, *V. encelioides*, and *S. munja* (grass) which were abundant. In the second year of growth, the tree species became dominant and undergrowth diminished to some species.

4.3.7 Initial Association

Initial plant associations appeared to benefit each other. These associations included *C. procera* with nitrogen-fixing *C. burhia*. Besides this at a later stage, the nitrogen-fixing *T. purpurea* was largely predominant along with other plants like *V. encelioides*, *A. scoparia*, *S. pauciflorum*, *S. cordifolia*, *C. burhia*, and *B. diffuse*. The biomass productivity ranged from 0.5 tons per ha (*C. colocynthis*) to 52 tons of dry matter per ha per annum (*Saccharum munja*). A combination of these plants could be used to form a three-tier system to colonize the wasteland and get productive biomass as an alternative model to the hydrocarbon-yielding plants.

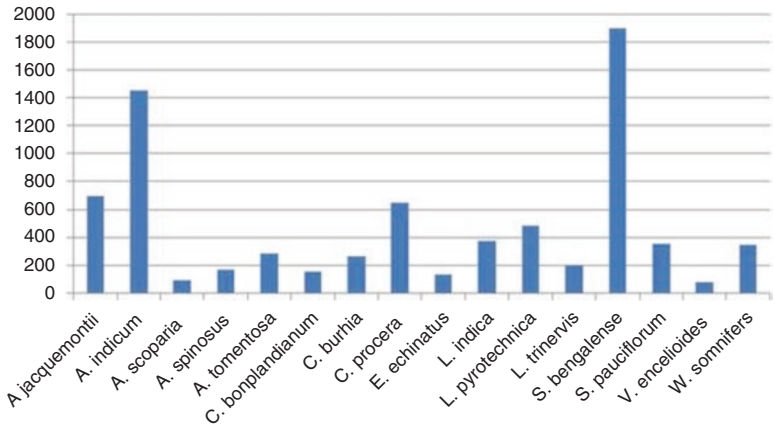

Fig. 4.1 Biomass of wasteland colonizer weight in gram per plant

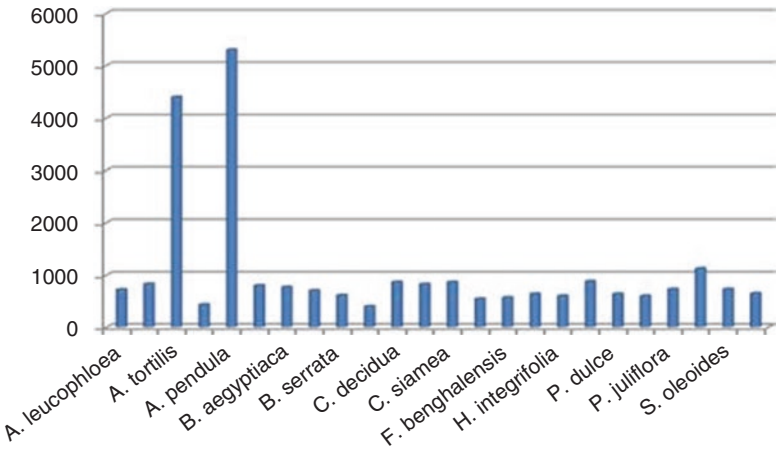

Fig. 4.2 Weight of tree species in kilogram per cube meter

4.3.8 Some Other Associations

A. scoparia, *B. diffuse*, and *C. colocynthis* largely cover the ground throughout the year due to their xerophytic adaptation which makes good association with these plants *C. bonplandianum*, *E. hirta*, *H. marifolium*, *P. angustifolia*, *P. corymbosa*, *R. purpurea*, *S. cordifolia*, *S. pauciflorum*, and *V. encelioides* (Figs. 4.1 and 4.2).

4.4 Conclusion

The present finding is based on the survey of plant biodiversity of different wasteland sites over a period of 5 years in arid and semiarid regions of Rajasthan state; regular and periodical visits to different sites and their seasonal appearances were also recorded (Kotia and Kumar 2001a, b, c, d). Around 200 plant species are suitable as an alternative sources of biomass. Among them, around 50 species are of high fodder and high medicinal value in Ayurvedic system (Jain 1963, 1991; Kotia and Kumar 2001a, b, c, d), while some of them are used as fodders and others provide edible fruits. Besides biomass, these plant species have great potential as nonedible oil, gums, resins, tannins, dyes, fibers and other uses.

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Predicting Biomass Production from Plant Robustness and Germination Efficiency by Calorespirometry

5

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Abstract

Respiration traits allow calculating temperature-dependent carbon use efficiency and can serve as biomarkers for the prediction of growth and biomass formation. While photosynthesis is responsible for capturing CO_2, respiration critically manages the destiny of structurally integrated CO_2 by regulating the use of energy and substances. The efficient interplay of cytochrome and alternative respiration pathways determines plant performance upon permanently changing and interacting abiotic and biotic environment. Thus, respiration traits are central for high biomass production and yield stability based on multi-stress tolerance. Hence, calorespirometry is a useful functional tool for pre-breeding that can

Dedicated to Lee Hansen in honor of his great contribution to science by driving technology development and supporting its application.

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discriminate plants based on genetic differences in respiration traits. Although it was earlier suggested that the methodology could be valuable in breeding programs to improve temperature-dependent growth performance, this concept had never been applied in global breeding on biomass production. This predictive tool can be applied as an efficient mean (1) to identify differences in germination efficiency among genotypes or through management practice in seed technology, (2) to select plants in conventional breeding, and (3) to identify relevant genomics-based functional markers for temperature-dependent multi-stress tolerance and yield stability. From respiration-related genes, alternative oxidase is a promising candidate for functional marker development. It relates to both germination efficiency and plant robustness linked to biomass yield stability.

Keywords
Calorespirometry • Alternative respiration • Germination efficiency • Genomics • Functional marker development

5.1 Introduction

Breeding on high-level and stable plant biomass production is important, when (a) biomass is directly used as harvest material for specific purposes, such as for fuels (see other chapters in this book), or (b) when biomass can serve as an indirect trait that is associated to final crop production, such as grain yield. The level of biomass production can strongly vary depending on environmental circumstances, which is threatening yield stability. Temperature is a major factor in this scenario. Thus, climate change as well as regional-specific temperature distribution are important issues. However, temperature needs to be seen in the context. It interacts permanently with the complex and variable diversity of abiotic and biotic factors with chaotic effects on plant growth performance. Depending on the region, individual factors, such as water and nutrient availability, can play a minor or major role and also vary in intensity during the plants life cycle. Plant genotypes that are more robust, which means they can tolerate a wider range of diverse and complex environmental conditions, will be more stable in growth performance. Therefore, it is important to recognize robustness as a novel breeding trait linked to yield stability (Arnholdt-Schmitt et al. 2006; Cardoso and Arnholdt-Schmitt 2013). Robustness is based on the capacity to respond upon environmental changes by highly adaptive, molecular-physiological plasticity.

In order to know whether a plant genotype is robust and shows ‘stability in biomass production’, testing of many different environments is required (e.g., Mühleisen et al. 2014). The term ‘environmental condition’ must consider also the availability and effectiveness of native endophytes, such as AMFs, *Rhizobia*, bacteria, and others. Also management practices need to be considered, such as pretreatment of seeds, soil management, irrigation, fertilizing, endophyte inoculation, and pesticide or other chemical inputs. Therefore, testing of environmental conditions is highly time-consuming, costly, and labor-intensive. So far, no reliable

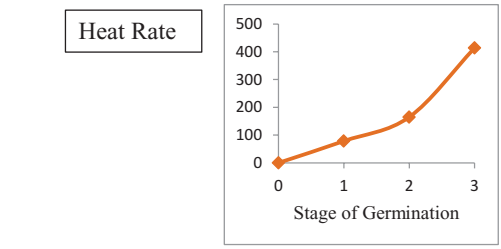
method exists that can reproducibly predict complex multi-stress tolerance that leads to robustness of plants in a satisfying way. Recently developed methods for high-throughput phenotyping platforms tend to be of high cost and therefore turn themselves to be questionable in view of practical significance for private breeders and agriculture. Translation of extremely expensive genomics data to plant improvement strategies remains a challenge with no clear perspective for efficient and low-cost options. Thus, tool development with high predictive power is supposed to lead to breakthrough advancement for the economic efficiency of prebreeding efforts.

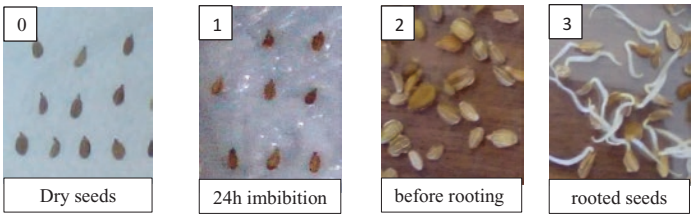
5.1.1 Calorespirometry for Prediction of Germination Efficiency and Seedling Vigor

Germination efficiency varies between plant species. It is supposed that evolutionary fitness is closely linked to germination efficiency (Parsons 2012). Also, it is widely known that germination efficiency in agriculture and horticulture practice can have relevant effects on final yields (e.g., Tian et al. 2014). Related to forestry, it was shown in *Pinus ponderosa* that metabolic heat rate measurements were the most important seedling characteristic that allowed predicting mature-tree performance. Increased metabolic heat rate in seedlings corresponded with greater vigor of mature trees (Momen et al. 2004). However, so far, it is not common to explore improvement in germination efficiency through breeding.

Germination efficiency concerns both (a) induction of germination linked to speed and (b) seedling growth and development. Recently, molecular research efforts are increased to identify genetic components linked to germination (e.g., Wang et al. 2016; Yuan et al. 2016a, b). In agricultural research, special focus is dedicated to improve germination efficiency through management practices via diverse physical pretreatment strategies that aim to substitute chemicals (reviewed in Paparella et al. 2015; De Sousa et al. 2016). Magnetic seed treatment, which provokes changes in energy status of seed cells and molecules, seems to be especially promising as a general strategy across species (e.g., De Souza et al. 2006, 2014; Matwijczuk et al. 2011; Paparella et al. 2015).

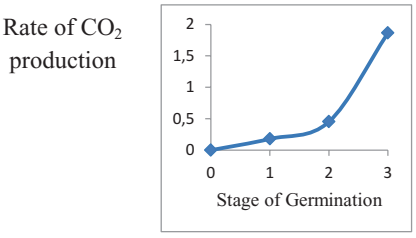
Water control and a temperature-dependent change in energy metabolism are of key importance for inducing germination. Germination speed depends on the species. In carrot, germination is slow. Thus, competition through rapidly growing weed can have serious effects on yield-determining carrot growth performance. After imbibition, mitochondria and thus respiration are immediately activated (Paszkiewicz et al. 2017). Therefore, it is reasonable to assume that calorespirometry provides an efficient tool for monitoring and predicting the germination process. Calorespirometry can measure temperature-dependent heat rate changes and CO_2 production (Hansen et al. 2005). Figure 5.1 demonstrates application of the methodology and shows calorespirometry data for carrot seed germination measured at 25 °C. From measured values of heat rate and the rate of CO_2 production, the oxycaloric equivalent and carbon use efficiency can be calculated, and the rate of

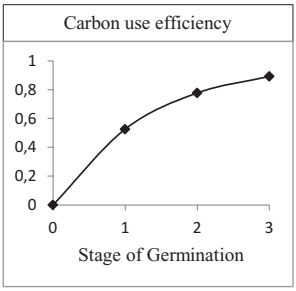


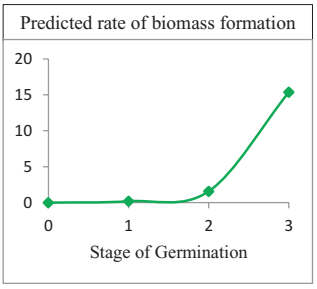


Rate of CO_2 production in dry seeds:

1. 40 seeds: 0.06
2. 100 seeds: 0.16
3. 200 seeds: 0.27
4. 400 seeds: 0.41







Method for calculating carbon use efficiency and rate of biomass formation described in Hansen et al. 2005; Arnholdt-Schmitt 2017

Fig. 5.1 Measuring metabolic heat rate (in μJ s^{-1}) and CO_2 production rate (in $mmols^{-1}$) of germinating carrot seeds by calorespirometry

biomass formation can be predicted (see detailed step-by-step protocol in Arnholdt-Schmitt 2017). Consequently, when performed under standardized conditions, calorespirometry can be used for both (1) developing biomarker for genetic discrimination and (2) evaluating seed technology strategies.

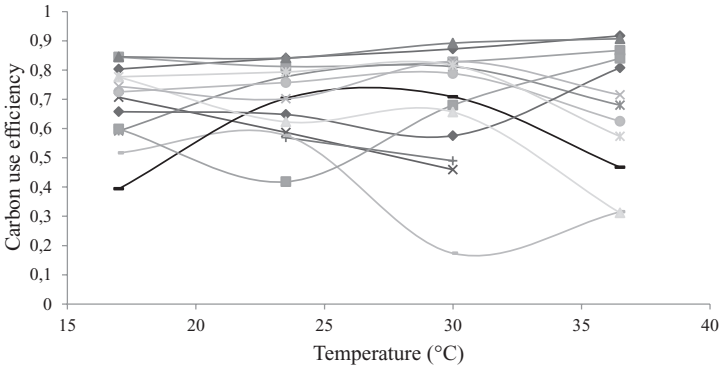
5.1.2 Calorespirometry for Genotype Selection on Stable Biomass Production

Calorespirometry was developed as a tool to understand how plant metabolism acclimates and responds to changes in environment by adapted growth performance (Hansen et al. 1997, 2005). A literature review showed the dominant role of temperature on carbon use efficiency when measured in growing plant material (Hansen et al. 2009). The congruency between temperature-dependent plant growth performance curves and the temperature distribution curve specific for a region can strongly influence the fitness of plants in view of biomass production in that region (Arnholdt-Schmitt et al. 2016). Thus, regional-specific selection of cultivars with defined temperature-dependent growth performance curves and climate change will affect regional biomass production. It was earlier suggested that calorespirometry could be helpful in selecting plants for temperature-dependent growth performance (Hansen et al. 1997; Taylor et al. 1998). No other method can do these measurements and prediction in a rapid way. However, the basic concept was never translated and applied into a breeding tool for major crops.

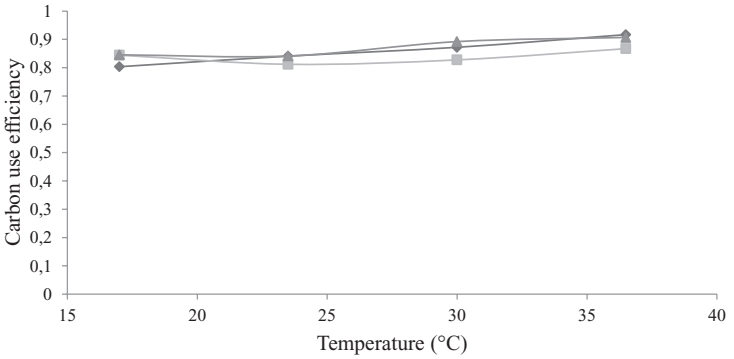
Biomass production is species-dependent. It relies on the response of target meristems for biomass production to developmental and environmental signaling. The development of calorespirometry as a pre-breeding tool was firstly promoted for carrot (Nogales et al. 2013, 2014). By using a small number of inbred lines, calorespirometry could be shown to identify genotype-specific optimum temperatures and low-temperature limits for tap root biomass growth. The approach was now advanced to diverse crop species, and the method is currently under final validation in the lab of the corresponding author. The '***T***emperature-dependent ***G***rowth ***E***fficiency pattern index' (TGE pattern index) is here introduced as a novel trait for the characterization of genotypes. Figure 5.2a, b gives examples, where calorespirometry was applied to spring wheat cultivars. Figure 5.2a demonstrates the potential of the methodology to indicate genotypes with higher yield stability. TGE pattern indices are compared between genotypes with differences in yield stability (Fig. 5.2a A, B). In Fig. 5.2b, it can be seen that calorespirometry data can help to identify strategies for within-cultivar improvement through selecting at both temperature extremes of the genotype-specific optimal growing temperatures. This confirms results obtained already for carrot tap root biomass yields (Nogales et al. 2014). Figure 5.3 shows that winter cereals (here winter barley) can be easily discriminated from spring cereals already at seedling stage by rapid TGE pattern indexing at higher temperatures (Fig. 5.2). It also seems to justify the stable standing of reference barley cultivars in the market through a higher and more stable temperature-dependent growth efficiency pattern in comparison to breeding lines.

a

A. TGE-Pattern-Index pointing to genetic variation among cultivars with known diversity in yield stability



B. TGE-Pattern-Indices of three cultivars with high yield stability in the field



b

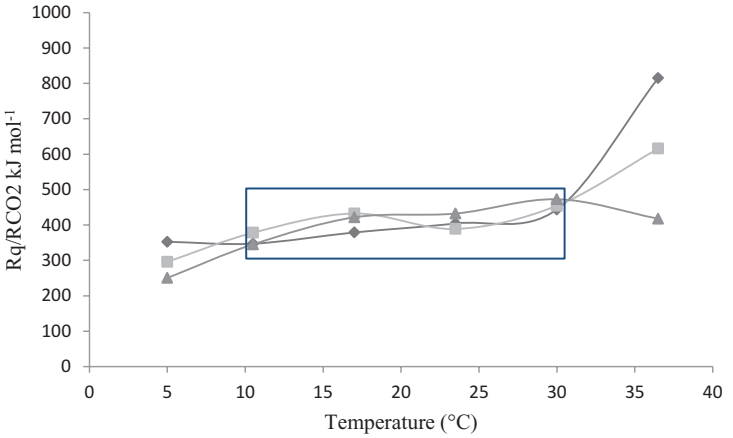
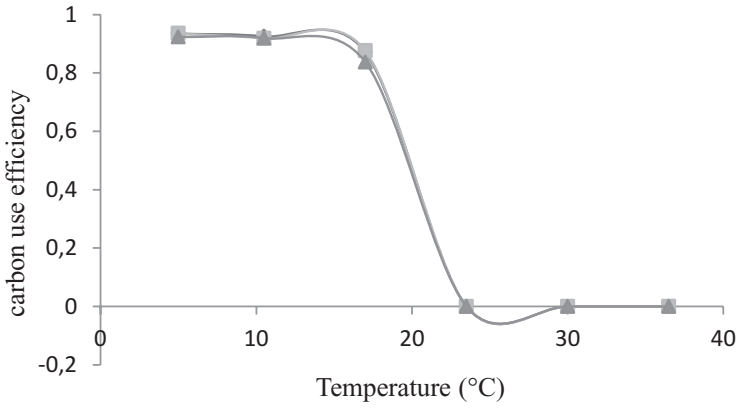


Fig. 5.2 (**a**) 'Temperature-dependent growth efficiency pattern indices' of spring wheat cultivars (TGE pattern indices). Unpublished preliminary results (Arnholdt-Schmitt) shown here to highlight the capacity of calorespirometry for predicting yield performance of cereal genotypes. (A) A TGE pattern index pointing to genetic variation among cultivars with known diversity in yield stability. (B) TGE pattern indices of three cultivars with high-yield stability in the field. (**b**) Temperature-dependent genetic variation identified within a spring wheat cultivar by the help of calorespirometry. The three curves present each a bulked sample of six seedlings (Arnholdt-Schmitt, unpublished)

A. TGE-Pattern-Index for three reference cultivars



B. TGE-Pattern-Index for breeding lines in comparison to the three reference cultivars

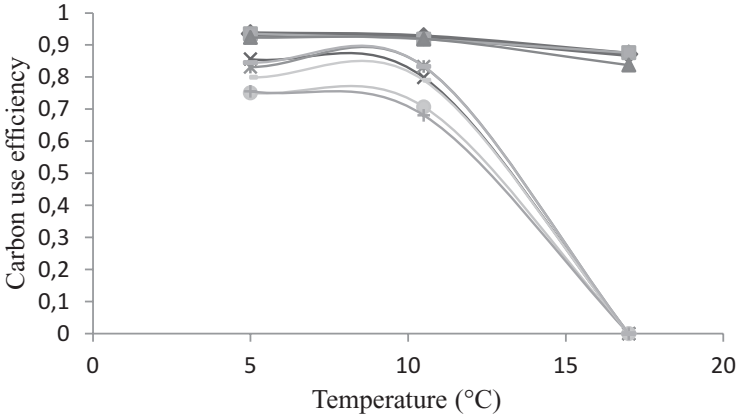


Fig. 5.3 TGE pattern indices of three reference cultivars (**a** and **b**: *upper curves*) and breeding material from winter barley (**b**: *lower curves*); unpublished preliminary results (Arnholdt-Schmitt) shown here to highlight the capacity of calorespirometry as a pre-breeding tool in cereal breeding; TGE pattern indexing discriminates genotypes by (1) level and (2) pattern of temperature-dependent carbon use efficiency. (**a**) TGE pattern index for three reference cultivars. (**b**) TGE pattern index for breeding lines in comparison to the three reference cultivars

5.1.3 Calorespirometry: A Tool for Evaluating the Relevance of Genomics-Based Data

As shown before, calorespirometry is appropriate to select plants in conventional breeding. Thus, the method allows selecting plants of any genetic background for effects on growth performance and yield stability. Consequently, it can be used as functional tool for validating all kinds of genomics-based candidate markers for their effect on biomass production. In the same way, calorespirometry could be applied for functional analysis of holobionts to confirm the effect of plant-microbiome interaction (Arnholdt-Schmitt et al. 2014; Nogales et al. 2016).

Most critical for applying calorespirometry is deep knowledge in physiology. This is important to establish a species-dependent, sophisticated strategy for choosing the correct target meristems and developmental stages for measuring. Data need to be retrieved from the same tissue simultaneously for genomics and for calorespirometry. For example, in order to link genomics data and predictive calorespirometry for carrot root biomass production, analyses need to be performed in the central tap root meristem, the root cambium, where 'the decisions' for secondary root growth are taken through cell identity and complex metabolic regulation in interaction with environment (Arnholdt-Schmitt 1995, 1999, 2004, 2005a, b; Arnholdt-Schmitt et al. 2006; Nogales et al. 2013, 2014). On the other hand, for predicting biomass production in cereals, calorespirometry needs to be performed in germinating seeds, when the focus is on germination efficiency or in growing tissue of seedlings in order to predict yield stability by the TGE pattern index.

Photosynthesis is the main focus when aiming to improve biomass production by breeding. However, it is becoming increasingly evident that mitochondria are the first to respond upon environmental signaling. Respiration with a special relevance of alternative oxidase seems to be critically involved in regulating the efficiency also of photosynthesis (Dinakar et al. 2016).

Respiration traits and, especially, the alternative oxidase (AOX) have been proposed as marker sources for molecular breeding on plant robustness (Arnholdt-Schmitt et al. 2006; Cardoso and Arnholdt-Schmitt 2013: Arnholdt-Schmitt 2017). It is increasingly accepted that adaptive respiration and the genes involved in normal and alternative respiration pathways have bottleneck function for molecular and metabolic cell reprogramming under abiotic and biotic stressful conditions (reviewed in Vanlerberghe 2013; Arnholdt-Schmitt et al. 2015). AOX is the key enzyme of alternative respiration. It consists of a small family of genes that belong either to subfamily *AOX1* or *AOX2* (Costa et al. 2014, 2017). Recently, it could be shown in primary carrot cultures that stress-induced accumulation of *DcAOX1* and *DcAOX2a* transcripts during the lag phase of adaptive cell reprogramming coincided with a critical time point for structural biomass prediction performed by calorespirometry (Campos et al. 2016).

Cytochrome c oxidase (COX) and AOX use natural oxygen isotopes O^{16} and O^{18} to a different extent (Guy et al. 1989; Ribas-Carbo et al. 2005). This allows calculating from oxygen consumption and isotope analysis the extent of normal and alternative respiration. Combining calorespirometry in a tool kit with oxygen consumption

measurements and oxygen isotope discrimination will enable to identify candidate genes from respiration for a given meristem or growing plant material that determine biomass growth (Arnholdt-Schmitt et al. 2016). Once the higher relevance of one or the other respiration pathways or of both is confirmed, functional markers can be identified in the candidate gene. Alternatively, gene editing could be applied to introduce target polymorphisms. In any case, selected plants that contain defined functional markers or marker patterns could then be evaluated by TGE pattern indexing as described before.

There are some indications in literature that *AOX* genes might also play a relevant role for regulating germination efficiency. Yentur and Leopold (1976) observed a transition from predominantly alternative respiration at the initial phase of germination to normal respiration after imbibition between 4 and 8 h in soybean. Alternative respiration was linked to germination itself but also to the rate of seedling growth and chlorophyll synthesis. Similar results were reported for other species also (Yentur and Leopold 1976; Esashi et al. 1981). Germination in its early stages was also shown to be sensitive to lower O_2 tension. This supports the relevance of alternative respiration at that time, since AOX has a lower affinity to O_2 than COX (Bonner 1973; cited in Yentur and Leopold 1976). In dry and mature seeds of *Arabidopsis thaliana* as well as during early germination, Saisho et al. (2001) and Clifton et al. (2006) found high expression of *AOX2*. The level of *AOX2* transcript accumulation decreased rapidly during germination (after 12 h) and remained low thereafter. Also in seedlings of *Hypericum perforatum* expression of *AOX2* was found to be stably low during the post-germinative phase (Velada et al. 2016). *AOX1* transcripts measured in the whole seedling without discriminating tissue-specific values increase in later stages in both *Arabidopsis* thaliana and *Hypericum perforatum*. *AOX1* was increased in *Arabidopsis thaliana* from 48 h after imbibition pointing to a differential role of both *AOX* genes. Nevertheless, the capacity of AOX was related to both AOX1 and AOX2 (Saisho et al. 2001). On the other side, cytochrome c oxidase mRNA levels increased continuously and corresponded to the increasing capacity of the COX pathway during germination and seedling growth. A bioinformatics search that we performed in recently published transcriptome data (Klepikova et al. 2016) underlines the importance of targeting tissue-specific transcription (Table 5.1). It confirms high expression of *AOX2* in dry seeds of *Arabidopsis thaliana* and also a rapid downregulation of *AOX2* early during germination, while *AOX1* genes and here especially *AOX1a* are simultaneously upregulated to the former high level of *AOX2*. This differential regulation of isoenzyme expression patterns might be linked to metabolite-specific control mechanisms through the change to an aerobic situation at germination (see in Costa et al. 2009). In dry seeds, fermentation is dominant (Botha et al. 1992), and the rate of CO_2 production is low (see Fig. 5.1), while after imbibition, mitochondria are activated. Whether there are differential impacts of both isozymes on the protective role of AOX against upcoming oxidative stress during mitochondrial respiration remains open. However, when seedling growth starts *AOX1* transcript levels explored at seedling meristems, cotyledons and hypocotyls are markedly decreased, and the lowest amount of *AOX* transcripts is found in the growing tissue, the seedling meristems (Table 5.1). This result is in

Table 5.1 Expression profile of alternative oxidase (AOX) genes of *Arabidopsis thaliana* in dry seeds, during seed germination, and in seedling tissues (meristem, cotyledons, hypocotyl, and root)

	Genes				
Conditions	*AtAOX1a*	*AtAOX1b*	*AtAOX1c*	*AtAOX1d*	*AtAOX2*
Dry seeds	33	2	0.00	33	2357
Germinating seeds 1 (first day after soaking)	155	0.39	99	0.39	216
Germinating seeds 2 (second day after soaking)	615	0.78	42	0.61	39
Germinating seeds 3 (third day after soaking)	2752	3	10	1	30
Seedling meristem	118	0.00	17	8	0.00
Seedling cotyledon	564	0.00	28	1	1
Seedling hypocotyl	767	0.00	0.00	5	0.00
Seedling root	511	0.00	4	3	3

agreement with former studies of Sieger et al. (2005) on transgene tobacco. These authors found that AOX1 regulates carbon use efficiency. They observed that increased AOX1 expression can be related to the suppression of growth. Suppression of yeast growth through AOX1 was observed also when yeast was transformed by the carrot *AOX1* gene depending on its concentration and a situation of mainly aerobic mitochondrial respiration (Arnholdt-Schmitt and Kumar Patil 2017). In summary, the available knowledge and presented bioinformatics data on respiration and respiration-related gene expression during germination allow hypothesizing that AOX genes can be interesting candidates for functional marker development linked to early steps in germination and to seedling growth. Recently, it was found that AOX might also have crucial function on optimizing structural interaction between plants and endophytes (Mercy et al. 2017), which can be especially important at seedling stage.

Data are read counts (derived from RNA-seq analyses) normalized by median-of-ratio method as described in Anders and Huber (2010). The data were accessed using the TRAVA (transcriptome variation analysis across different organs and developmental stages in *Arabidopsis*) webserver [http://travadb.org (Klepikova et al. 2016)] as well as the gene Ids of *AtAOX1a* (AT3G22370), *AtAOX1b* (AT3G22360), *AtAOX1c* (AT3G27620), *AtAOX1d* (AT1G32350), and *AtAOX2* (AT5G64210).

5.2 Conclusion

Seed germination efficiency and plant robustness are important for high-level and stable biomass production. For both traits, respiration plays essential role that links to evolutionary fitness as driver for biodiversity. Genetic variation in respiration traits can be explored to support sustainable agriculture with low chemical input. Calorespirometry is promising as a predictive functional tool to advance early plant

selection for biomass production in conventional and molecular breeding. Genomics-based markers of any kind can be easily evaluated. Functional markers developed from *AOX* genes seem to be promising for improving biomass production. A vast number of studies across different species have been published during the last decade that indicate high genetic sequence variability in *AOX* genes (see publications at www.eu_chair.uevora.pt). Additional data from a diversity of species can be retrieved and explored from public and private data banks. The predictive power of calorespirometry can be used to validate whether AOX activity and *AOX* gene polymorphisms have relevant effects on biomass production.

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Appropriate Rural Technologies: Agricultural Waste to Charcoal and Strategies for Biogas Production from Organic Garbage

6

Anand D. Karve

Abstract

Abstract for Sect. 6.1

Agricultural Waste to Charcoal: Two cottage scale processes have been described for pyrolysing agricultural waste into charcoal. One of the processes uses a kiln working on the "oven and retort" principle, and the other one uses the "top-lit, updraft" principle. Because charred agricultural waste is powdery in nature, it is mixed with a binder and extruded into briquettes. Currently, more than 100 organizations in India and abroad are making charcoal by using these processes. A team of four persons can produce daily about 70–80 kg briquettes. Working for about 200 days in a year, in the post-monsoon period, this team can make about 15 tons of char briquettes having a market value of about Rs.400,000, but the process can be easily scaled up without increasing the manpower component. Sugarcane trash was found to be ideal for continuous supply of raw material, but one can also use leaf litter from roadside trees, plantations and forests. The charcoal briquettes made by this method can be used as cooking fuel, as industrial fuel or also to replace metallurgical grade coke. The charcoal can also be applied to the soil for raising soil fertility. Efforts are on at the author's organization to convert the charcoal into value-added products like active charcoal and water gas.

Abstract for Sect. 6.7

A Strategy for Biogas Production from Organic Garbage: In spite of new knowledge gained since the advent of the twenty-first century, the biogas researchers still use some of the older concepts. The new concepts pointed out in this article are:

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1. Because the biogas-producing microbes reside in the intestines of animals, they eat what the animals eat.
2. Breeding super-methanogens can be achieved, but their use in a biogas plant would not be practical, as it would be impossible to maintain them in an open system like a biogas plant.
3. All animals represent living biogas plants. The faecal matter of animals is therefore effluent slurry of a biogas plant. The biogas-producing microbes occur in the faecal matter of animals because they exit the animal body along with dung.
4. The anaerobic microbes extract the chemically bound oxygen from their feedstock and use it in their metabolism. Therefore, feedstock of a biogas plant must have oxygen in its chemical makeup. Since animals represent living biogas plants, the same is also true of food eaten by animals.
5. The value called % volatile solids actually represents the % digestibility of the concerned substance.
6. The digestibility of the feedstock is more important than the C/N ratio of a feedstock.
7. In the case of material which is difficult to be digested under anaerobic conditions, use is made of biphasic digestion, in which the feedstock is first treated aerobically and then subjected to anaerobic digestion. This is wrong, because most of the organic carbon, which would have yielded methane in the anaerobic phase, gets oxidized in the aerobic phase itself, leading to drastic reduction in the methane yield.

Keywords
Keywords for Sect. 6.1
Char briquettes • Pyrolysis • Use of char
Keywords for Sect. 6.7
Biphasic digestion • C/N ratio • V.s.%

6.1 Introduction

India generates annually about 800 million tons of agricultural waste, having more or less the same energy as an equivalent quantity of wood. It cannot be used directly as fuel, because of its low density. Currently, this waste is compressed into briquettes, which are sold as boiler fuel at Rs.10,000 per ton. Although commonly called “white coal”, these briquettes resemble wood in their physico-chemical properties. There is good demand for biomass briquettes in the industrial belt of Western Maharashtra. As boiler fuel, 3 kg briquettes, worth Rs.30, can replace a litre of fuel oil costing about Rs.60. It is logical to expect farmers who have agricultural waste to convert it into briquettes, but the high capital cost of the briquetting machinery and the requirement of a three-phase, 50 kW electricity connection prevent farmers from doing so.

6.2 Charring and Torrefaction of Agro-Waste

Industry also needs mineral coal, and charcoal can substitute it. Scientists of the Appropriate Rural Technology Institute (ARTI) developed in the year 1996 a cottage scale process for converting agricultural waste into charcoal. In this process, biomass filled into metallic barrels is pyrolysed by heating the barrels from outside. At a temperature >300 °C, biomass undergoes a process called pyrolysis, whereby about 70% of it decomposes into a volatile fraction called pyrolysis gas, leaving behind 30% as a solid fraction, called charcoal. In the process developed at ARTI, it was possible to control the degree of pyrolysis by controlling the period of time over which the barrels are heated (Karve et al. 2001). The practice of terminating pyrolysis prematurely is called torrefaction. Torrefaction yields more charcoal than complete pyrolysis, but torrefied charcoal is of an inferior quality because it still retains some of the volatiles. Because agricultural waste is light, its charcoal too is powdery and light. Using starch paste as a binder, the char is densified into briquettes, by means of an extruder. Although extruders of industrial scale are available in the market, ARTI developed a small one which uses a 1 h.p. motor, running on a 220 volt single-phase domestic electrical connection. The kiln for charring the biomass and a small briquette extruder together cost only about Rs.25,000. At this cost, charring agricultural waste and briquetting the char become a viable business for a rural family. Two persons in the family, operating two kilns in tandem, and two other persons operating the extruder can produce daily about 75–80 kg charcoal briquettes. Excluding 4 months of the rainy season, it is possible to conduct this operation for about 200 days in a year, to produce about 15 tons of char briquettes, saleable at about Rs.400,000. Availability of agricultural waste in sufficient quantity can be a limiting factor in some areas, but at least in the sugar belt of Maharashtra, sugarcane trash is plentifully available in the sugarcane harvesting season. Leaf litter from roadside trees, plantations, and forests is also a potential source of raw material for this business (Figs. 6.1, 6.2, 6.3, 6.4, 6.5, and 6.6).

Fig. 6.1 An "oven and retort"-type kiln for charring agricultural waste

Fig. 6.2 Kiln working on TLUD principle (Photograph courtesy of Samuchit Enviro Tech, Pune, India)

Fig. 6.3 A briquette extruder in operation

6.3 The TLUD Process of Making Charcoal

In 2006, a new type of wood burning stove was personally demonstrated to the staff members of ARTI by Prof. Paul Anderson from the United States of America. The fuel chamber of the stove is filled with wood chips, and the pile is ignited at the top. Air required for burning the wood is provided through holes at the bottom of the combustion chamber. The heat generated by the burning wood pyrolyses the wood chips, and the pyrolysis gas produced by the wood is provided with additional fresh air to burn it as fuel in the same stove. The charcoal that is produced due to pyrolysis

Fig. 6.4 Sarai cooker system for using char briquettes as fuel (Photograph courtesy of Samuchit Enviro Tech, Pune, India)

Fig. 6.5 Individual components of Sarai cooker system (Photograph courtesy of Samuchit Enviro Tech, Pune, India)

Fig. 6.6 An urban domestic biogas plant (Photograph courtesy of Samuchit Enviro Tech, Pune, India)

is left behind in the fuel chamber. Because of good mixing of air with the pyrolysis gas, this stove gives a surprisingly clean fire, without any smoke at all. This stove gives a charcoal yield of about 30% of the weight of the wood chips. Because the wood chips are ignited at the top of the heap, and because air is supplied from the bottom, this stove is called top-lit updraft stove, or TLUD stove. Based on this principle, ARTI developed a stove named Sampada (wealth), which uses waste woody biomass as fuel (e.g. coconut shells, coconut fronds, corn cobs, mango kernels, hulls of legume pods, twigs, seeds, bark of trees, etc.). While cooking the food, the stove simultaneously generates charcoal, a high-value fuel. The disadvantage of Sampada is that the fire intensity cannot be easily controlled. It is therefore primarily used for heating bath water.

In the year 2013, the author developed a charring kiln based on this principle. Its fuel chamber has a capacity of 100 liter. If filled with wood, it takes about 45 min to char it, whereas light biomass like leaf litter, sugarcane trash, twigs, hulls of legumes, etc. is pyrolysed in just about 15 min. A clear and smokeless flame emerges from this kiln, so that one can also use the kiln as a stove for cooking a meal. A video showing the operation of this kiln can be seen by activating the YouTube link provided in the website, www.samuchit.com. More than 100 kilns of this type are currently in operation in India and abroad.

6.4 Using Briquetted Charcoal as Cooking Fuel

Charcoal burns very cleanly, without producing any smoke or soot. It is therefore valued as cooking fuel. Along with the kiln and the extruder, ARTI also developed a non-pressurized steam cooker, which uses just 100–150 g charcoal for cooking a complete meal consisting of rice, dal, vegetables, meat, etc. for a family of five. The cooker became an instant success, because once the cooker has been placed on its built-in charcoal stove, the housewife can do any other chore, or even leave the house for an hour or two, because once the charcoal in the stove has burned itself out, the fire gets extinguished. This cooker comes in 4 models, small for 2 persons, medium for 4–5 persons, large for 8–10 persons and Jumbo for 25 persons.

In the year 2002, Appropriate Rural Technology Institute received the Ashden Award for Renewable Energy, for developing the technologies described above, namely, charring kiln, briquette extruder and the steam cooker.

6.5 Other Uses

The fully pyrolysed charcoal produced by either of these methods is devoid of volatiles, and it burns very cleanly, without any smoke or soot. Being free from volatiles, it is equivalent to metallurgical grade coke, which costs about Rs.35 per kg in Pune. The industries using coke require it in relatively large quantities. The present process of charring and making char briquettes has been designed for operation on a small scale. The reason for opting for the small size of the kilns was to make them

portable. It was assumed that the kilns would be taken to the sites where agricultural waste would be available. With larger kilns, one has to transport the biomass to the site where the kilns are situated. But the example of white coal has shown that if farmers get a remunerative price, they are willing to transport their agricultural waste to the processing factory at their own cost. The white coal manufacturers currently pay farmers Rs.2000–2500 per ton for the biomass delivered at the factory gate. With the present price of Rs.35,000 per ton of the metallurgical grade coke, one can very well afford to have large kilns and to pay farmers for the waste biomass delivered at the factory gate.

In the Himalayan Region, our process is being used for making charcoal briquettes from pine needles. These briquettes are used in special stoves, which burn day and night during winter, for room heating. In the forested areas of Eastern Maharashtra, attempts are being made under the aegis of the Department of Forests to use our process for charring fallen leaves of teak and to sell this char to manufacturers of *agarbatti* (incense sticks). Several of our kilns have been exported to Germany, where people use them for charring leaf litter, which is applied to the soil for increasing soil fertility.

If the charring process can be conducted at a higher temperature of about 800 °C, the resultant char can serve as active charcoal. Efforts are on at the author's organization to develop commercially viable methods of producing activated charcoal.

6.6 Future Prospects

Charcoal consists of molecular carbon and some of the minerals found in the original biomass. Before the modern process of iron smelting was introduced to India, iron was extracted from iron ore by heating powdered iron ore together with powdered charcoal, whereby the oxygen in the iron oxide combined with carbon in the charcoal to release metallic iron. Iron smelting was conducted on a cottage industry scale. Today, we export iron ore from Peninsular India, because of paucity of mineral coal in this region. However, with the availability of the technology of making charcoal from agricultural waste, we can reintroduce iron smelting as a cottage industry in India.

Char briquettes are currently sold primarily as cooking fuel, but by scaling the process up, the briquettes can also be sold to industries to supplement mineral coal, of which very little is available in Maharashtra. Maharashtra has 100,000 ha of sugarcane. Assuming that each ha produces 10 tons of trash, Maharashtra can supply 1 million tons of trash for making char briquettes. It should be noted that sugarcane trash is normally burned *in situ* by the farmers. By using our process, one can produce about 300,000 tons of charcoal from sugarcane trash alone. Our process can also be used for converting combustible urban solid waste into charcoal. One unit in Pune City uses our process to produce charcoal briquettes from empty hulls of green coconuts left behind after people have drunk the coconut water. The author was told that Pune City generates daily 30 tons of this material. In addition, the city also generates shells of mature coconuts in all households, restaurants and temples. There are many other sources of combustible dry waste such as seeds and kernels of

various fruits, bamboo and furniture waste from respective artisanal workshops, sugarcane bagasse from sugarcane juice vendors, corn cobs and husk from vendors of roasted cobs, leaf litter and pods from avenue trees, etc. They can all be used for making charcoal.

If steam is passed through charcoal heated to about 700 °C, it yields a combustible gas called water gas, which consists of a mixture of carbon monoxide and hydrogen. It can be used as fuel in internal combustion engines. If this process is properly developed, we can completely stop using petroleum-based fuels in transport.

6.7 Introduction

It had always intrigued the author as to why cattle dung is used as feedstock in biogas production. Other industrial fermentations (e.g. antibiotics, citric acid, as well as alcohol) use sugar. Trials in the year 2003, in which sugar was used as feedstock, showed that while one needed 40 kg cattle dung and 40 days time to produce 1 kg (about 800 l) biogas, just a kilogramme of sugar was enough to produce a kilogramme of biogas and that the microbes completed this reaction within just 24 h. Assuming the dry weight of dung to be 20 kg, my biogas system turned out to be 800 times as efficient as the traditional dung-based biogas system. Also starch, cellulose, powdered milk, vinegar, flour of cereals, flour of legumes, oilcake, etc. yielded 1 kg biogas per kg dry weight. I could thus show that anything that served as food for humans or animals proved to be a highly efficient feedstock for biogas production. Since my biogas plant did not use dung, I had a system which could be used as an urban domestic biogas plant which would yield biogas by consuming food waste. Dung was applied only once, at the beginning, as an inoculum. During the years 2003 and 2004, we installed about 40 biogas plants in and around the city of Pune. At that time, the traditional biogas scientists believed that anything that goes into a biogas plant must be accompanied by dung and therefore nobody believed in my results. Therefore, I could not publish my findings in any formal journal but wrote popular articles and gave talks on my concepts. Unfortunately, nobody in India paid heed to me, but my ideas found an echo abroad. In the year 2005, our Institute (Appropriate Rural Technology Institute) received, for this work, an award from the US Environmental Protection Agency, and in the year 2006, the Ashden Award for Renewable Energy. Later, Yale University (USA) invited me to interact with its final year MBA students who wanted to commercialize the urban domestic biogas plant. Invitations were also received by me to give lectures in Sri Lanka, South Africa, East Africa and Nepal.

6.8 Why Everybody Used Dung

Because microorganisms producing biogas are found universally in the dung of animals, it was assumed in the past that dung was the food of these organisms. But it is now known that being residents of the intestines of animals, these microbes eat

what the animals eat. They are found in dung because they exit the animal body along with the dung. While most of the bacteria in the guts of animals are facultative anaerobes, the methanogens are obligate anaerobes. The latter belong to a very ancient group of unicellular microbes called *Archaea*, which appeared on the earth about 4 billion (4×10^9) years ago, when there was no oxygen in our atmosphere. The photosynthetic organisms that evolved about half a billion years (5×10^8 years) later started to produce oxygen in its gaseous (or molecular) form, which proved to be toxic to the *Archaea*. The latter then retreated to sites having no free molecular oxygen. Guts of animals was one of the locations where these organisms found a safe refuge.

6.9 Utility of Super-Methanogens

It is quite common in the fermentation industry to develop highly productive microbes by using mutation breeding. Similar efforts are being made also in the case of methanogens, and many strains of so-called super-methanogens have been developed and patented. Such organisms are offered commercially by their respective developers as super-methanogens. This concept works in the case of antibiotics, because the antibiotics are produced under strictly sterile conditions. But it does not work in the case of biogas, because a biogas plant is not operated under sterile conditions. One cannot afford to spend money every day on sterilizing the garbage that goes into the biogas plant. As a result, the super-methanogens have to compete with the local microbes, which would soon outnumber and eliminate the super-microbes because nature does not care whether an organism produces more methane or less. It favours those organisms which have a high rate of multiplication.

6.10 Anaerobes Too Need Oxygen

Although a biogas plant represents an anaerobic system, the biogas-producing organisms do need oxygen like all living organisms. However, instead of obtaining it from air or from the oxygen dissolved in water, they obtain oxygen from their feedstock itself. Thus, by removing oxygen from carbohydrates, a biogas plant gives back to us methane, which is a hydrocarbon. A corollary of this statement is that a substance devoid of oxygen in its chemical makeup cannot serve as feedstock of a biogas plant. Since it was shown by us that the biogas-producing organisms eat what the animals eat, the rule of oxygen in the food is applicable even to animal food. That is why hydrocarbons and plastics cannot be digested either by a biogas plant or by an animal. The oxygen extracted from the feedstock is used by the methanogens in their own metabolism, resulting in the formation of carbon dioxide, which combines with water to form carbonic acid (H_2CO_3). Methanogens are so hungry for oxygen that they remove oxygen not only from a part of the H_2CO_3 but also from the nitrate and sulphate in the feedstock to convert them into their reduced forms, namely, ammonia (NH_3) and hydrogen sulphide (H_2S), both of which contribute to the foul odour of faecal matter. Ammonia and hydrogen sulphide are also

present in biogas, but when biogas burns, the malodorous substances get oxidized, and the foul odour vanishes.

6.11 Methanogens Are in Contact with Other Bacteria

The methanogens, being very primitive organisms, can digest only small organic molecules having just two atoms of carbon. The non-methanogenic organisms in the intestine degrade relatively complex organic substances which ultimately end up as acetic acid (CH_3COOH), having only two carbon atoms. In the presence of oxygen, these organisms would convert acetic acid into carbon dioxide and water, but in the absence of oxygen, this reaction stops at the stage of acetic acid. Methanogens generally attach themselves like parasites to the cells of the intestinal microflora and, taking up the acetic acid directly from the cells of the hosts, convert it into one molecule each of carbon dioxide and methane.

6.12 Animals Represent Living Biogas Plants

All animals act as living biogas plants, and their faecal matter represents their effluent slurry. It had always intrigued me why rural householders smear dung paste on the flooring, walls and even on their stoves. The rural householders assured me that dung plaster repelled flies. The fact however is that dung, being the end product of the process of digestion, has nothing in it that would serve as food to attract flies. This also explains why dung of ruminants is inefficient as feedstock for producing biogas, because there is practically nothing left in it to digest. 1 kg dung (dry weight) produces only about 50 g biogas, having an energy content of only about 200 kcal, whereas the same quantity of dry dung, if burned directly, would yield about 4000 kcal energy. One thus recovers only 5% of the original energy from dung, if it is transformed into biogas. Dung cakes, having a calorific value of about 4000 kcal per kg, are saleable as fuel in India. A family-sized biogas plant requires daily about 40 kg dung. In the form of dung cakes, it would fetch a daily income of Rs.70. Thus, only a fool would use dung as feedstock in his biogas plant. This explains why, out of an estimated 168 million rural households in India, only 2 million, or less than 2%, have working biogas plants.

6.13 Percent Volatile Solids Means Percent Digestibility

The percectage of biogas that can be obtained from any substance is denoted as its "volatile solids percentage" (v.s.%). It changes from feedstock to feedstock. Sugar, starch, cellulose, digestible proteins, fats, vegetable waxes and mucilage get completely converted into biogas, giving a v.s.% of 100%. Green leaves, on the other hand, have 80% moisture, and out of the moisture free dry matter, only 50% is digestible. Therefore, they exhibit v.s.% of only 10% on the basis of fresh weight.

These examples show that the v.s.% of a feedstock depends on the % digestibility of the feedstock. Laboratory methods are now available for estimating the in vitro dry matter digestibility (IVDMD) of any substance. The figures representing IVDMD match those of v.s. %.

Energy lost in the process of conversion from feedstock to biogas is relatively low in the case of substances having a high degree of digestibility. Thus a kg of sugar, having a calorific value of 4000 kcal/kg, yields 370 g methane, also having a calorific value of 4000 kcal. Dung, having low digestibility, would yield per kg dry weight only 18.5 g methane, having only 200-kcal energy. Therefore, a rule of thumb for selecting a feedstock for biogas generation is to verify if an animal would be able to digest that substance. If the answer is "yes", one can use that material as feedstock in a biogas plant. The "animal" in this case can even be an insect.

6.14 How Important Is C/N Ratio

Textbooks lay a lot of emphasis on a value called carbon/nitrogen (C/N) ratio of the feedstock. The textbooks state 25 to be the ideal C/N ratio. This is the C/N ratio of dung, but it is a relic of the past when dung was universally considered to be the ideal feedstock. It has already been discussed how dung, representing the effluent slurry of a living biogas plant, can hardly be considered to be the ideal feedstock. Sugars, starches, cellulose, fats and digestible proteins show 100% digestibility. Of these substances, sugars, starches, cellulose and fats have C/N = ∞, whereas the C/N ratio of proteins is just 4 or 5. And yet, all of them show the v.s.% value of 100. One should realize that as a living system, a biogas plant requires all the inorganic components that a living cell needs and that it is illogical to take only the nitrogen content of the system into consideration.

6.15 Biphasic Systems

The realization that some of the substances in the feedstock are digested only under aerobic conditions has given rise to using the so-called biphasic digestion. This system has two digesters, one aerobic and the other anaerobic. The biomass is first introduced into the aerobic digester, from where it is led into the anaerobic digester. It is argued that the material that does not get degraded under anaerobic conditions is degraded in the aerobic part of the system, reducing thereby the indigestible ballast that would unnecessarily enter the anaerobic digester. Experience however shows that the carbon, which would normally have yielded methane, gets converted into carbon dioxide in the aerobic digester, resulting in drastic reduction in biogas yield. Thus, if at all one has to use a biphasic digester, it is advisable that both the phases should be anaerobic.

The account given above covers mainly some traditional beliefs and practices with which the author does not entirely agree. The author feels that the biogas researchers take into consideration the new knowledge that has been gained since

the year 2000 and discard some of the misconceptions that are still being carried over from the last century.

6.16 Conclusions

Present paper tries to clear some misunderstandings about the biogas production in order to drive forward the movement initiated by Khadi and Village Industries Corporation in the last century. New concepts and clear understanding of biogas plants using organic garbage mentioned here are likely to solve many problems like disposal of urban biodegradable garbage, clean energy from biomass, etc.

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Lignocellulosic Feedstock Improvement for Biofuel Production Through Conventional Breeding and Biotechnology

7

Yuan-Yeu Yau and Mona Easterling

Abstract

Biofuels are the class of fuels derived from biological sources. Plant-based fuels are derived from renewable sources, produce less greenhouse gas (GHG) emissions than fossil fuels, and provide an attractive alternative to fossil fuels for future energy security. By using a variety of materials as biofuel-producing feedstocks, several generations of biofuels have been created. Lignocellulosic biomass is the most abundant organic material for biofuel production. Both the biomass yield of a biofuel crop and the cell wall components of the lignocellulosic biomass will determine the efficiency of overall biofuel production. This chapter presents details of biofuel production from its launch through three generations and includes discussion of their respective advantages and disadvantages. Carbon capture and storage (CCS) technology promises carbon-negative results in fourth-generation production, with the hope of mitigating climate change. Research involving conventional breeding, marker-assisted breeding, and transgenic breeding to improve quantity and quality of lignocellulosic biomass is explored. Advanced CRISPR/Cas9 technology for modification of cell walls in plants used as biomass sources is also highlighted. Case studies from credible, scientific journals are provided in all discussions.

Keywords

Algae • Biofuel • CCS • Cell wall • Cellulosic ethanol • CRISPR/Cas9 • Genetic transformation • Genome editing • GHG • Lignin • Lignocellulosic biomass • Metabolic engineering • Monolignol

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7.1 Introduction

Greenhouse gas (GHG) including O_3 and other species (e.g., black carbon) traps heat and triggers global warming, which effects global climate. Global climate change induces stress on crop and livestock production (Nelson et al. 2014; http://nca2014.globalchange.gov/report/sectors/agriculture). Reduction of GHG emissions can benefit global climate change (Unger et al. 2010). According to US Environmental Protection Agency (EPA), the largest source of GHG emissions from human activities in the United States is from burning fossil fuels for electricity, heat, and transportation. Available data shows primary sources of GHG emissions in the USA for 2014 were electricity (30%), transportation (26%), industry (21%), commercial/residential (12%), and agriculture (9%) (https://www.epa.gov/ghgemissions/sources-greenhouse-gas-emissions). The co-emitted air pollutants from burning fossil fuels are sulfur dioxide (SO_2), nitrogen oxides (NO_x), ground-level ozone, particulate matter (PM), and carbon monoxide (CO), which threaten public health by contributing to cardiorespiratory which can cause death (Rohde and Muller 2015).

Biofuels are fuels produced from biological sources, which can be used as alternative energy for producing electricity, heat, and transportation. There are several types of biofuels, including solid biofuels, liquid biofuels, and gaseous biofuels (Petrou and Pappis 2009). The two main liquid biofuels are bioethanol and biodiesel. Currently, approximately 80% of the global liquid biofuel production is in the form of ethanol. In 2012–2014, global bioethanol and biodiesel production reached 108 and 28 billion liters, respectively (Popp et al. 2016). The two world's largest ethanol producers are the USA and Brazil. Gaseous biofuels aroused less frequently than other biofuels (Petrou and Pappis 2009). Biofuels are carbon-neutral, renewable, and environmentally friendly alternative forms of energy. Because they are clean burning, there is a growing interest in replacing depleted fossil fuel sources with biofuels. The development and use of biofuels can secure our energy future by reducing both dependence on imports and environmental impacts. Use of biofuels can reduce GHG emissions and other major air pollutants. Although challenged by some groups and studies (Althor et al. 2016), the general consensus remains that GHG emissions can be reduced by burning biofuels, as CO_2 from biofuel combustion is offset by the CO_2 consumption of crops or other plant feedstocks, which rely on CO_2 to drive their own photosynthetic process (Ragauskas et al. 2006). Washington-based consultancy ICF International, in a 2017 report prepared for the USDA, finds that typical corn-based ethanol production has attained a 43% reduction in GHG emissions compared to 2005-era gasoline. Their analysis also projects that GHG emissions from corn-based ethanol reductions could rise to 50% by 2022 and could reach 76% in 2022 with widespread adoption of optimal crop production and continued or improved biorefinery efficiency (https://www.usda.gov/media/blog/2017/01/12/innovation-driving-down-greenhouse-gas-emissions-corn-based-ethanol).

Some industries have been gradually substituting fossil fuels with biofuels in portions of their operations. According to the American Public Transportation

Association (APTA) 2016 statistical data, 7.4% of US transit buses are now running on biodiesel (http://www.apta.com/mediacenter/pressreleases/2016/Pages/160422_Earth-Day.aspx). Hamburg Airport (HAM) in Germany has become the first major airport to use Neste's *Renewable Biodiesel* (https://www.neste.com/fi/en/neste-oil/sustainability/cleaner-solutions/renewable-solutions-traffic) in its diesel-powered ground fleet, powering aircraft tugs and firefighting vehicles. This further reduces the airport's reliance on fossil fuels and carbon footprint (https://bioenergyinternational.com/biofuels-oils/renewable-diesel-to-decarbonize-hamburg-airports-ground-fleet). Other smaller airports such as Kiruna and Bromma airports in Sweden have already tested 100% renewable diesel in their ground fleet (https://www.neste.com/). *USA TODAY* (2017) reported US Navy fighter planes are testing and flying 100% biofuel. According to that report, the cost to fill training planes with biofuel supplied by AltAir Fuels in California has fallen to $2.15 per gallon compared to $26 per gallon during the first green run of 2012 (http://www.usatoday.com/story/money/columnist/2016/09/14/skys-limit-navys-biofuel-focus/90326310/).

Three generations of biofuels have been created using variations in two factors, feedstock and processing. Feedstock refers to materials used as or converted to biofuels. Across the decades several feedstocks and processing methods have been used to affect change in three generations of biofuels (Aro 2016). In this chapter, we will first discuss the different generations of biofuel, including basic production processes, feedstocks used, as well as the benefits and challenges of producing each. Then, the discussions will focus on lignocellulosic biomass-based biofuel production. We will look into the three largest cellulosic ethanol producers in the USA and how they operate their factories. Conventional breeding with molecular analysis and transgenic breeding using modern tools for genetic manipulation can be used to increase genetic diversity and develop new cultivars. These techniques can also be used to improve lignocellulosic biomass yield and modify cell-wall quality for better biofuel production efficiency. We will look into different approaches for increasing the quantity and quality of lignocellulosic biomass for biofuel production. Although there are several potential biomass crops (woody and grass species) which can be used for lignocellulosic biomass-based biofuel production, the main energy crop used as an example for conventional breeding in this chapter is the grass species giant *Miscanthus*. Two approaches are the focus for yield increase: (1) growth rate and yield of biomass produced from energy crops and (2) development of new cultivars capable of being grown on marginal lands due to tolerance of stressful environments. Modern biotechnology has been used to design plant cell walls used for lignocellulosic feedstocks. These modified cell walls are more amenable to chemical degradation, which reduces the hefty cost of pretreatment. Research results from using biotechnological approaches to knock down or knock out several key lignin-biosynthetic genes will be described.

7.2 Different Generations in Biofuel Production

First-generation (1G) biofuel uses energy-rich food-based feedstocks. These feedstocks include starch and sugar. Corn grains are a major source for starch. Another high-starch grain source is grain sorghum (also known as Milo). On the other hand, sugarcane, sugar beet, and sweet sorghum are sugar sources for biofuel production. In the USA, corn is the least expensive first-generation biofuel feedstock (source: USDA Rural Development). Three examples of first-generation biofuel products are starch-based corn-grain ethanol, sugarcane ethanol, and biodiesel. In the production of corn-grain ethanol, the first process is to convert corn starch into fermentable sugars with enzymes (e.g., cellulase, β-glucosidase, and hemicellulose). The resulting sugars are then fermented into ethanol using yeast or other microbes (such as bacteria and fungi). Yeast, *Saccharomyces cerevisiae*, is one of the most widely studied microbe and the workhorse in the current biofuel industry, as many consider it superior to other ethanol-producing microorganisms (Buijs et al. 2013). It tolerates a wider range of pH and ethanol parameters better than other strains. Liquid ethanol is distilled and dehydrated (from 190 proof from 200 proof) and then denatured (by adding ~2% denaturant, such as natural gasoline) prior to loadout for offsite sale. In the USA, produced ethanol is transported through train and tanker truck (90%), barge (10%), or pipelines (minimum) for distribution (Source: U.S. Department of Agriculture: http://www.afdc.energy.gov/fuels/ethanol_production.html). By-products, such as *distillers grains* and *corn-distillers oil*, are produced. Distillers grains are sold as high-protein feed for livestock (especially ruminants). Two types of distillers grains (DG) are available, wet (WDG) and dry (DDG). According to the Renewable Fuels Association (RFA), one bushel of corn grain (56 lb) processed by a dry mill ethanol biorefinery produces an average of 2.85 gal denatured ethanol, 16.5 lb of distillers grains animal feed, 0.65 lb of corn-distillers oil, and 17 lb of biogenic carbon dioxide (http://ethanolrfa.org/how-ethanol-is-made/). Sugarcane ethanol is derived from sucrose in sugarcane juice and molasses. Brazil is the world's largest sugar producer and sugarcane ethanol producer, yielding 8 billion gallons in 2015/2016 (http://sugarcane.org/sugarcane-products/ethanol). Schematic of process of sugarcane to produce sugarcane ethanol was shown in Fig. 7.1. Sugarcane juice is extracted through an extractor and used for either sugar or bioethanol production through several steps. The leftover dry fiber is called bagasse. Bagasse was considered an agricultural waste and mostly used for combustion to generate heat and power (https://www.e-education.psu.edu/egee439/node/647). However, it is now an important lignocellulosic feedstock for second-generation biofuel production as well (see next paragraph).

Second-generation (2G) biofuels are produced from lignocellulosic biomass. Second-generation biofuels are also known as "advanced biofuels," as opposed to the conventional corn-grain biofuel. Second-generation biofuels can be manufactured from various types of biomass. Typical sources of lignocellulosic feedstock are crop, forest, or wood process residues or purposely grown energy trees. Crop residues include corn stover, cereal straw (e.g., barley, rice, and wheat straw), grain sorghum stubble, etc. Examples of purpose crops and trees are giant *Miscanthus*

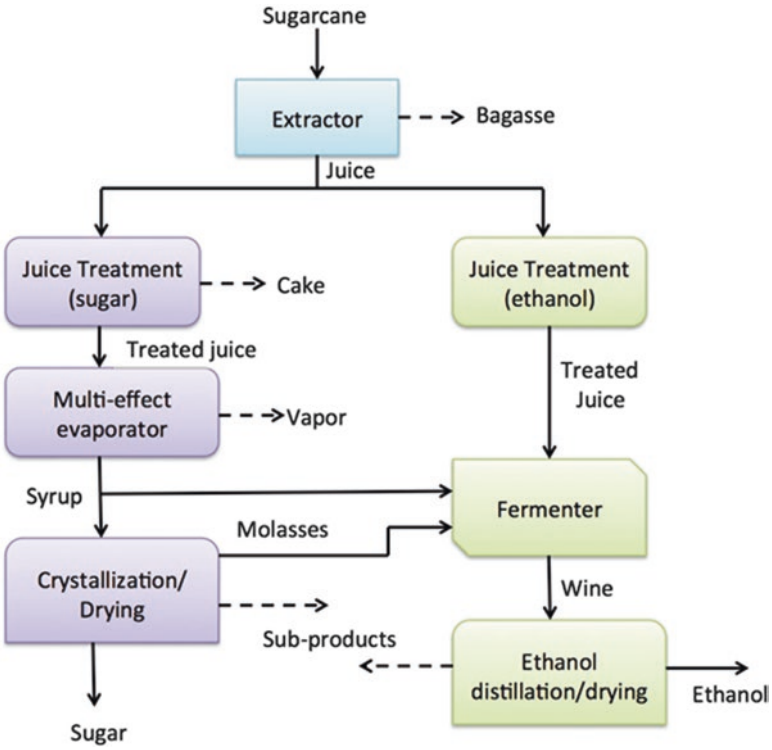

Fig. 7.1 Schematic of process of sugarcane to produce ethanol and sugar (Figure was reproduced from EGEE 439: *Alternative Fuels from Biomass Sources*, https://www.e-education.psu.edu/egee439/node/647; with permission from Dr. Caroline Clifford, EMS Energy Institute at Penn State University)

(*Miscanthus* x *giganteus Greef* et Deu.), switchgrass (*Panicum virgatum* L.), poplar (*Populus* spp.), and others. Sugarcane, a first-generation biofuel feedstock, provides fibrous residues, called bagasse, which can also be used as lignocellulosic feedstock for second-generation biofuel production. Bagasse represents about 25% of total sugarcane weight and contains 60–80% carbohydrates (Betancur and Pereira 2010; Rezende et al. 2011). One of the world's largest bagasse-based ethanol-producing facilities is under construction in Thailand. The plant will produce 1400 tons of cellulosic sugar from bagasse, and the sugar will be used for bioethanol and other commercial by-product production. This takes advantage of the fact that Thailand is one of the largest sugarcane-producing nations in the world (http://www.biofuelsdigest.com/bdigest/2017/01/05/toray-and-mitsui-to-invest-up-to-51-million-in-thai-cellulosic-ethanol-plant/). One example of second-generation lignocellulosic biofuel is cellulosic ethanol.

Compared to grain ethanol, industrial cellulosic ethanol production remains a challenge due to high processing costs associated with required novel technology. According to Sorek et al. 2014, before publication, only two commercial-scale

lignocellulosic biofuel facilities existed in the world. One in Crescentino, Italy, owned by Beta Renewables (http://www.betarenewables.com/en), became the first facility used for global commercial-scale production of cellulosic ethanol, when launching operations in the fall of 2013. The other facility is KiOR in Columbus, Missouri, USA. However, KiOR filed bankruptcy in late 2014 and changed its name (http://fortune.com/kior-vinod-khosla-clean-tech/). By that time, at least a dozen commercial-scale lignocellulosic refineries were under construction in the USA (Sorek et al. 2014). *Ethanol Producer Magazine* cited 17 US biorefineries using cellulosic biomass-based platforms for ethanol production in February 2017 (http://www.ethanolproducer.com/plants/listplants/US/Existing/Cellulosic).

Here, we describe three 20–30 MGPY (million gallons per year) cellulosic ethanol-producing biorefineries in the USA: (1) POET-DSM Advanced Biofuels' biorefinery plant (named "Project Liberty") in Emmetsburg, Iowa. The investment for building the plant was at least $275 million dollars, with a grant of $105 million dollars from the US Department of Energy (DOE). It officially opened in September 2014, as the first commercial-scale cellulosic ethanol plant in the USA. According to its website, the plant consumes 285,000 tons (770 tons a day) of biomass to produce 20 million gallons of cellulosic ethanol annually. The biomass is corn stover and is gathered locally from farms within a 45-mile radius of the plant. Farmers remove approximately 1 ton (~25%) of farm residue per acre for the plant (http://poet-dsm.com/pr/first-commercial-scale-cellulosic-plant). (2) Abengoa Bioenergy Biomass of Kansas (ABBK) plant is operated by Abengoa Bioenergy in Hugoton, Kansas. It began operations in October 2014. The refinery was fueled with 100% biomass and was expected to produce 25 MGPY of cellulosic ethanol (http://www.abengoabioenergy.com/web/en/2g_hugoton_project/). According to reports, due to Abengoa's bankruptcy, this facility was shut down at the end of 2015 and sold out to Synata Bio at the end of 2016. (3) DuPont's Cellulosic Ethanol Biorefinery in Nevada, Iowa, is by far the largest facility of its kind in the world. The facility opened in October 2015. According to its website, DuPont is collaborating with more than 500 farmers to collect, store, and deliver 375,000 dry tons of corn stover each year from 190,000 acres of farm land within 30 miles of the plant to produce 30 MGPY of cellulosic ethanol (http://www.dupont.com/products-and-services/industrial-biotechnology/advanced-biofuels/cellulosic-ethanol/nevada-iowa-cellulosic-ethanol-plant.html). Corn stover is identified as the primary feedstock used by these biorefinery plants. The plants are built at locations (Iowa, USA) surrounded by huge farmlands, especially corn farms. Iowa State is centered in the Corn Belt of the USA (Midwest of the USA). This allows for feedstock collection from nearby farms to reduce transportation costs. The overall cost of harvesting, collecting, storing, and delivering corn stover biomass to the Midwest (USA)-based 30 MGPY cellulosic biorefinery is estimated to be $82.40/ton, with feedstock transport accounting for 40% of the cost (http://ohioline.osu.edu/factsheet/fabe-660).

Meanwhile, edible oil derived from oilseed crops (e.g., soybean, rapeseed, canola, mustard, sunflower, oil palm, coconut, etc.) and non-edible oils derived from certain oilseed plants have been used as sources for biofuel, such as biodiesel (Demirbas et al. 2016). Jatropha tree (*Jatropha curcas*), karanja tree (*Pongamia*

pinnata), mahua tree (*Madhuca indica*), and castor bean seed (*Ricinus communis*) produce non-edible oil and can grow on marginal lands (e.g., *Jatropha*) or forests (e.g.., mahua). Depending on area, climate, and land use, different countries or regions grow preferred oilseed plants. In the USA, soybean oil is the most popular oil source for biodiesel, while rapeseed oil is the preferred source in many European countries. 2013 US data shows 75% of major biodiesel feedstocks were crop based, with soybean oil accounting for more than 50% of that number. The remaining 25% was from animal fat, poultry fat, and yellow grease (used cooking oil) (https://www.eia.gov/todayinenergy/detail.php?id=15451). Oilseed feedstocks originate from rural areas, but animal fats and used cooking oil are more urban in origin. In India, the deciduous mahua tree is considered a potential biofuel plant. Its kernel constitutes 70% of the seed and contains 50% oil, which can be extracted at levels of 34–37% (https://www.eurekalert.org/pub_releases/2013-01/ip-tso010913.php). Another tree, karanja, in India is also intensively studied for its potential (Raheman and Phadatare 2004; Baiju et al. 2009). Karanja seeds contain 30–40% oil (Scott et al. 2008).

The *third-generation (3G)* biofuels are produced using unicellular photosynthetic algae, including macroalgae and microalgae. The discussion of microalgae-based biofuel production is available in another chapter of this book by the same authors (Dr. Yuan-Yeu Yau and Mona Easterling).

The future *fourth-generation (4G)* biofuel production practices the concept of "bioenergy with carbon storage (BECS)." Research involving first- to third-generation biofuel production is ongoing, but research for future generations of biofuel production is emerging. It represents the combination of bioenergy technology and carbon capture and storage (CCS) technology. For fourth-generation biofuel production, not only the feedstocks are tailored to improve processing efficiency (e.g., through genetic engineering), but also the CCS technology is employed to capture CO_2 emissions from biorefineries (http://www.isaaa.org/kc/cropbiotechupdate/article/default.asp?ID=1008). Therefore, the production of the fourth-generation biofuels is not just carbon neutral; it is carbon negative (http://www.isaaa.org/ kc/cropbiotechupdate/article/default.asp?ID=1008). Carbon negativity is defined as to remove CO_2 from the atmosphere rather than adding it.

Genetic engineering including its subset, metabolic engineering, and synthetic biology can be used to design microorganisms or plants to make them become more efficient in biofuel production processes. Examples are as follows: (1) yeast has been genetically modified to enhance their role in bioethanol production (Lam et al. 2014). The role of synthetic biology in yeast metabolic engineering for fuel production and synthetic biology tools used for improving yeast for biofuel production has been reviewed by other authors (Madhavan et al. 2017; Tsai et al. 2015). (2) The metabolic pathways in algae have been modified to increase lipids production. (3) Genetic engineering is used to modify cell wall components. One example involves gene suppression of lignin-producing enzymes to reduce lignin content in lignocellulosic-based feedstock.

CCS technology involves the capture and storage of CO_2 from factories, before CO_2 is released into the atmosphere. Carbon dioxide capture and geologic

sequestration (also referred to as storage) can greatly reduce CO_2 emissions and mitigate climate change (Szulczewski et al. 2012). CCS technology is currently at a demonstration phase, and researches are mainly focusing on huge fossil fuel burning power plants and coal powered. However, CCS can be applied to a wide range of energy plants in the future. CCS technology involves three steps: (1) trapping emitted GHG, including CO_2, (2) separating CO_2 from other gases, and (3) transporting CO_2 for storage. The captured CO_2 can be transported through pipes or other means to specific sites for storage and use for other applications later. Inspired by natural CO_2 storage of natural gases and other petroleum fluids in underground geologic formations over millions of years, storage sites are usually placed deep underground. The ideal sites are geological formations such as saline aquifers, depleted gas fields, oil wells, and unmineable coal seams (http://socalcarb.org/storage.html). Concept of capturing CO_2 is not new, which have been practiced since decades ago. However, the long-term storage of CO_2 in a specific site is a relatively new idea. A lot of unknown details are involved. CCS relies on the verified safe and effective long-term storage of CO_2. Leakage of stored CO_2 can result in fatalities of human beings and animals. The biomass of large forests is another valuable resource for carbon storage. For example, the Himalayan nation, Bhutan, is one of few countries with negative carbon emissions, due to its large forests. According to *EcoWatch*, the nation is currently 72% forested. The amount of CO_2 emitted (1.5 million tons) annually is much less than the CO_2 absorbed by the forests (5 million tons) (http://www.ecowatch.com/this-country-isnt-just-carbon-neutral-its-carbon-negative-1882195367.html).

CCS technology is already deployed at industrial scale in the USA to capture CO_2. Three examples are described below: one from a coal-fired power plant and two from biofuel-producing plants. The world's largest carbon-capture facility came online in 2016 – the Petra Nova commercial-scale post-combustion carbon-capture facility at the W.A. Parish Generating Station in Thompsons, Texas, USA (http://www.nrg.com/generation/projects/petra-nova/?iid=RPR_NH6_2017January_PetraNova_HPBanner). The Station is one of largest coal-fired power plants. The captured CO_2 is transported by pipelines to an oil field 80 miles away and injected into depleted oil wells to squeeze out residual oil and sequester CO_2 underground at the same time. This is an operation called "enhanced oil recovery" (EOR) (https://energy.gov/fe/petra-nova-wa-parish-project). EOR accounts for ~2% of the USA's current oil production. At the end of last year, it captured about 90% of the CO_2 released from the power plant. The Illinois Industrial Carbon Capture and Storage (IL-CCS) project at Decatur, Illinois, USA, is another example. This demonstration project is supported by the US DOE (Department of Energy), and CO_2 is captured from Archer Daniels Midland Company's corn-to-ethanol biofuel plant using Alstom's amine process. Captured CO_2 is injected into the ground at ~7000 ft depth in Mt. Simon Sandstone for storage, where researchers believe it can be safely stored (Source: 7th IEA CCS Network Regulatory Meeting: ADM CCS Projects, UIC Class VI Permitting Experience). The Mount Simon Sandstone in the Illinois Basin is one of the largest saline aquifers in the world. The CO_2 is stored in a natural saline aquifer (http://herald-review.com/business/energy/

adm-begins-carbon-dioxide-injection-process/article_41d91184-6c60-5d16-a7bd-23d23ec898db.html). For the first phase, 1 million tons of CO_2 were successfully injected and stored over a 3-year period (between November 2011 and November 2014). No leakage from the storage site has been observed to date. The captured CO_2 seems to remain sealed in place. It is the world's first large-scale CCS project from a biofuel source. Another company employing CCS technology is the biofuel company Aemetis. Aemetis is one of the largest bioethanol producers in the USA (http://www.aemetis.com/). Aemetis, California (USA)'s largest ethanol producer, is adding a CO_2-capture facility next to its ethanol biorefinery in Keyes, California, using the CCS concept. The CCS system at Keyes will capture CO_2 produced from its ethanol biorefinery fermentation process and convert it into liquid CO_2. Liquefied CO_2 can be used to carbonate soft drinks and beverages or to make dry ice (frozen carbon dioxide, with surface temperature of −109.3°C) (http://www.biofuelsdigest.com/bdigest/2014/10/27/liquid-co2-or-liquid-gold-maybe-both-as-aemetis-adds-co2-liquefaction-at-its-keyes-ca-plant/). According to Global CCS Institute, there are 22 large-scale CCS projects in operation or under construction globally (Source: The Global Status of CCS: 2016 Summary Report; https://www.globalccsinstitute.com/projects/large-scale-ccs-projects#overview). Globally, CCS could remove 10 billion tons of CO_2 from the atmosphere every year by 2050 when available sustainable biomass is used [Biomass with CO_2 Capture and Storage (Bio-CCS), European Technology Platform for Zero Emission Fossil Fuel Power Plants (ZEP)].

7.3 Pros and Cons of Different Generation of Biofuels

Every generation of biofuel production has its advantages and disadvantages. For first-generation biofuel production, the process of converting starch and sugars to liquid biofuels is easier than that of producing second- and third-generation biofuels. However, first-generation production generated a food-/water-vs-fuels dilemma, due to its heavy reliance on food crops as feedstock (Tenenbaum 2008). In a recent report, results indicated that first-generation biofuels rely on about 2–3% of the global water and land used for agriculture, which could feed about 30% of the malnourished population (Rulli et al. 2016). Since they are crop-based, feedstock supplies can be volatile. During a drought year, the yield of feedstock can be dramatically reduced. This supply factor can affect feedstock commodity price volatility. Farmers might also be more likely to devote their croplands to more profitable energy corns over growing corns for human or animal consumption. For example, more growers could opt for growing Enogen hybrid corn (or amylase corn) developed for bio-ethanol production by Syngenta Seeds, Inc. (http://www.syngenta-us.com/corn/enogen). The biotech corn, approved by USDA in 2011, has α-amylase enzyme directly built into each grain (Kumar and Singh 2016). This dramatically reduces the viscosity of corn mash, so no external liquid enzyme needs to add, thereby increasing the efficiency of ethanol factories. Recent studies showed the combined use of amylase corn and superior yeast (an engineered *Saccharomyces cerevisiae* which produces high ethanol yields in the

dry-grind processing industry) can reduce total external enzyme usage more than 80% (Kumar and Singh 2016). Corn growers are encouraged to grow amylase corn, which generates additional revenue. Farmers can earn on average a 40-cent premium per bushel for delivering contracted Enogen corn grain to ethanol plants. This incentive can motivate farmers to grow biotech energy corn instead of food crops. This can drive up the price of food corn, due to fewer producers. *Time magazine* has reported that tens of thousands of people marched on Mexico City in 2007 to protest the skyrocketing cost of tortillas, a development linked to increased demand for corn for ethanol (http://content.time.com/time/specials/2007/environment/article/0,28804,1602354_1596572_1604188,00.html). In 2013, *The New York Times* also reported that the increasing cost of tortillas in Guatemala was due to the high demand of energy corn in an article titled "As Biofuel Demand Grows, So Do Guatemala's Hunger Pangs" (http://www.nytimes.com/2013/01/06/science/earth/in-fields-and-markets-guatemalans-feel-squeeze-of-biofuel-demand.html). Therefore, research and use of alternative feedstocks for biofuel production are becoming important.

For second-generation biofuel production, lignocellulosic biomass was used. Lignocellulosic biomass is by far the most abundant organic material for biofuel production. Many materials used are considered agriculture and forest "wastes" and not for human consumption. Food-based feedstocks (first-generation biofuels) were replaced with non-food material feedstocks in second-generation biofuels, to remove the food-/water-vs-biofuel dilemma. Despite this, using lignocellulosic feedstocks requires extensive pretreatment to make them useful. Lignocellulosic biomass is comprised of cellulose, hemicellulose, lignin, pectin, and other components (Sorek et al. 2014). Pretreatment is used to remove the unfavorable components, especially the lignin, in the cell walls. Lignin, an inert phenolic polymer, provides structural integrity and stiffness to secondary cell walls (Bonawitz and Chapple 2010). However, lignin resists enzymatic hydrolysis and degradation and impedes the *saccharification* (hydrolysis of polysaccharides to soluble fermentable sugars) process (Zhang and Lynd 2004; Himmel et al. 2007). Lignin also induces the forming of cellulose crystallinity during saccharification (Chang and Holtzapple 2000). The degree of cellulose crystallinity is a major factor affecting enzymatic hydrolysis (Yoshida et al. 2008). In an experiment with *Miscanthus sinensis*, Yoshida et al. (2008) observed that a reduction of crystallinity in substrates, such as cellulose, increased glucose production in saccharification process. This suggests crystallinity inhibits enzymatic hydrolysis of cellulose to glucose. Degree of cellulose crystallinity can be determined by X-ray diffraction. Delignification or decreasing lignin makes the carbohydrate more accessible to enzymes and enhances saccharification (Fig. 7.2; Yoshida et al. 2008; Saballos et al. 2012). Pretreatment is the most expensive step in production of lignocellulosic biofuels due to use of expensive chemicals, high temperature, and pressures. How to improve cell wall components of energy crops to make them more suitable for lignocellulosic biofuel production through breeding or genetic manipulation becomes important.

Another factor affecting second-generation biofuel production is growth inhibition of yeast cells during fermentation. A side effect of pretreatment is forming of

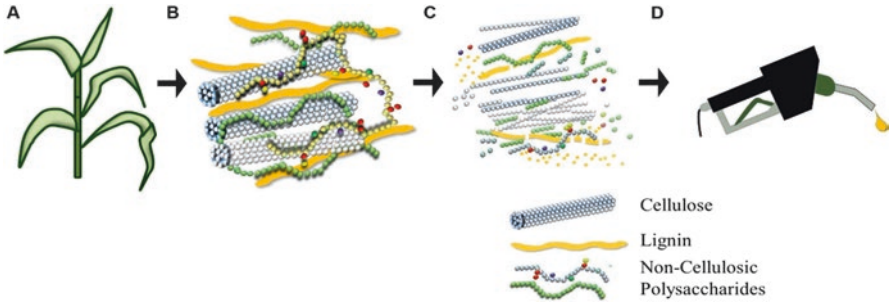

Fig. 7.2 Plant biomass can be pretreated to liberate its constituent sugars to produce ethanol (**a**–**d**). Plant cell walls (**b**) consist of an intricate and recalcitrant network that must be degraded for the conversion of the polymeric lignocellulosic biomass to be fermentable precursors for liquid fuel production. Cellulose is a homopolymer of glucosyl units that coalesces to form condensed microfibrils. Non-cellulosic polysaccharides are usually substituted or branched heterogeneous polymers composed of hexoses (glucose and galactose), pentoses (xylose and arabinose), and a wide variety of other sugars. Burton et al. (2010) provide details of the branching of backbone and the diversity of types of non-cellulosic polysaccharide. Lignin is a polymer of phenylpropanoid units that is shown here as elongated units, but which in reality is distributed throughout the cell wall matrix, where it entraps the polysaccharides to create an extremely strong and dense biocomposite that is difficult to degrade (Figure from Tan et al. 2016, an Open Access article distributed under the terms and conditions of the Creative Commons by Attribution (CC-BY) license, https://creativecommons.org/licenses/by/4.0/)

lignocellulose-derived microbe inhibitors (Jönsson and Martín 2016). Inhibitors can also be present in degradation products (hydrolysate) from hydrolysis of lignocellulosic biomass. Those inhibitors inhibit yeast cell growth and sugar consumption during later *S. cerevisiae* cultivation for fermentation (Keating et al. 2006; Tesfaw and Assefa 2014). Fermentation can also be completely inhibited in hydrolysate generated from drought-stressed lignocellulosic biomass (Ong et al. 2016). Ong et al. observed that hydrolysate from switchgrass (collected in a major drought year) inhibited the growth of *S. cerevisiae* during fermentation. Drought caused switchgrass plants to accumulate soluble sugars, which subsequently degraded to pyrazines and imidazoles during saccharification and inhibited growth of the yeast (Ong et al. 2016).

In the meantime, use of edible oil (from oilseed crops) as biofuel-producing stocks can drive up the price of the edible oils. Oilseed shrub *Jatropha curcas* produces non-edible oil and was once recognized as a "miracle crop" for biofuel production a decade ago. It was promising because (1) it is inedible (so, it wouldn't divert food supplies to produce fuel), (2) it can grow on harsh environment, and (3) it can serve as a living fence for crop protection in the rural area (Achten et al. 2014; Hunsberge 2016). Today, as more and more disappointing research results appear, this dream crop has fallen from grace. Although *Jatropha curcas* can grow in water-scarce regions, producers used a large amount of irrigation to increase the yield (Gerbens-Leenes et al. 2009). The reasons for failure of *Jatropha curcas* projects can be found from a case study in Kenya documented by Hunsberger

(2016). The report states that farmers found the yield low in dry conditions. Plants also suffered from insect and disease attacks. The situation was worsened by a lack of buyers or a few buyers offering very little money to buy seeds, causing many farmers to stop growing this plant (Hunsberge 2016). This changed attitude in markets from farmers happened in a very short time of 4 years, between 2009 and 2013. Companies also completely or partially dropped *Jatropha Curcas* to opt for other plants.

The third-generation biofuel refers to biofuel derived from algae (Behera et al. 2014). This includes macroalgae and microalgae. These photosynthetic algae use sun energy to convert CO_2 into carbon-rich lipids. Additionally, algae can grow on non-arable lands and will not compete for agricultural lands. Their high productivity and ability to make and store energy-rich compounds [e.g., triglycerides (TAG)], which can be extracted and converted to biofuels, make them a very attractive biofactory for biofuel production (Hu et al. 2008). The major barrier preventing their use in large-scale biofuel production is difficulty finding suitable algae and prohibitive operational cost. After building ponds to grow the algae, a huge investment is also required to supply the expensive inputs (nutrients, CO_2, water, light, etc.) to support algae growth. Recently, a 300-million-dollar research project, financed by ExxonMobil, has shown the process of producing biofuel through synthetic genomic engineering of algae is harder than expected (http://www.erneuerbareenergien.de/another-setback-for-biofuels-from-algae/150/482/68662/).

Developments using lignocellulosic-based and algae-based feedstocks for biofuel production are still in the early stages compared to first-generation biofuel production. Much of the data and relevant information about second- and third-generation products are experimental studies from demonstration facilities. Despite hefty investment and vigorous studies, the use of lignocellulosic-based and algae-based feedstocks for large-scale production still faces barriers, such as high processing cost. For example, only a handful of biorefineries produce cellulosic ethanol using biomass in the US. By far, only 17 commercial-scale biorefineries out of more than 200 fuel ethanol-producing plants are biomass-based [(http://www.ethanolproducer.com/plants/listplants/US/Existing/Cellulosic), (https://www.eia.gov/petroleum/ethanolcapacity/)]. Today, the largest biofuel product, bioethanol, is still produced through directly converting starch and sugars from food crops into biofuels (Mussatto et al. 2010).

7.4 Breed *Miscanthus* for Fertility

Due to its widespread availability, corn stover is currently considered the primary feedstock choice of cellulosic biorefineries. It was used by early-generation cellulosic biorefineries at both POET-DSM's Project Liberty and DuPont's Cellulosic Ethanol plants. However, there are other sources of lignocellulosic biomass with potential for future exploration, including *Miscanthus*. *Miscanthus* is a rhizomatous grass genus, comprised of C4 perennial warm-season rhizomatous grasses (Lewandowski et al. 2003). Currently, cultivars of *Miscanthus* used for planting and

testing are propagated by clonal methods of rhizome division or tissue culture. Some *Miscanthus* species are widely studied worldwide, especially the hybrid cultivar "giant" *Miscanthus* (*Miscanthus* x *giganteus Greef* et Deu.) owning to its high biomass yield and a potential energy crop (Smeets et al. 2009). The average lignocellulosic biomass yield (dry matter) for *Miscanthus* species is 12–40 tons/ha/year, compared to switchgrass (7–35), sugarcane (22.9), *Erianthus* (40–60), and *Eucalyptus* (15–40) (Hoang et al. 2015). The sterile *M.* x *giganteus* plants are triploid (2N = 3× = 57), which derive from interspecific hybridization between cross-pollination species *M. sinensis* (2N = 2× = 38) and tetraploid *M. sacchariflorus* (2N = 4× = 76).

The high biomass yield of *M.* x *giganteus* results from hybrid vigor (or *heterosis*) (Greef et al. 1997). *M.* x *giganteus* requires low nutrient input and very little from the environment. *M.* x *giganteus* needs only minimal nutrient input (e.g., fertilizer) for growth, because of high efficacy relocating nutrients, such as nitrogen, from stem to the rhizomes. *Miscanthus* can produce more biofuel than other feedstocks. For example, *M.* x *giganteus* yields two to three times more biomass than, another potential bioenergy crop, switchgrass in the Midwestern United States. According to a report from the US Department of Energy (DOE), ethanol production from corn grains, corn stover, switchgrass, and *Miscanthus* is 456, 300, 421, 1198 gal/acre, respectively (http://articles.extension.org/pages/26625/miscanthus-miscanthus-x-giganteus-for-biofuel-production). Low nutrient input, low environmental requirements, rapid growth rate, and high biomass yield make it the most attractive *Miscanthus* species as a bioenergy crop.

Due to seed sterility, the propagation of *M.* x *giganteus* requires labor-intensive vegetative propagation by clonal propagation of rhizome cuttings. Difficulties in sexual hybridization have also restricted development of genetically improved cultivars and use of germplasms/accessions for genetic improvement. Currently, a single genotype, obtained by clonal propagation, accounts for virtually all commercial production in most countries (Dwiyanti et al. 2014).

Artificial chromosome doubling in plants can be achieved using antimitotic agents. Antimitotic agents prevent cell mitosis in treated plant tissues by inhibiting microtubule formation and the polar migration of chromosomes (Hansen and Anderson 1996). Artificial chromosome doubling can be used to generate polyploidy plants containing larger cells, higher vegetative biomass yields, and enhanced compound production (Birchler et al. 2003; Yan et al. 2016). Through microspore or anther tissue culture, artificial chromosome doubling can also be used to produce double-haploid (DH) lines for plant breeding (Yuan et al. 2015). Fertile plants can be generated from sterile interspecific hybrids through artificial chromosome doubling. Nimura et al. (2006) had successfully induced amphidiploids from a sterileinter-specific hybrid (diploid) between carnation (*Dianthus caryophyllus* L.) and *Dianthus japonicus* Thunb. by artificial chromosome doubling. The amphidiploids exhibited a larger flower size and demonstrated restored fertility of both pollen and seed (Nimura et al. 2006). In theory, doubling the chromosomes of sterile triploid (2N = 3×) *M.* x *giganteus* to hexaploid (2N = 6×) could restore its fertility. Similar artificial-chromosome doubling work was performed on *Miscanthus* using

antimitotic agents (Yu et al. 2009). In this study, calli derived from *M.* x *giganteus* immature inflorescences were treated with chemicals colchicine and oryzalin. Chromosome-doubled calli were obtained through flow cytometry detection. However, analysis reports on these chromosome-doubled plants have not yet been completed (Yu et al. 2009). In 2012, Researchers Darren H. Touchell and Thomas G. Ranney from N.C. State University (USA) reported that pollen viability staining rose from 34% to 88% in the hexaploid cytotypes of triploid *M.* x *giganteus* at the 2012 ASHS annual conference (HORTSCIENCE 47(9) (SUPPLEMENT). The authors predicted that "the restoration of fertility to *M.* x *giganteus* may allow this valuable germplasm to be incorporated into future breeding programs for bioenergy crop improvement."

7.5 Genetic Studies for *Miscanthus* Breeding

The narrow genetic basis, mentioned above, and sterile characteristics of *M.* x *giganteus* have limited its utilization and adaptation in extreme climate conditions (Nie et al. 2016). Ideally, energy crops (such as *Miscanthus*) can be grown in a broad range of agricultural settings and use marginal or degraded lands under extreme climate conditions. Decreasing the competition for fertile lands intended for growing food crops is a goal for energy crops. To this end, developing new genotypes or cultivars of feedstocks is desirable.

To develop new genotypes or cultivars, diverse germplasms of *Miscanthus* are needed. Exploration and evaluation of natural genetic variation in *Miscanthus* germplasms are vital. A variety in genome sizes and chromosome numbers among *Miscanthus* accessions has been published (Chae et al. 2014). Ribosomal sequences were the basis of determining the phylogenetic relationships in *Miscanthus* and related taxa. Natural genetic variation in *Miscanthus* was also studied using molecular markers. Several marker systems were employed for genotyping to explore genetic variation and phylogeography of this species, including restriction fragment length polymorphism (RFLP) markers, randomly amplified polymorphic DNA (RAPD) markers (Cichorz et al. 2014), amplified fragment length polymorphism (AFLP) markers (Greef et al. 1997), sequence-related amplified polymorphism (SRAP) markers (Nie et al. 2014, 2016), and inter-simple sequence repeats (ISSR)-polymerase chain reaction (ISSR-PCR) markers (Cichorz et al. 2014). Cichorz et al. (2014) analyzed 18 accessions of three *Miscanthus* species, namely, *M.* x *giganteus*, *M. sinensis*, and *M. sacchariflorus*, using ISSRs and RAPD. As expected, they found that *M.* x *giganteus* clones had the highest genetic similarity coefficient (0.94), indicating the genetic diversity within this species was very low (Cichorz et al. 2014). Newer marker system such as single nucleotide polymorphism (SNP) was explored as well (Swaminathan et al. 2012; Clark et al. 2014). SNP is a tool for genotyping quantitative trait locus (QTL) analysis and association mapping (Swaminathan et al. 2012). Swaminathan et al. (2012) used SNP markers (discovered partly by deep transcriptome sequencing or RNAseq) to evaluate genetic variation and to construct a dense genetic map for *Miscanthus*. A dense map of all 19

linkage groups in *M. sinensis* was constructed. Clark et al. (2014) employed tens of thousands SNP markers to survey 767 *Miscanthus* accessions (collected from China, Japan, Korea, and the USA). Their results indicated that Southeastern China was the origin of *M. sinensis* populations, found in temperate eastern Asia, and *M. sinensis* migrated directly from Southeastern China to Japan before migrating to the same latitudes in China and Korea (Clark et al. 2014). Nie et al. (2016) focused study on *Miscanthus sinensis*, one of the progenitors of *M.* x *giganteus*, because *Miscanthus sinensis*, which was originally distributed in East Asia, has abundant genetic resources. The authors found traits associated with biomass yield production. For example, plant height was a highly stable trait correlated with biomass yield. They thereafter identified four markers for plant height and one for biomass yield (Nie et al. 2016). All these studies will facilitate germplasm conservation, association analyses, identification of potential heterotic groups, and biological discovery for breeding effort to improve *Miscanthus* as a bioenergy crop (Swaminathan et al. 2012; Clark et al. 2014; Nie et al. 2016).

Increased sequence information will help promote research into *Miscanthus* species' biology, physiology, and breeding. Ma et al. (2012) used genotyping by sequencing (GBS) to create a high-resolution linkage map (covering all 19 linkage groups) of *Miscanthus sinensis*. Results demonstrate that diploid *M. sinensis* has a tetraploid origin consisting of two sub-genomes. The complete and high-resolution composite linkage map will also serve as an important resource for novel QTL discoveries (Ma et al. 2012). Kim et al. (2014) have sequenced the entire genome transcriptomes of two *Miscanthus* species, *M. sinensis* and *M. sacchariflorus*. In the studies, hundreds of thousands expressed sequence tags (ESTs) were produced from leaf and rhizome tissues and sequenced. The results revealed the functional specificity in rhizomes and clarified evolutionary relationships. The genomic data also permits researchers to discover candidate genes capable of enhancing biomass production (Kim et al. 2014).

7.6 Transgenic Breeding to Improve Lignocellulosic Biofuel Feedstocks

As mentioned earlier, lignocellulosic biomass is the most abundant and low-cost feedstock for second-generation biofuel production. However, pretreatment step is a needed step to remove the lignin barrier and crystalline structure of cellulose in order to allow efficient enzymatic hydrolysis. There are a number of different pretreatment regimes that are being explored (Fig. 7.3; Tan et al. 2016). Pretreatment methods include physical, chemical, and biological approaches. Typical pretreatment processes use acids (such as sulfuric acid, phosphoric acid, maleic acid) and alkalis (such as sodium hydroxide, ammonia, ammonium sulfite) (Mosier et al. 2005; Zhu et al. 2009; Maurya et al. 2015). Biological approach employs the use of microorganisms (bacteria and fungi) and is considered a "green" pretreatment (Capolupo and Faraco 2016). For example, many types of white-rot fungi can be used for efficiently delignification. The cost of the pretreatment for lignin removal

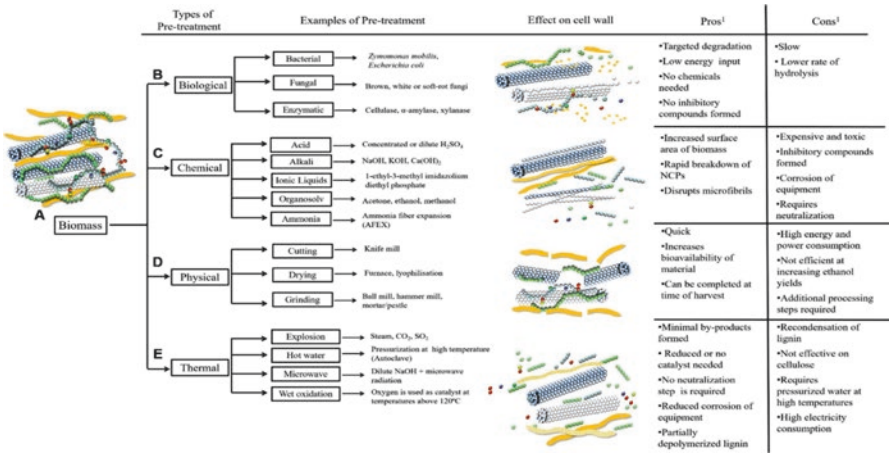

Fig. 7.3 Selected pretreatment methods differentially affect the breakdown and liberation of polymers from the cell wall. Lignocellulosic biomass is a complex network of carbohydrate and non-carbohydrate polymers (**a**). In its native form, plant biomass is usually recalcitrant to conversion and fermentation processes. Pretreatment is the initial processing step used to convert raw biomass into a form that can be more readily hydrolyzed. Biological pretreatments are efficient at removing lignin from the network, leaving a carbohydrate-enriched fraction (**b**). Chemical pretreatments may result in complete breakdown and fragmentation of cell wall components (**c**). Physical pretreatments are used to reduce the particle size of the biomass (**d**). The use of thermal pretreatments may loosen bonds between and within polymers, but lignin is not completely removed (**e**) (Mosier et al. 2005; Kumar et al. 2009; Takara and Khanal 2012) (Figure reproduced from Tan et al. 2016, an Open Access article distributed under the terms and conditions of the Creative Commons by Attribution (CC-BY) license, https://creativecommons.org/licenses/by/4.0/)

is the most expensive procedural step in the production of lignocellulosic feedstock-based biofuel, due to chemical price. Chemicals, high temperature (100–200 °C), and pressures are all currently used to speed up the process (Zhu et al. 2009). In the future, biotechnology (e.g., genetic engineering) can be used to design plants with fewer lignins or lignins more amenable to chemical degradation to reduce the hefty cost of pretreatment (Weng et al. 2008). Genetic manipulation could also provide an alternative to sugar recovery during pretreatment, which uses chemicals like sodium hydroxide.

The abundance and connectivity of cell wall components are distinctive between dicots and monocots, specifically the relative amounts of hemicellulose and pectin. Despite differences, the biosynthesis pathway for lignin is highly conserved across plant species (Li et al. 2014). Lignin is typically produced through polymerization of three monomers, also called monolignols. These three monolignols are coniferyl alcohol, sinapyl alcohol, and typically minor amounts of *p*-coumaryl alcohol (Boerjan et al. 2003). Coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol are also called G, H, and S monolignol, respectively. They are used to synthesize G lignin, H lignin, and S lignin, respectively (Boerjan et al. 2003). The principal monolignol biosynthetic pathways for *Arabidopsis*, poplar, and switchgrass have been documented in the review paper by Li et al. (2014). More than ten enzymatic steps are

involved in monolignol (lignin building blocks) synthesis. The genes catalyzing each step of monolignol biosynthesis had been cloned and identified (Vanholme et al. 2012). Enzymes involved in monolignol synthesis include phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate:coenzyme A ligase (4CL), *p*-hydroxycinnamoyl-CoA:shikimate *p*-hydroxycinnamoyl-transferase (HCT), *p*-coumaroyl shikimate 3-hydroxylase/coumarate 3-hydroxylase (C3H), caffeoyl-CoA *O*-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), coniferaldehyde 5-hydroxylase/ferulate 5-hydroxylase (CAld5H/F5H), caffeic acid/5-hydroxyconiferaldehyde 3-*O*-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD) (Fig. 7.4; Li et al. 2014). Monolignols are then transported to cell walls for synthesizing lignin polymers using peroxidases (POS) and laccases (LACs) (Smith et al. 2013; Lu et al. 2013). The impact of these genes on lignin production and composition has been studied through mutants or reverse genetics in various species, mostly *Arabidopsis* (*Arabidopsis thaliana*), maize (*Zea mays*), alfalfa (*Medicago sativa*), and poplar (*Populus* spp.) (Weng et al. 2008). Different plant species use different types of monolignols for lignin synthesis. For example, lignins from gymnosperms and related species are rich in G units and contain low amounts of H units (Weng and Chapple 2010).

Lignin is the most significant limiting factor in the conversion of plant lignocellulosic biomass to fermentable sugars. As the crucial genes for lignin-biosynthetic pathway are known, modifying genes to decrease lignin content or to change its composition to one better suited for lignocellulosic feedstock through genetic engineering is becoming an attractive strategy (Van Acker et al. 2014). A naturally occurring *Arabidopsis* mutant (*ref8*) displays *reduced epidermal fluorescence* phenotype with reduced lignin content. Authors demonstrated that the *ref8* mutant's C3H gene is defective and has no activity. C3H is a crucial enzyme in the monolignol biosynthetic pathway (Fig. 7.4; Li et al. 2014). Lignin content of *ref8* mutant stems is 20–40% less than wild-type levels. *Ref8* mutants also deposit a lignin formed primarily from *p*-coumaryl alcohol, usually a very minor monomer in the lignin polymers in the wild-type plants (Franke et al. 2002). Due to this change in lignin quantity and composition, the authors found *ref8* cell walls more completely degraded by enzymes (Franke et al. 2002).

Cinnamoyl-CoA reductase (CCR) is a key enzyme for monolignol synthesis, which has been engineered. CCR catalyzes the first step of the lignin-biosynthetic pathway's monolignol-specific branch (Fig. 7.4; Li et al. 2014). Wadenbäck et al. (2008) reported that CCR-deficient Norway spruce (through overexpression and antisense construct expression) has 8% less lignin than wild type. Another study with pine (*Pinus radiata*) shows similar results. In 2010, Tu et al. (2010) reported the use of dsRNAi-mediated gene silencing technology to knock down expression of CCR1 gene in ryegrass (*L. perenne* cv Grasslands Impact 566). Among transgenic lines, one line showed a 94% decrease in endogenous CCR1-specific expression (Tu et al. 2010). This line had reduced levels of all three (S, G, and H) lignin subunits. Dramatic reduction of lignin subunits caused no significant physical difference in this transgenic line compared to control plants (Tu et al. 2010). In another case study, also using RNAi technology, Wagner et al. (2013) demonstrated (1) the

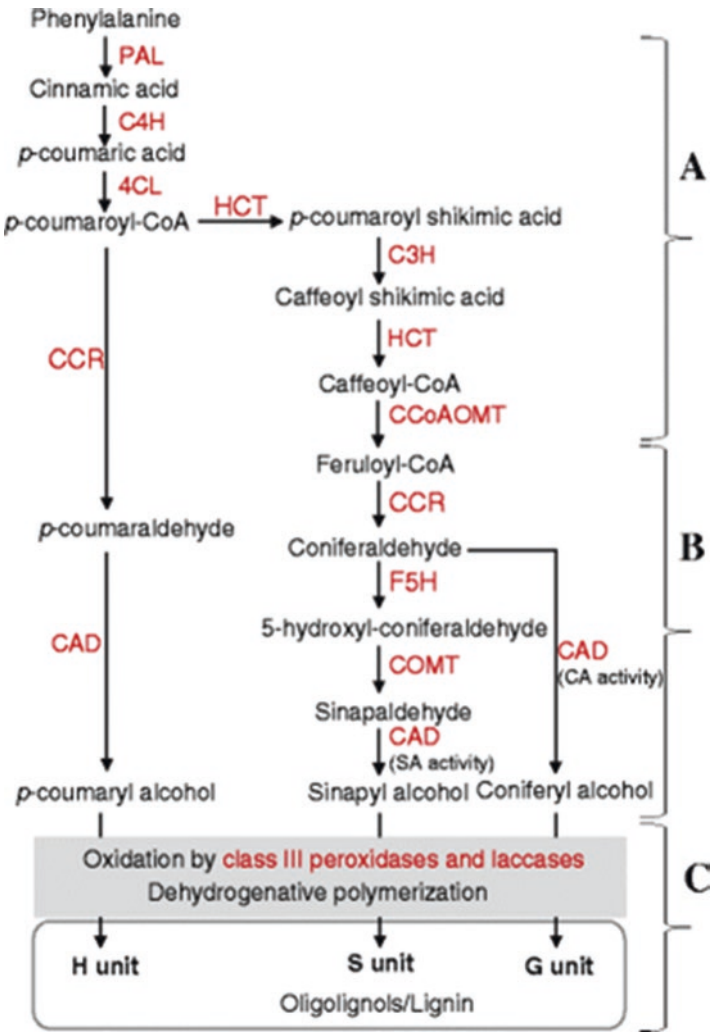

Fig. 7.4 Lignin biosynthesis pathway in plants. The monolignols (coniferyl alcohol, sinapyl alcohol, and p-coumaryl alcohol) synthesized from phenylalanine through the general phenylpropanoid pathway (**a**) and monolignol-specific pathway (**b**) are oxidized and incorporated into the G (guaiacyl), S (syringyl), and H (hydroxyphenyl) units, respectively, in the complex and three-dimensional polymer of lignin (**c**). Oligolignols, which are formed during lignin polymerization, are racemic radical coupling products of monolignols. *PAL* phenylalanine ammonia-lyase, *C4H* cinnamate 4-hydroxylase, *4CL* 4-coumarate:CoA ligase, *C3H* p-coumarate 3-hydroxylase, *HCT* p-hydroxycinnamoyl-CoA:quinate/shikimate p-hydroxycinnamoyl transferase; *CCoAOMT* caffeoyl-CoA O-methyltransferase, *CCR* cinnamoyl-CoA reductase, *F5H* ferulate 5-hydroxylase, *COMT* caffeic acid O-methyltransferase, *CAD* cinnamyl alcohol dehydrogenase (Figure reproduced from Nguyen et al. 2016, an Open Access article distributed under the terms and conditions of the Creative Commons by Attribution (CC-BY) license, https://creativecommons.org/licenses/by/4.0/)

redirection of metabolite flow within phenylpropanoid metabolism and (2) severe CCR suppression reducing lignin content by almost 50% in tracheary elements from *CCR*-RNAi transgenic lines (Wagner et al. 2013). Researchers Van Acker et al. (2014) generated poplar tree lines with CCR gene downregulation by genetic transforming "sense" or "antisense" CCR gene constructs. CCR-deficient plants showed red coloration of the xylem, as a sign of lignin reduction. Some plants had red patches on the wood, while others showed uniform red coloration throughout. Differing degrees of red coloration indicated that uneven gene silencing had occurred. These woody tissues were subjected to different saccharification and fermentation assays, and results showed wood had increased ethanol yield as high as 161% from the most affected transgenic trees (Van Acker et al. 2014). In another recent report, downregulation of the CCR gene in rice reduces lignin up to 10.8% (Ponniah et al. 2017). These results reconfirm the vital role CCR gene regulation plays in plant lignin biosynthesis.

Another key lignin-biosynthetic pathway enzyme is *4-coumarate:coenzyme A ligase* (or 4CL) (Fig. 7.4; Li et al. 2014). Studies show that isoforms for the 4CL gene exist in different plant species and belong to a small gene family. In hybrid poplar, *Populus trichocarpa* x *Populus deltoides*, there are at least three 4CL isozymes (Allina et al. 1998). In switchgrass, there are at least two 4CL isozymes: Pv4CL1 and Pv4CL2 (Xu et al. 2011). *Arabidopsis thaliana* contains four 4CL isozymes, At4CL1, At4CL2, At4CL3, and At4CL4 (Li et al. 2015). However, not every single isozyme is devoted to lignin synthesis pathways. Some of these isoforms are involved in flavonoid biosynthesis. Therefore, use of genetic engineering (e.g., RNAi or CRISPR/Cas9 system) to reduce lignin content in plants requires advance confirmation of key isozyme responses beforehand. In *Arabidopsis*, 4CL suppression yielded significant decrease in G lignin units (without much change in the S lignin units) and dramatically decreased the G/S ratio (Lee et al. 1997). Hu et al. (1999) generated transgenic aspen (*Populus tremuloides Michx.*) trees with downregulated *4CL* gene using an antisense technique. These transgenic aspen lines had a 45% reduction in lignin content. However, the loss of lignin was compensated for by a 15% increase of cellulose (Hu et al. 1999). Serious 4CL reduction in coniferous gymnosperm *Pinus radiata* substantially affected plant phenotype and resulted in dwarfed plants with a "*bonsai tree*-like" appearance (Wagner et al. 2009). Xu et al. (2011) generated 4CL-reduced switchgrass transgenic plants using RNA interference technology. Published results showed successful 80% reduction in extractable 4CL enzyme activity (Xu et al. 2011). Reduced 4CL enzyme activity led to reduced lignin content. Transgenic biomass with low lignin content significantly increases cellulose hydrolysis (saccharification) efficiency. However, the most important observation was stable biomass yield without yield penalty in the transgenic plants (Xu et al. 2011). Similarly, Min et al. (2012) generated three 4CL-downregulated transgenic cottonwood (*Populus trichocarpa*) plant lines, and lignin content was reduced from 21.3% (wild type) to 16.7–19.3% (transgenic lines). In another research, Li et al. (2015) showed that At4CL1 accounted for the majority of total 4CL activity in *Arabidopsis*. Loss of At4CL1 activity leads to reduction in lignin content but no growth defect (Li et al. 2015).

One other enzyme involved in lignin-biosynthetic pathway, which can be manipulated to reduce lignin, is *caffeic acid O-methyltransferase* (COMT; EC 2.1.1.68). The enzyme catalyzes *O*-methylation of the C5 hydroxyl moiety of suitably hydroxylated phenolic rings of monolignols, leading to the preferential formation of S subunits (Tu et al. 2010). Transgenic ryegrass lines with RNAi COMT enzyme showed decreased lignin (Tu et al. 2010).

Recently, Vanholme et al. (2013) identified a new step and a new central enzyme, previously undocumented, in the *Arabidopsis* lignin-biosynthetic pathway. The work was published in *Science* and revised currently accepted models of the lignin-biosynthetic pathway. The enzyme, *caffeoyl shikimate esterase* (CSE), catalyzes synthesis of caffeate. When the CSE gene was the target knockout, lignin content per gram of stem material was reduced by 36%. As a result, the conversion of cellulose to glucose from un-pretreated plant biomass increased from 18% (control plants) to 78% (*cse* mutant plants). However, inflorescence stems of *cse* mutant plants were 37% smaller and 42% lighter compared to wild type and had collapsed vessel elements (one cell type found in xylem) (Vanholme et al. 2013). The collapsed vessel element is an indication of a weakened secondary cell wall. Vargas et al. (2016) further engineered the *cse* mutant (*cse-2*) plants to restore vessel integrity by using vessel-specific complementation approach (by overexpressing a vessel-specific-promoter-driven CSE gene). The resulting plants had improved glucose release efficiency, with yields 25–36% higher than *cse-2* mutant and 134–154% higher than wild-type (Vargas et al. 2016). Research results also showed that CSE enzyme is critical for lignin that CSE enzyme is critical for lignin production in barrel medic (*Medicago truncatula*, dicot, Leguminosae). Transposon (TNT1)-mutated *cse* mutant lines of *M. truncatula* showed severe dwarfing, altered development (e.g., significantly delayed flowering time), and reduced lignin content (Ha et al. 2016). At full maturity, inflorescence stem heights are 115 cm and 10 cm for wild-type and *cse* mutant plants, respectively (Ha et al. 2016).

7.7 New Genome-Editing Tools to Improve Lignocellulosic Biofuel Feedstocks

Genome-editing (GE) technology is a recently developed tool for gene targeting. These tools can be used to precisely modify a target allele. Although GE and RNAi technologies both can be used to suppress gene expression of a specific gene, GE technology is different from RNAi technology. RNAi usually does not completely eliminate the gene product (enzyme/protein) but only "knocks down" the gene. By contrast, GE technology can completely silence a gene by knocking it out completely. GE systems include ZFN, transcription activator-like effector nucleases (TALEN) (Bogdanove and Voytas 2011), and the most recent groundbreaking technology, *clustered, regularly interspaced short palindromic repeat/CRISPR-associated proteins*, known as CRISPR/Cas9 system (Jinek et al. 2012). These GE systems have a similar feature: to precisely create a double-stranded break (DSB) at a target allele *in vivo*. Created DSBs have many applications, such as knockout of a

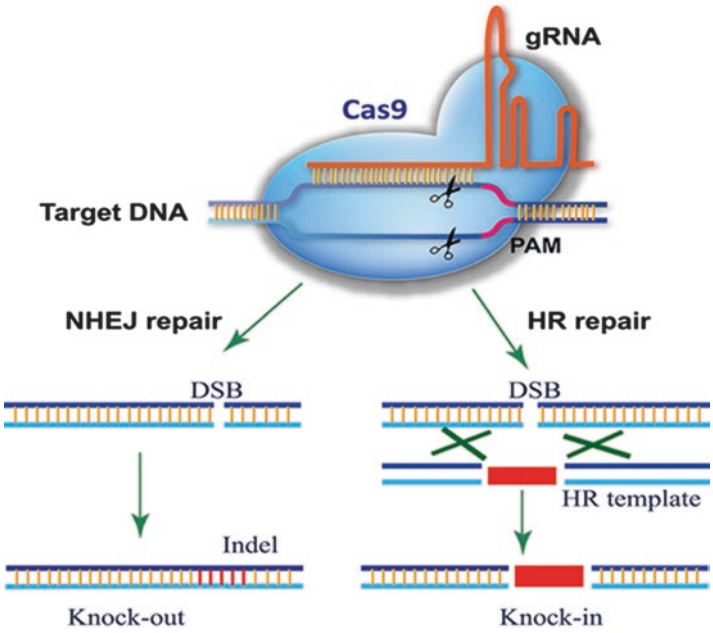

Fig. 7.5 Schematic of Cas9/gRNA genome editing. Cas9 is directed to its DNA target by base pairing between the gRNA and DNA. A PAM motif downstream of the gRNA-binding region is required for Cas9 recognition and cleavage. Cas9/gRNA cuts both strands of the target DNA, triggering endogenous DSB repair. For a knockout experiment, the DSB is repaired via the error-prone NHEJ pathway, which introduces an indel at the DSB site that knocks out gene function. In a knock-in experiment, the DSB is repaired by HDR using the donor template present, resulting in the donor DNA sequence integrating into the DSB site (Figure reproduced from Ding et al. 2016, an Open Access article distributed under the terms and conditions of the Creative Commons by Attribution (CC-BY) license, https://creativecommons.org/licenses/by/4.0/)

specific allele, repair of a mutated allele, insertion of a gene at the DSB site, and so on, through the use of the cellular DSB repair pathway (either HRD or NHEJ). Among them, CRISPR/Cas9 system is the most popular tool by far, due to its simplicity of construction and efficiency of causing mutation, compared to ZFN and TALEN. The CRISPR/Cas9 system only needs a single-guide RNA (sgRNA) and Cas9 enzyme to finish the job (Fig. 7.5). First, sgRNA and Cas9 form a protein-RNA complex. Then, sgRNA-guided targeting occurs, which induces a DSB at a specific locus. Due to the high demand of this technology, many web-based sgRNA-design platforms have been developed (Xie et al. 2014; Doench et al. 2016; Mohr et al. 2016). A list of useful web-based sgRNA-design tools is located at the link below: http://www.clontech.com/US/Products/Genome_Editing/CRISPR_Cas9/Resources/Online_tools_for_guide_RNA_design. For sgRNA design, the "guide sequence (~20 nucleotides)" is only the part that needs to be designed by users. Usually the remaining part, tracrRNA scaffold, is already built in the vector. Users only need to ligate the designed short "guide sequence" into the vector containing

the tracrRNA scaffold to form a complete sgRNA for downstream CRISPR experiments. Many previously published vectors (for different organisms) are deposited at Addgene – a nonprofit global plasmid repository. Researchers can just purchase a suitable vector and ligate the guide sequence into the vector. A simple restriction map with a detailed cloning strategy to ligate in the guide sequence for a specific vector usually comes with the purchased vector (https://www.addgene.org/crispr/).

In plants, CRISPR/Cas9 technology has been used in many projects to successfully edit alleles (Noman et al. 2016; Puchta 2017). As a result, a null allele has been generated, a genetically abnormal allele was repaired, or a targeted allele site was created for insertion of other genes for different research purposes. In addition to editing single locus-specific genes, CRISPR/Cas9 is also able to edit multiple genomic sites (multiplex genome targeting) by the simultaneous expression of two or more sgRNAs (Cong et al. 2013; Li et al. 2013; Xing et al. 2014; Zhang et al. 2016; Shen et al. 2017). Shen et al. (2017) multiplexed gene editing in rice by targeting eight genes with eight sgRNAs on a single vector and generated differing mutants, including double mutations, quintuple mutations, sextuple mutations, septuple mutations, and octuple mutations. Among these were two homologous octuple mutant lines. Quick generation of a genetically diverse (mutant) population with various gene combinations in the T0 generation is useful for preparing breeding stock. In another study, Peterson et al. (2016) used CRISPR/Cas9 to simultaneously target 14 genomic loci at once using the multiplexed CRISPR/Cas9 system and obtained a 33–92% success rate in *Arabidopsis*. Generation of multiple gene mutations is also necessary for investigating the relationship and function among several related genes or genes from gene families.

Recently, researchers used CRISPR/Cas9 technology to efficiently induce multiallelic mutagenesis in polyploid (tetraploid) potato (*Solanum tuberosum*) using protoplasts as start materials (Andersson et al. 2017). In that study, *granule-bound starch synthase* (GBSS) gene function was fully knocked out by mutating four allelic alleles simultaneously. Reduction of GBSS enzyme activity leads to altered amylose synthesis (Andersson et al. 2017). These results suggested that CRSPR/Cas9 not only is a powerful tool for diploid plant gene modification, but it is also an efficient tool for polyploid plant genome manipulation. The other advantage of using CRISPR/Cas9 technology is for generation of biallelic homozygous gene mutants as early as the first-generation or in T0-generation transgenic plants (Zhang et al. 2014; Fan et al. 2015; Shen et al. 2017). For example, high frequencies of homozygous biallelic mutants have been detected in tomato T0 plants (Pan et al. 2016). The mutations were stably transmitted to T1 and T2 generations without revision. Researchers will not be required to wait for T1 plants to obtain homozygous gene mutants. In addition to basic research use, CRISPR/Cas9 technology has been used for translational researching agriculture. For example, it was used to create a common white button mushroom (*Agaricus bisporus*) resistant to browning by gene knockout of the browning enzyme: polyphenol oxidase. Recently, tomatoes capable of flowering and ripening weeks earlier than usual were created in Cold Spring Harbor Laboratory (CSHL, USA), using CRISPR/Cas9 technology. CRISPR/Cas9 introduces small mutations to anti-florigen SP5G (SELF PRUNING

5G) gene in domestic Roma and cherry tomato varieties forcing earlier flowering and fruit ripening (Soyk et al. 2017). Horns of dairy cattle are routinely removed manually to protect other cattle and dairy-farm animal handlers from injury. Manual dehorning is painful for animals. Genetic dehorning is considered a humane way to dehorn cattle. Scientists have successfully used both TALEN and CRISPR/Cas9 as non-meiotic allele introgression tools to generate cattle without horns (Tan et al. 2013; Carlson et al. 2016; http://www.sciencefriday.com/segments/scientists-develop-a-hornless-cow-through-gene-editing/).

Other applications, in addition to gene editing, can be accomplished using CRISPR/Cas9 technology, such as:

1. Generating virus-resistant plants (Zaidi et al. 2016; Romay and Bragard 2017). CRISPR/Cas9 was used to inhibit *beet severe curly top virus* (BSCTV) accumulation in *Nicotiana benthamiana* through transient assays (Ji et al. 2015). *Arabidopsis* and *N. benthamiana* plants overexpressing sgRNA/Cas9 showed highly resistant to virus infection (Ji et al. 2015).
2. CRISPR-based genome imaging tool. Imaging can be done at a targeted genomic locus or simultaneously at multiple genomic loci. Chen et al. (2016) used *S. aureus* CRISPR-Cas9 system for CRISPR imaging. A dCas9-EGFP stably expressed cell line has been transformed with sequence-specific sgRNAs for imaging, with dCas9 containing catalytically inactive Cas9. Labeled genomic elements (such as telomeres, centromere alpha satellite, and 5S rDNA) were clearly observed through fluorescent protein expression. Most importantly, this system has an ability to resolve genomic elements at the scale of ≈100 kb. Dual-color CRISPR imaging was also observed, when both dCas9-EGFP and dSaCas9-mCherry were used for labeling (Chen et al. 2016). This technique would enable many applications for chromosomal studies, including tracking translocation events and visualization of chromatin contacts in living cells (Chen et al. 2016).
3. Using as a synthetic transcriptional repressor and activator. These synthetic transcriptional factors can be used to activate or repress transcription of an endogenous genomic target gene. These technologies are termed CRISPR interference (or CRISPRi) and CRISPR activation (or CRISPRa), respectively. To perform CRISPRi, a synthetic transcription factor "dCas9 with a guide RNA (sgRNA)" is designed. The dCas9-sgRNA complex binds to DNA site (~20 bps) complementary to the sgRNA, which blocks and halts transcriptions, resulting in repression of the target gene (Larson et al. 2013; Qi et al. 2013). CRISPRi has been used for prokaryotic metabolic engineering for rapid assessment of metabolic engineering interventions (Cress et al. 2017). In another study using CRISPRa, *phytoene desaturase* (PDS) gene expression levels were dramatically increased in tobacco *N. benthamiana* cells when the PDS gene promoters were targeted with synthetic transcriptional activators dCas9::EDLL and dCas9::TAD constructs (Piatek et al. 2015).

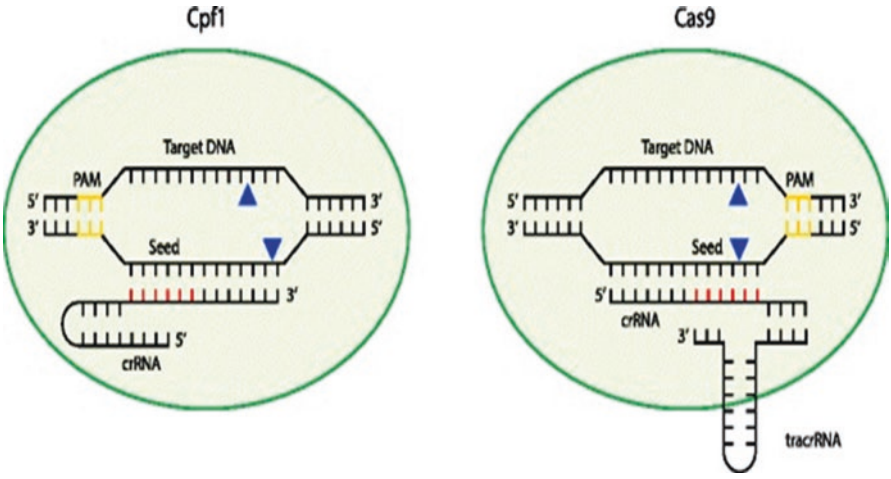

Fig. 7.6 Schematic comparison of target recognition and degradation by Cpf1 and Cas9. An R-loop is formed as a result of protospacer adjacent motif (*PAM*) recognition (*yellow*), and subsequent base-pairing interactions occur between the CRISPR RNA (*crRNA*) and its cognate target sequence. Note that the guide RNA in Cas9 is an RNA duplex involving crRNA and trans-activating CRISPR RNA (*tracrRNA*), whereas Cpf1 uses a single crRNA. Upon sufficient complementarity in the seed region (*red*), Cpf1 and Cas9 nucleases will make two single-stranded cuts (*blue triangles*) resulting in a double-stranded break. DNA and crRNA lengths and cleavage positions are schematic only and are not drawn to scale (Figure reproduced from Fagerlund et al. 2015, an Open Access article distributed under the terms and conditions of the Creative Commons by Attribution (CC-BY) license, https://creativecommons.org/licenses/by/4.0/)

New CRISPR systems with new features are continually being tested and added into the GE toolbox. Ran et al. (2015) used Cas9 (SaCas9) from *Staphylococcus aureus* CRISPR/Cas9 system, instead of the commonly used *Streptococcus pyogenes* Cas9 (SpCas9), for efficient gene editing in mammalian cells. Both SaCas9 and SpCas9 provided similar efficiency in gene editing. However, smaller-sized SaCas9 is much easier to package in a single adeno-associated virus (AAV) vector for delivery into cells. Recently, a newly characterized RNA-guided endonuclease (~1,300 amino-acid protein), Cpf1 (CRISPR of *Prevotella* and *Francisella* 1), was found to display cleavage activity in mammalian and plant cells (Zetsche et al. 2015, 2017; Fagerlund et al. 2015; Lowder et al. 2016; Tang et al. 2017). CRISPR/Cas9 and Cpf1-based CRISPR systems have similar functions, but some features differ (Fagerlund et al. 2015). A major difference between Cas9 and Cpf1 proteins is that Cpf1 does not utilize tracrRNA. It only requires the crRNA (Fig. 7.6). Endo et al. (2016) used *Francisella novicida cpf1* (FnCpf1) to effectively induce mutations in various target genes in tobacco and rice. Results showed average mutation frequencies on targeted loci of 28.2% and 47.2% for tobacco and rice, respectively (Endo et al. 2016).

For many years, RNAi technology was used as a tool to suppress lignin-biosynthetic genes. Zhou et al. (2015) reported the first application of CRISPR/Cas9 for biallelic mutations of 4-coumarate:CoA ligase (4CL) gene family in stably

transformed *Populus*. The lignin content in the transgenic plants was reduced by 23%, and the ratio of S/G decreased by 30%. The most important observation was the telltale uniform reddish-brown wood observed in every single 4CL1 transgenic line (Zhou et al. 2015). Reddish-brown color usually is an indication of lignin insufficiency. This is different from transgenic plants generated using RNAi technology. In transgenic plants generated from RNAi, the reddish-brown wood colors were not uniform. Discoloring appeared as patches on the wood, presumably due to the unstable nature of RNAi-mediated gene silencing. RNAi technology is considered an effective tool for suppressing gene expression (McManus and Sharp 2002). However, huge variation in gene silencing efficacy is reported using RNAi (McGinnis 2010). Recent paper also reported that CRISP/Cas9 uncovered potential errors in data collected by using RNAi (Lin et al. 2017). The problem behind this discrepancy can be tracked back to RNAi's potential for off-target effects. The 2015 paper (Zhou et al. 2015) is the first paper to demonstrate using GE tool CRISPR/Cas9 to successfully knock out a lignin-biosynthetic gene and suppress its expression in the wood; we expect that more such research of this nature will appear in the near future (Tsai and Xue 2015).

7.8 Final Remarks and Future Perspective

Fossil fuels used today are nonrenewable and limited in supply and will one day be depleted. There is an increased interest in searching for alternative renewable fuels. Biofuels derived from biological materials are such an alternative. The use of biofuels can reduce GHG and air-pollutant emissions and decrease environmental impact and improve public health. For the past several years, different generations of biofuel production were created based on feedstocks used. In first-generation biofuel production, grain or food-based feedstocks were used; in second generation, lignocellulosic biomass was used; and in third generation, algae were used as biorefineries. Fourth-generation biofuel production is under way now. Energy crops are designed to be a better source of biofuel-producing feedstocks. In addition, CO_2-emitting factories (e.g., bioethanol plants) are designed to capture, store, and reuse CO_2. Fourth-generation biofuel production combines bioenergy technology and CCS technology. This generation of biofuel is not only carbon neutral but also carbon negative.

There are advantages and disadvantages for each generation (first to third) biofuels. The use of lignocellulosic feedstock, a non-food-based feedstock, for generating biofuels can resolve the food-water-oil dilemma of first-generation biofuels. However, lignin in cell walls decreases efficiency of saccharification process of biofuel production. Lignin must be removed beforehand through pretreatments to increase the efficiency of biofuel production process, which includes saccharification (polysaccharides to sugars) and fermentation (sugars to ethanol). Costly pretreatment becomes a barrier for large-scale, commercial production of lignocellulosic feedstock-based biofuel. Genes controlling lignin production are well studied (Fig. 7.4). Employment of metabolic engineering to modify expression of specific

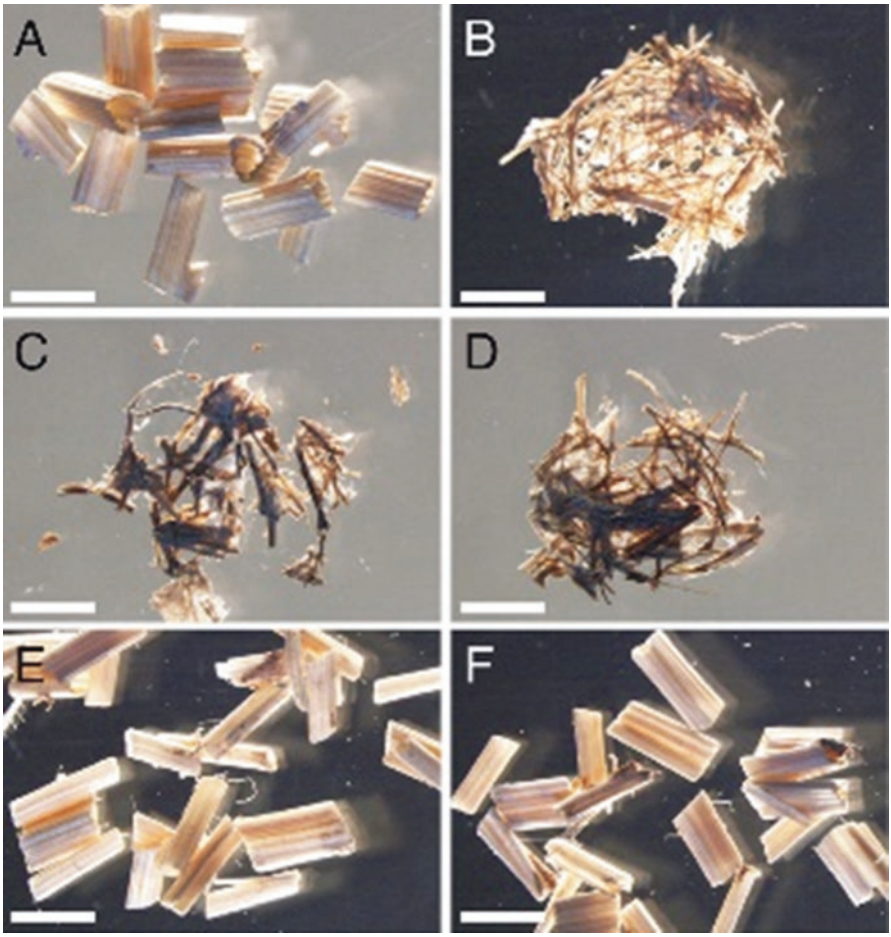

Fig. 7.7 Cell wall degradation after saccharification. Stem material after 48 h of saccharification (including acid pretreatment). (**a**) Wild type. The structure of stem segments of the mutants *c4h-2* (**b**), *ccr1–3* (**c**), and *ccr1–6* (**d**) are fully degraded as a consequence of the almost complete conversion of cellulose into glucose. Although the cellulose conversion was also improved in other mutants, such as *c4h-3* (**e**) and *4cl1–1* (**f**), the stem structure remained intact. Scale bar = 2 mm (Figure reproduced from Van Acker et al. 2013, an Open Access article distributed under the terms and conditions of the Creative Commons by Attribution (CC-BY) license, https://creativecommons.org/licenses/by/4.0/)

genes in the lignin-biosynthetic pathway is an effective strategy for reducing lignin. Several research groups have used RNAi or CRISPR/Cas9 technologies to successfully suppress monolignol biosynthetic-pathway gene expression and reduce lignin content in transgenic plants. And, indeed, many generated mutants show decreased lignin contents and increased saccharification efficiency (Van Acker et al. 2013; Fig. 7.7).

Silencing and knockout methods can reduce lignin content. However, since lignin is an important component in the cell walls, a severe decrease in lignin content can have a detrimental impact on normal plant growth and development. Studies have shown that a strong reduction in lignin content resulted in a significant loss of total biomass yield (or yield penalty) (Van Acker et al. 2013). Some plants have a severe dwarfing phenotype, and others lose vascular integrity. For example, the final height of the inflorescence stems for *ccr1-3* mutant was reduced by 83%, and the weight of their inflorescence stem was reduced by 77% compared to wild type (Mir Derikvand et al. 2008; Van Acker et al. 2013). *Arabidopsis cse-2* mutant plants were one third smaller than the wild-type ones and showed collapsed vessel elements (Vanholme et al. 2013). Evidence showed that collapsed vascular tissue was the direct cause of yield penalty in *cas-2* mutant *Arabidopsis* plants (Vargas et al. 2016). CSE mutant lines of *M. truncatula* also showed severe dwarfing phenotype (Ha et al. 2016). Ideally, lignin-content-modified plants should have the same yield as wild-type unmodified plants. Design of a plant with less lignin content, which grows normally without yield penalty, becomes the next important research goal.

In another item of note, many crop residues on farms can be used as lignocellulosic materials for biofuel production. However, excessive farm crop residual (e.g., corn stover) removal can also remove the soil nutrients, affect nitrogen availability, lead to increased soil erosion, and decrease soil organic carbon (SOC) and soil fertility (Wilhelm et al. 2007). Data from a 6-year field research has shown corn stover harvest increased N, P, and K removal by an average of 6.5, 0.6, and 7.6 $kg \cdot Mg^{-1}$, respectively, compared to harvesting only corn grain (Karlen et al. 2015). This can have a negative impact on future grain yields and sustainability. Therefore, farmers who collaborate with biofuel plants to supply crop residuals need to contemplate the appropriate ratio of crop residuals to remove while keeping future soil impacts in mind. For now, farmers are advised to supply 25% crop residuals per acre of their farms to POET-DSM Advanced Biofuels' plant.

Pretreatment and saccharification are important for second-generation biofuel production, and fermentation is another important factor affecting overall biofuel production efficiency. Researchers are testing and engineering different microorganisms, such as bacterium, yeast, and fungi, to improve fermentation efficiency (Ha et al. 2011; Wang et al. 2016). For example, Wang et al. (2016) observed that ethanol fermentation from both inulin and Jerusalem artichoke tuber powder was dramatically improved for most engineered *S. cerevisiae* strains. This testing also includes the hopeful bacterium *Zymomonas mobilis* (He et al. 2014). *Zymomonas mobilis* is a natural ethanologen with some advantages over *S. cerevisiae*. It consumes glucose faster than *S. cerevisiae*, leading to higher ethanol productivity. It is a preferentially anaerobic microorganism, meaning it can produce ethanol efficiently and economically without the costly advanced aeration control during fermentation process (Yang et al. 2016). Only after pretreatment, saccharification, and fermentation each reach their own optimal conditions can producing maximum yield of biofuel from lignocellulosic feedstock-based platform be achieved.

During the development of advanced biofuels, we have seen some setbacks, such as the failure of *Jatropha curcas* projects, the shutdown of lignocellulosic

biomass-based biorefinery KiOR, and discouraging results of ExxonMobil-backed algae-as-biofuel projects. However, we have also seen some encouraging successes, such as several commercial-scale second-generation biofuel plants currently open and running. CCS technology coupled with bioenergy technology is carefully being researched. Some biofuels have replaced fossil fuels in industrial setting and reportedly reduced GHG. Looking toward the future of biofuels, there is no greater potential than that of genetic engineering to maximize the potential of feedstock and microbial drivers of the production process. CRISPR technology holds great promise in this regard. All of us can hope for a future where biofuels provide a safe and economical alternative to fossil fuels.

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A Review on First- and Second-Generation Biofuel Productions

8

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Abstract

Renewable energy resources are in great urge to reduce dependability on fossil fuels as well as to minimize greenhouse gas emission. Since more than a decade, biofuel industries especially bioethanol and biodiesel have been highly expanding in conjugation with agriculture crop production. First generation biofuel production is highly relied on the agriculture crops such as corn, sugarcane, sugar beets, soybean, and canola. Therefore, inherent competition between foods versus fuels remained debatable in the society from the last few years. Current technological advances in the research and development opened an avenue for next-generation biofuel production from different feedstock such as agriculture waste products, crop residues, and cellulosic biomass from high-yielding grass species. This review explains the current status of first-generation biofuel production and their challenges at net energy benefit as well as competition of feedstock for food and fuel production. This chapter also focuses on recent advances in research and development of the second-generation biofuel production from different feedstocks. Future direction of agriculture industries and energy

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industries has been discussed to feed the ever-increasing world population and to fuel the world's highest energy demanding sector, transportation.

Keywords
Bioenergy • Bioethanol • Biofuels • Biodiesel • Biogas • Fossil fuel • Renewable • Feedstocks • Energy

8.1 Introduction

Fossil fuel utilization primarily relates to anthropogenic emission of greenhouse gases (e.g., carbon dioxide, methane, and nitrous oxide) in the atmosphere that alters the Earth's energy budget and causes warming of the climate system (IPCC 2013). The largest effect on the climate system is linked to the atmospheric concentration of CO_2 which is 40% higher from preindustrial era (IPCC 2013). Lower CO_2 emission is linked to renewable and carbon neutral fuels; therefore, it is promoted in many parts of the world (Soetaert and Vandamme 2009; World Energy Council 2013).

About 52.5% of the world's population live in urban areas (The World Bank 2014). This urbanization percentage will increase to 70% by 2050 (Zhang et al. 2014). Globally, major cities consume about 76% coal, 62% oil, and 82% natural gases (Sullivan 2011). Fossil fuel is exclusively being used by urban transportation. As a fact, cities are becoming major emitters of CO_2, one of the most significant greenhouse gases (Sullivan 2011).

The molecules of plant oils and petroleum fuels consist largely of chains of reduced carbons. The similarity of chemical properties of plant oils and fossil fuels was one major innovative factor of early versions of a diesel engine that was designed to run on peanut oil (Durrett et al. 2008). Biofuels referred to as bioethanol and biodiesel can be produced from agricultural crops and other recycled waste products. Bioethanol is produced through fermentation process using wheat, corn and other cereals, sugarcane, sugar beets, and potatoes. Biodiesel is mainly derived from plant oil or animal fat through esterification with a primary alcohol (Knothe 2005). This so-called first-generation biofuels inherently compete with food and their use is hence hotly debated. The life cycle analysis (LCA) of bioethanol derived from maize, sugarcane, sugar beet, and wheat indicates some advantage with greenhouse gas emission prospective which levels off when land use is considered to produce this feedstock (Munoz et al. 2014). Biodiesel derived from canola which has net energy balance with land use to produce canola seed is the major area of concern (Sanz Requena et al. 2011). Sustainable production of next-generation biofuels from agricultural crop waste such as straw, cornstalks, or agricultural debris has been proposed and pursued (Economist 2006).

8.2 Types of Biofuels

Renewable energy is one of the most important factors in the transportation world. Biofuels are classified as any alternative fuels derived from biomaterials such as grain crops (corn, wheat, sugarcane, sugar beet, cassava, and other cereals), crop residues (rice straw, rice husk, corn cobs, wheat straw, corn stover), and waste biomass (food waste, livestock waste, other waste products). Globally, its gaseous forms such as biogas and liquid forms such as bioethanol and biodiesel have been produced and utilized as renewable resources of energy alternative to fossil fuels.

8.2.1 Biogas

Naturally, the gas generated from organic digestion under anaerobic conditions by a wide range of microorganisms is commonly known as biogas, and it is an alternate source of energy produced at both small scale on farm levels and large scale at industrial levels. Biogas is comprised of mixtures of gases including methane (55–65%), carbon dioxide (35–40%), nitrogen (0–3%), hydrogen (0–1%), and hydrogen sulfide (0–1%) (Balat and Balat 2009a). Biogas production and utilization has begun both in rural and industrial areas since 1958. Biogas has been produced from a wide range of feedstock including agricultural waste, animal manures, sewage sludge, municipal solid waste, landfill, and industrial organic waste. The animal manure is primary the source of microorganisms for biomass biodegradation of feedstock. Yadvika et al. (2004) reviewed various techniques for biogas production enhancement from solid substrates. Biogas production is also reported from goat manure that was co-digested with three crop residuals, wheat straw, rice straw, and cornstalk; these three crop residuals comprise over 80% of agriculture waste in China (Zhang et al. 2013). In the European countries, over 1500 million tons (MT) of biogas is produced annually (Balat and Balat 2009a). In the USA, biogas production is estimated about 7.9 million tons yearly from different feedstocks.

8.2.2 Bioethanol

For the ever-increasing world energy consumption which is predicted to increase to 54 % by 2025, research and technology has been diverted toward sustainable production of renewable energy sources to mitigate surging future demands. Bioethanol is one of the largest produced and consumed liquid forms of fuel being used by transportation industries in the world by blending it with fossil fuel. As a complete renewable biofuel, bioethanol has relatively lower energy density and poor storage properties compared to gasoline; therefore, it is required to blend with gasoline to provide optimum energy density during combustion (Radakovits et al. 2010).

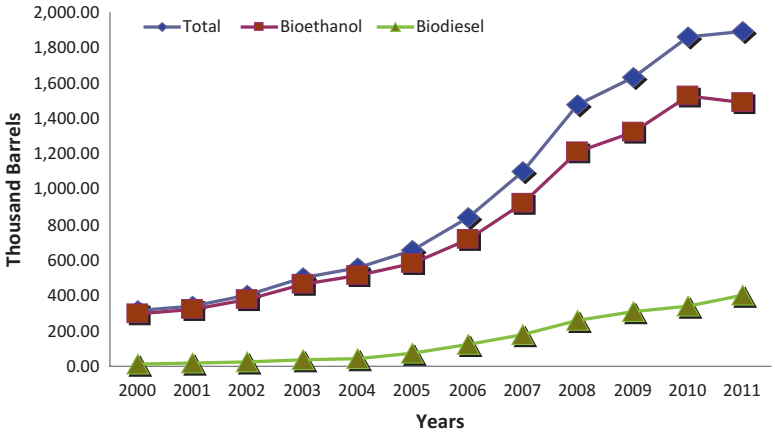

Fig. 8.1 World total biofuel, bioethanol, and biodiesel production from 2000 to 2011 (The US Energy Information Administration 2014)

Fermentation and subsequent distillation of sugars directly from crops like sugarcane or sugar beets or indirectly from starch-rich crops like corn, wheat, potatoes, and cassava are carried out for ethanol production (Carere et al. 2008). During the fermentation process by yeast, about 10% of sugar is converted into glycerol and succinic acid, which are by-products of most bioethanol production plants. Recently, research has been conducted for developing genetically engineered yeast for efficient fermentation process to maximize ethanol production; however, these novel strains of yeast have not been tested at commercial scale production (Zaldivar et al. 2001).

8.2.3 Biodiesel

Biodiesel is the second most important liquid fuel after bioethanol that has been used as renewable environmentally friendly alternative to fossil fuel in any diesel engine without modification. Biodiesel production was initiated in the early 1990s, and it has been widely adapted as a renewable source of energy for transportation industries; since then, its production has been steadily increased (Fig. 8.1).

8.3 First-Generation Biofuels

Globally, several industrial and developing countries have been strongly promoting biofuel production and utilization to reduce greenhouse gas emissions and to diversify energy resources. Currently, majority of biofuels including bioethanol and

biodiesel are produced through first-generation technology in which biofuel is made mainly from agricultural commodities such as corn, canola, soybean, sugarcane, sugar beets, etc.

8.3.1 Feedstock for First-Generation Biofuels

In the biofuel production, feedstock alone represents more than 75% of the overall bioethanol as well as biodiesel production cost. Selection of the best feedstock is very crucial to ensure low production cost that can give competitive edge to fossil fuel. First-generation biofuel that is currently being commercialized in different countries is primarily produced from grain crops as feedstock.

Bioethanol is a liquid biofuel that can be produced from several different types of biomass as feedstocks using a variety of conversion technologies. Biomass with appreciable amounts of sugar or materials that can be converted to sugar such as starch, cellulose, and hemicelluloses can be fermented to produce bioethanol. For the first-generation bioethanol production, feedstocks can be classified into two types:

- Sucrose-containing feedstocks such as sugar beet, sweet sorghum, and sugar cane
- Starchy materials such as grains of wheat, corn, barley, and rice

Globally, about 350 oil-bearing crops have been identified as potential sources for the first-generation biodiesel production; this is the most significant factor of the steady increase in the biodiesel production (Shahid and Jamal 2011; Atabani et al. 2012). Biodiesel feedstock can be classified into four different groups depending on their source (Pinto et al. 2005):

- Edible vegetable oil crops: rapeseed, soybean, coconut, palm, peanut, sunflower, safflower, corn, cottonseed, false flux (*Camelina*), etc.
- Nonedible vegetable oil crops: castor, jatropha, seashore mallow, algae, halophytes, Chinese tallow tree, etc.
- Recycled oil or waste cooking oil
- Animal fats: tallow, yellow grease, chicken fat, fish oil, and other products

Production of first-generation energy-efficient biodiesel from oilseed crops is currently not economically feasible, so more research and development is required for their production. Biodiesel properties are strongly associated with the properties of individual fatty esters of the feedstock oil. It is therefore important to improve fatty ester properties to improve biodiesel suitability and performance (Pinto et al. 2005).

8.3.2 Current Global Production of First-Generation Biofuel

Bioethanol is the most widely used liquid biofuel in transportation industries in combination with fossil fuel. Global bioethanol production is reported to be about 41 billion liters in 2004. The major producers in the world are the USA (39%), Brazil (38%), and Asia (17%). The USA is the world's largest producer of bioethanol (18 billion liters). Brazil is the second largest bioethanol producer country with a production capacity of 16.4 billion liters in 2004, accounting for nearly 18% of the country's automotive fuel needs.

The world biodiesel production was about 0.8 billion liters in 2000 and it increased to 4 billion liters in 2005. In 2010, the total production reached to over 16 billion liters. A similar steady increase in biodiesel production reported 22.4 billion liters in 2011 and 22.5 billion liters in 2012. The European countries account for more than 80% of the world's biodiesel production followed by the USA (REN21 2014). List of the countries with current and future mandates in the bioethanol and biodiesel production is presented in Table 8.1.

8.4 Second-Generation Biofuels

Recent advances in the research and development have made second-generation biofuels available for commercial-scale production. Second-generation biofuels is the term designated to those biofuels including bioethanol and biodiesel produced from nonfood materials as feedstocks. Ultimately, these biofuels will have no or least competition with food crops, and crop residues and other non-crop plant species will be exclusively grown for bioenergy production.

Bioethanol production from lignocelluloses is a multistep process. The processed biomass is pretreated typically using a combination of heat and chemical treatments. Pretreatment altered native cellulose fibers to induce hydrolysis activities of cellulose. The sugar produced by the enzymatic hydrolysis is fermented by *Saccharomyces* yeast which is the most preferred industrial microorganism for bioethanol production. In some cases, enzymatic hydrolysis and fermentation are carried out in a single step called simultaneous saccharification and fermentation (SSF). The SSF is more efficient for ethanol conversion from available sugar and which also enhanced efficiency of enzymatic hydrolysis process through reducing end product accumulation. However, SSF is high depending on suitability of biomass and availability of comestible enzyme and yeast strains.

8.4.1 Feedstock for Second-Generation Biofuels

8.4.1.1 Lignocellulosic Biomass

Naturally occurring abundant and renewable resources are essential for the functioning of industrial societies and vital for the development of a sustainable global economy through production of ethanols for transportation industries around the

Table 8.1 List of the countries with current and future mandates in the bioethanol and biodiesel production

Countries	Percent current mandate		Percent future mandate	
	Bioethanol	Biodiesel	Bioethanol	Biodiesel
Angola	10	–	–	–
Argentina	10	7	–	–
Australia	4	2	10	10
Brazil	20	5	25	–
Canada	5	2	–	–
Chile	–	–	5	–
China	10	–	10	10
Columbia	8	–	10	–
Costa Rica	7	20	–	–
Ethiopia	5	–	–	–
European Union	10	10	–	–
Fiji	–	–	10	–
India	10	–	20	20
Indonesia	3	2.5	–	–
Jamaica	10	–	–	–
Kenya	10	–	–	–
Malawi	10	–	–	–
Mexico	2	–	–	2
Mozambique	10	–	–	–
Nigeria	–	–	10	–
Panama	2	–	10	–
Paraguay	24	1	–	–
Peru	7.8	2	–	5
Philippines	10	2	–	–
South Africa	10	–	–	–
South Korea	–	2.5	–	–
Sudan	5	–	–	–
Taiwan	–	1	3	–
Thailand	–	5	–	–
USA	10	5	15	10
Uruguay	–	2	5	–
Vietnam	5	–	–	–
Zambia	–	–	10	5
Zimbabwe	–	–	10	–

GRFA (2014)

world. As wood and paper products, lignocellulose has played a significant role in the evolution of civilizations. Wood and wood by-products derived from hybrid poplar, saw dust, wood chips, and wood pellets can be subjected to hydrolysis, and the resulted sugar can be further processed through fermentation to produce bioethanol. Wood contains about 30–45% cellulose, 25% hemicelluloses, and 20–30% lignin and remaining contains protein and pectin. Efficient utilization of these three

components, cellulose, hemicellulose, and lignin, would play a significant role in the economically viable bioethanol production. Globally, numerous efforts are being made to increase bioethanol production from lignocellulosic materials from plant debris. Biomass conversion processes involved five crucial steps to produce bioethanols: selection of suitable biomass, pretreatment, fermentation of hexoses and pentoses by various enzymes, and further processing. Menon and Rao (2012) reported an extensive review of different methods of pretreatment of the biomass for the efficient production of bioethanol from plant residues.

8.4.1.2 Biomass of Cellulosic Crops

Switchgrass

Switchgrass is a perennial herbaceous C4 grass species (*Panicum virgatum* L.) native to prairies of North America. It requires low input compared to other annual crops for high biomass production with sustainable agronomic traits and is known for its efficient soil carbon sequestration and relatively high net energy benefits, making it highly suitable as bioenergy crop for soil conservation of arable lands (Cleveland 2005; Lewandowski et al. 2003; Sanderson et al. 2006; Vogel et al. 2010). Schmer et al. (2008) reported extensive analysis of switchgrass total biomass production, total bioethanol production, and net energy values per hectare. The study suggested one of the best feedstocks for efficient bioethanol production from biomass. Dien et al. (2013) studied upland and lowland switchgrass species for conversion of biomass to bioethanol and suggested efficient production of bioethanol 80–150 g/kg switchgrass dry biomass.

Miscanthus

This is a perennial sterile grass hybrid and propagates through rhizomes. It has potential to be used as feedstock to produce 2.5 times more ethanol compared to maize on the same area. It grows on marginal lands and does not compete with cash crops and is being perennial in nature; it also protects soil erosion and fixes more atmospheric carbon into soil compared to annual cash crops (Heaton et al. 2008). Different species of *Miscanthus* have high yield potential with appreciable carbohydrate and lignin content to be used for future biofuel production (Jørgensen 2011; Brosse et al. 2012).

Crop Residues (Straw and Corn Stover)

Crop residues, for example, corn stover, wheat straw, rice straw, and sugarcane bagasse, are the most abundant by-products of crop production used as biomass throughout the world. Utilization of these crop residues for bioethanol production can be a great net energy benefit together with greenhouse gas reduction. In the USA, it is estimated that 367 × 106 Mg/year residues are produced for 9 cereal crops, 450 × 106 Mg/year for 14 cereals and legumes, and 488 × 106 Mg/year for other 21 crops (Lal 2005). The crop residue produced in the world is about 2802 × 106 Mg/year for major cereal crops, 3107 × 106 Mg/year for 17 other cereals and legumes, and 3758 × 106 Mg/year for 27 food crops (Lal 2005). The crop residues

are easily available, cheap feedstock for bioethanol production. Bioethanol produced from crop residues will have higher net energy benefit compared to grain-based bioethanol.

8.5 Government Policies on Biofuels

Various promotions and incentives by many governments have enhanced bioethanol production alone from 17,000 million liters in 2000 to 41 billion liters in 2004 using first-generation technology, and it is increasing yearly around the globe (Balat 2007; Balat and Balat 2009b).

In order to reduce reliance on fossil fuels due to uncertainty on price and future availability as well as to reduce greenhouse gas emission, governments of different countries promoted utilization of biofuels through establishing targets and/or mandates for blending biofuel components in the fossil fuel. Currently, about 5–20% blending of biofuels with fossil fuel is mandated, with increasing blending percentages established as target every year by the USA, Brazil, Canada, China, European Union (EU), India, Japan, Malaysia, South Africa, and Thailand.

In the USA, the Energy Independent and Security Act of 2007 contained a renewable fuel target of 36 million gallons by 2022 (Martin 2010). The European Parliament and the Council of the European Union required about 6% transport fuels to be derived from renewable biomass by 2010, and further increase of renewable energy is given the most priority. The second most important step toward the promotion of biofuel industries through subsidies has been initialized by several countries in the world. Globally, renewable energy was subsidized by $88 billion in 2011 (REN21 2014).

8.6 Impact of Biofuels on Global Economy

Biofuel industry has been growing significantly in recent years from first-generation commercial production to research and development for third-generation biofuels. Both bioethanol and biodiesel industries have crucial contribution to the individual economies of producing countries and global as a whole. The primary driving factors of the global biofuel industry are alternative renewable sources of energy to soaring fossil fuel prices, reducing greenhouse gases to mitigate climate changes, stimulating agriculture production, and increasing revenue for farmers through the production of value-added biofuel-producing crops or crop products. In addition to that, biofuel industry has been promoting state and/or national policies in the form of mandate of renewable form of energy blended with fossil fuels. Various national- and state-level policies have given direct support to the steady growth of biofuel industry around the globe. Promotion by individual countries to biofuel industry through policies also reflects precautionary steps toward the changing climates. Due to first-generation biofuel production, agriculture crop production has been diverted from food crop production to fuel crop production; in order to overcome this

problem, significant efforts have been made by different countries for research and development for next-generation biofuel production from waste or crop residuals so that biofuel production has no or less impact on food channels.

8.7 Impact of Biofuels on Environment

Biofuels are renewable fuels that replace petroleum fuels that have several benefits for the environment, economy, and end users. The main advantages of biofuels including bioethanol, biodiesel, biogas, biomethanol, and vegetable oil are ready availability, renewability, higher combustion efficiency, lower sulfur and aromatic content, and biodegradability (Demirbas 2009). Higher oxygen level from 10 to 45% in biofuels compared to fossil fuels has better advantage to minimize greenhouse gas emissions. Biodiesel significantly reduces greenhouse gases such as N_2O, CO_2, hydrocarbons, carbon monoxide, and nitrogen oxides. Balat and Balat (2008) reviewed reduction in overall greenhouse gas emission by soybean oil-based biodiesel compared to petroleum fuel.

8.8 Food Versus Fuels

The food crisis of 2007–2008 triggered by increased food commodity prices sparked the immense contested food versus fuel debate (Timilsina 2012). The primary objective of agricultural commodity production is to feed the increasing global population. Abbott et al. (2009) identified three primary drivers of flux of food prices: first, increase in agricultural commodity consumption, lowering global agricultural commodity inventories; second, weakening of the US dollar against other global currencies; and third, energy generation from agricultural commodities.

Production of biofuels from agricultural feedstock and using this environmentally friendly fuel as an alternative to fossil fuel are becoming central to over 50 jurisdictions of the world (Javed et al. 2014). Among other drivers of climate change, emission of CO_2 is contributed by urban transport sector which is exclusively fuel by fossil fuel. Reduction of CO_2 emissions to 450 ppm by 2050 would be challenging and require difficult choices to make for reduced carbon emission for sustainable environment.

Historically, energy and agricultural commodity production/consumption has remained an independent path (Abbott et al. 2009). This relationship has been changed with production of biofuel from agricultural feedstock. Mitchell (2008) concluded that in spite of increased crop production before 2008, the production of biofuels from agricultural commodities caused appreciable decline in global food stock inventories and increased food prices to about 35–40% from 2002. In contrast, Gilbert (2010) reported no direct influence of biofuel production from grains and oilseeds on food prices. Instead, investment in agricultural future markets contributed to food price rises in 2007–2008. However, investments in speculative buying in agricultural markets in response to rising commodity prices may not have been

triggered without large-scale diversion of grains and oilseeds toward biofuel production (Mitchell 2008). It has lowered global agricultural commodity inventories to produce first-generation biofuels such as cereal grains; plant oil inherently competes with food.

The Food and Agriculture Organization of the United Nations reports that one in nine of the 795 million individuals in the world is chronically undernourished. Majority of these people live in the developing countries that constitute to about 13% of their population (FAO 2015). The global demand for food is projected to be doubled by 2050 (Timilsina 2012). Due to the interplay between food commodities and bioenergy, the diversion of food commodities toward large-scale production of biofuel is not supported by several studies (Timilsina 2012). In contrast, intensification of agricultural activities is forecasted to lessen food versus fuel debate in developing countries, whereas some studies suggest that an increment in agricultural production must be used toward meeting the growing food demand (Timilsina 2012). Poudel et al. (2012) provided a quantitative estimate of the available grain for biofuel production under different scenarios of dietary requirements in the world in 2050. In case of dietary requirement increase by more than 20% from 2007, then crop yield must increase by more than 60%, and additional 16% conversion of pastureland for crop production would be sufficient to dietary requirements. This would also leave enough grain to meet the 23% liquid fuel demand (Poudel et al. 2012).

Challenges faced by global fuel ethanol industry are multidimensional (Jolly and Rocha 2009). Profitability in ethanol fuel industry is linked to higher crude oil prices and supportive government policies toward biofuels (Jolly and Rocha 2009). Regardless, biofuel future seems bright and global energy scenario is forecasted to be mix of renewable energy with 70% share of fossil fuel by 2050 (IPCC 2013). Fuel versus food debate has started impacting government policy toward biofuels, but for a brighter biofuel future, three dimensions have to be addressed: first, food security concerns; second, net energy gain from and reduced greenhouse gas emission balance of biofuels; and third, sustainability (Jolly and Rocha 2009).

Singh et al. (2010) describe that increasing energy consumption is exerting stretching natural resources; this has led to a quest of renewable energy resources not only to improve security of supply but also to reduce greenhouse gas emissions. Adaptation of biofuel as renewable energy resource has inherent weakness, it competes for land use, and produced grain is further diverted away not for food consumption but for biofuel production. The associated net energy balance with the use of different biofuels is suggesting further diversification in feedstocks to produce biofuels. Sustainable biofuels such as from wastes and lignocellulosic materials have significantly better greenhouse gas balances and do not directly compete with food commodity (Singh et al. 2010). Compared with biodiesel strategy using first-generation biofuel derived from rapeseed oil, Singh et al. (2010) suggest that biomethane production from animal wastes, slurries, and slaughter waste together and from energy crops such as grass can substitute 33% of natural gas by 2020 and can save 23% of Irish arable area. Energy production is critical for the development of human societies.

8.9 Conclusion

Globally, first-generation biofuel production has significant impact on both economy and environment. However, competition of agricultural commodities as food supply or fuel supply remains challenging until novel technological advancement unravels the paths for next-generation biofuels from agriculture waste. Second-generation biofuels especially bioethanol from biomass and agriculture crop residues and green algae-based biodiesel production have been highly hopeful to resolve food versus fuel debate in the society as well as to produce biofuels with high net energy balance to be considered biofuels and renewable sources of energy. Tremendous research and development studies are being undertaken from next-generation biofuels; however, its implementation for commercial scale production has many challenges to overcome. Overall, the energy industry is one of the most important industries especially biofuel that has been in co-alliance with the agriculture industry to make a better world by producing and utilizing more produce efficiently for food and fuels. Globally, largest produced cereal crops such as wheat, rice, and corn can be used for food, and by-products such as wheat straw, rice straw, and corn stover remain available for biomass-based bioethanol production. Biomass-derived bioethanol is ultimately sustainable and cost-effective compared to the currently produced bioethanol from grains of cereal crops. Yet numerous challenges are required to be tackled to make successful commercial scale production of second-generation biofuels from biomass as feedstocks.

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Critical Evaluation of Biodiesel Production Initiatives in India

9

Chigullapalli Sreenivas, Anand B. Rao, and Anand Patwardhan

Abstract

Biofuels are receiving increased attention due to their potential to enhance the energy security of the energy-deficit countries with simultaneous climate change mitigation by reducing GHG emissions. In addition to these, they also offer a pathway for low-carbon inclusive growth for India. Fluctuating imported crude oil prices create a dilemma about the production of biodiesel in India. The objective of the present study is to understand the need for biodiesel production in India while focusing on both forward linkages (such as current status of commercial biodiesel production in India, market linkages and economics of biodiesel production) and backward linkages (such as feedstock availability and their technological implications, policy implications on the economy, supply chain analysis). A critical understanding of the externalities associated with

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biodiesel production through many government initiatives is also carried out. To be able to make biodiesel production in India a commercial success, we may need to have strong technological base supported by policy support mechanisms. If produced sustainably, biodiesel may offer a comprehensive solution for problems such as energy security, import dependence for energy, rural employment generation and climate change mitigation.

Keywords
Biodiesel • Transesterification • Forward and backward linkages • Climate change • GHG emissions

9.1 Introduction

India is one of the fastest growing economies in the world today and consequently is one of the largest and fastest growing energy consumers. India is the third largest consumer of oil and petroleum products in the world in 2016, after the United States and China. India imports around 70% of its crude oil and petroleum product requirement. The oil import expenditure has increased by more than six times in the last 25 years due to an increasing population, demand and escalation in global prices. Figure 9.1 shows that over the last two decades, the proven total oil reserves in India are stabilized in the range of 5.5–5.9 thousand million barrels, mostly in the western part of the country. Domestic production has stagnated in recent years, and Indian national oil companies increasingly purchase equity stakes in overseas oil fields. The country depends heavily on imported crude oil, mostly from the Middle East.

The domestic crude oil production in India grew at 18% from 34.9 million tons in 2005 to 41.2 million tons in 2015, whereas crude oil consumption grew at 60.4%

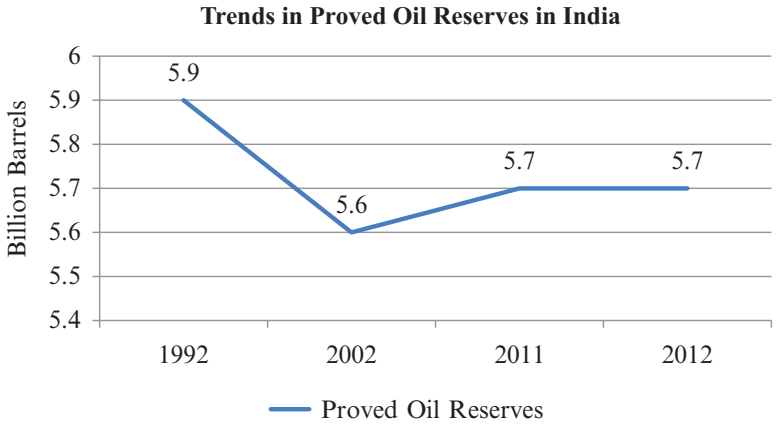

Fig. 9.1 Trends in total proved oil reserves in India

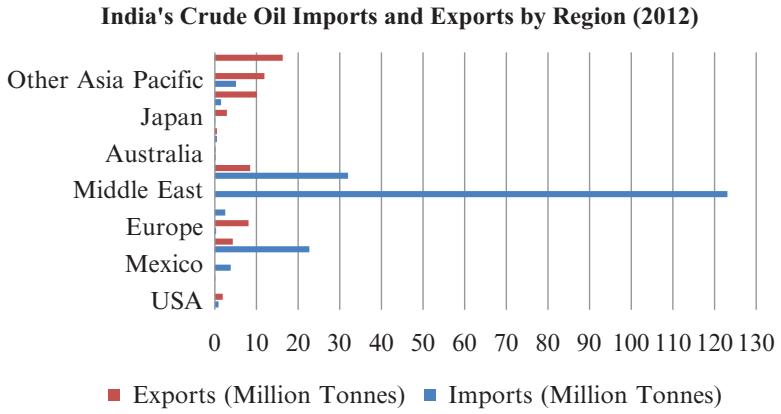

Fig. 9.2 India's crude oil imports and exports by region (2012)

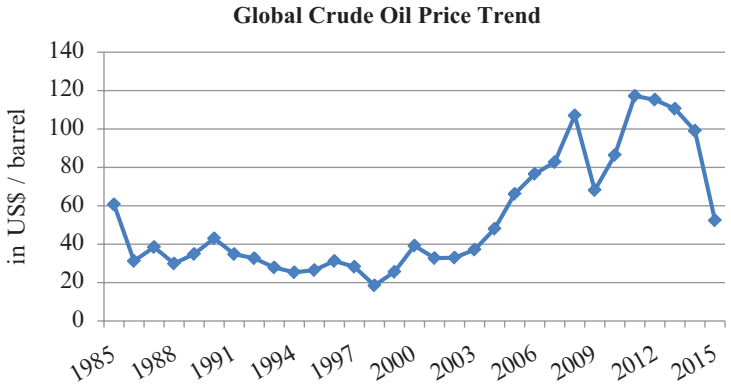

Fig. 9.3 Global crude oil price trend

from 121.9 million tons in 2005 to 195.5 million tons in 2015. It is evident that India is always dependent on oil imports to meet its demand for oil and oil products. The crude oil imports in India grew from 71% in 2005 to 79% in 2015. India's transport sector's energy demand was expected to grow by 6–8% per annum during the 11th Five-Year Plan period (2007–2012) (British Petroleum 2016). The major portion of India's crude oil imports are concentrated to the Middle East region amounting to 65% of its total imports in 2012 (shown in Fig. 9.2).

Figure 9.3 shows that the global crude oil price has been fluctuating over the past two decades and has been falling sharply since the last 3 years, reaching US$44.49 per barrel as of August 13, 2016 (oil-price.net 2016). Although the price of crude oil is not a direct component of the cost of biodiesel production, it provides the baseline (i.e. price of diesel) against which the cost of biodiesel production must be compared. From the perspective of the biodiesel producer, the price received for biodiesel output will most likely bear a close relationship with the price of diesel and therefore will be a direct influence on the profitability of the producer's operation.

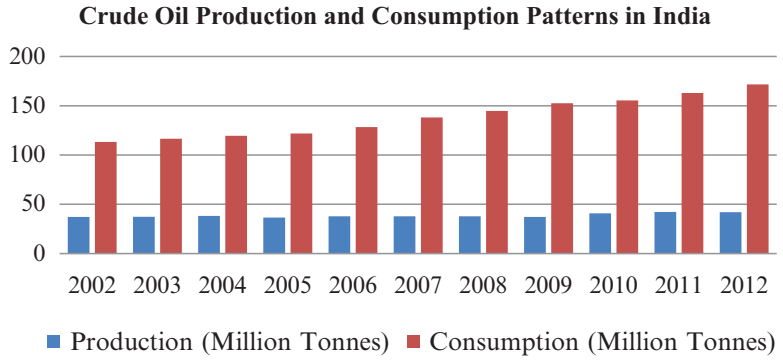

Fig. 9.4 Crude oil production and consumption patterns in India

In Indian transport sector, over 80% of passengers and 60% of freight are transported by road. India's phenomenal economic growth has increased not only the income and expenditure of consumers but also the use of personal modes of transport such as cars and two wheelers. In India, the automotive vehicle population is growing by 12–15% per annum. This will, in turn, impact the transport sector's energy demand. Diesel and gasoline (petrol) contribute to 98% of the energy consumed in the transport sector.

India's transport sector's energy demand was expected to grow by 6–8% per annum during the 11th Five-Year Plan period (2007–2012) (MoP&NG 2013). Diesel constitutes 45% of the total crude-based products consumed in India amounting to 68.5 million tons in the year 2012. In India, annual diesel consumption constitutes 83.5% of the total middle distillate consumption in 2012, which grew from 72% in 2005 (MoP&NG 2013).

It is imperative from the above statistics that:

- There exists limited pool of proven oil reserves in India
- Consumption of petroleum products surpasses the production over the last decade
- There is import dependency to meet the increasing demand for crude oil and products
- Energy insecurity as imports is concentrated from few regions/countries
- Diesel forms 72% of the middle distillates and 33% of the total crude oil-based products
- Considerable amount of CO_2 emissions contribute to the total GHG emissions in India (Figs. 9.4 and 9.5)

The growing consensus about the anthropogenic climate change across the globe is leading the attention of scientific community towards the alternative energy sources which will offer effective response in terms of mitigation. Renewable energy derived from solar, wind and biomass sources has great potential for growth to meet our future energy needs. Fuels such as biodiesel, ethanol, methane and

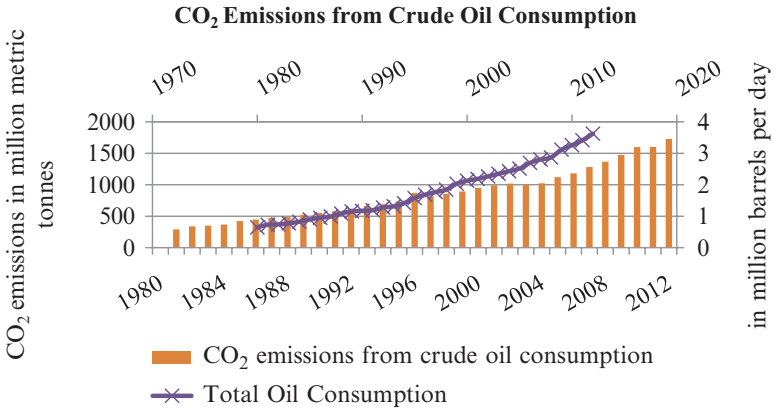

Fig. 9.5 CO_2 emission trends from crude oil consumption in India

hydrogen are characterized as biofuels because they can be produced by the activity of biological organisms. Which of these fuels will play a major role in our future? The answer is not clear, as factors such as land availability, future technical innovation, environmental policy regulating greenhouse gas emissions, governmental subsidies for fossil fuel extraction/processing and public support for alternative fuels will all affect the outcome.

9.2 Current Status of Biodiesel in India

According to the "National Policy on Biofuels", the goal is to ensure that minimum level of biofuels become readily available in the market to meet the demand at any given time. An indicative target of 20% blending for both biodiesel and bioethanol by 2017 is proposed. The observed blending rate for the biodiesel in India is far below the pre-decided target of 20%. Figure 9.6 describes the achieved biodiesel blend rate over the past decade. The blending rate achieved during 2015 is 0.08% (Aradhey 2016). Table 9.1 summarizes the current status of the National Biodiesel Mission in India.

India is having limited production capacity for commercial biodiesel manufacture since biodiesel has to compete with diesel for its commercial success. Administered prices for crude oil products were heavily subsidized, whereas biodiesel purchase price is far below the cost of production in most of the cases. Table 9.2 provides a statistical description about the biodiesel production and consumption in India (Aradhey 2016). The demand for diesel is five times higher than the demand for petrol in India. But while the ethanol industry is mature, the biodiesel industry is still in its infancy. Since the demand for edible vegetable oil exceeds supply, the government has decided to use nonedible oil from *Jatropha curcas* seeds as biodiesel feedstock.

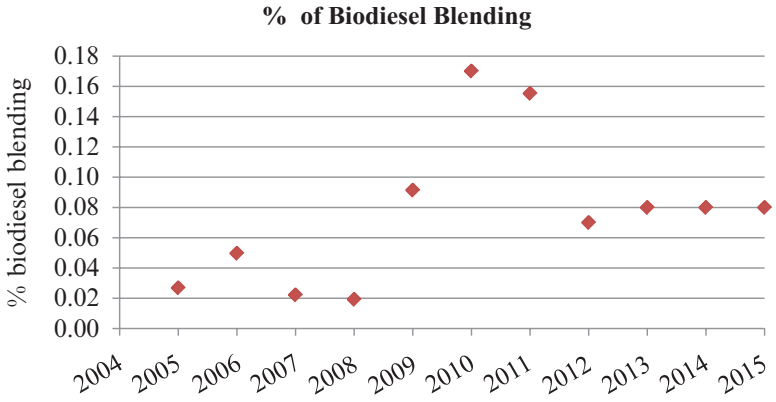

Fig. 9.6 Observed blend rate for biodiesel in India

Table 9.1 Current status of the National Biodiesel Mission in India

Date	Action	Comments
April 2003	Demonstration phase (2003–2007): Ministry of Rural Development appointed as nodal ministry to cover 400,000 hectares under *Jatropha* cultivation	Public and private sector, state government, research institutions (Indian and foreign) involved in the programme achieved varying degrees of success
October 2005	The MoP&NG announced biodiesel purchase policy in which oil marketing companies (OMCs) would purchase biodiesel across 20 procurement centres across the country to blend with high-speed diesel with effect from January 2006	Purchase price set at Rs 26.5 per litre. Cost of biodiesel production was higher (20–50%) than purchase price. No sale of biodiesel
Calendar year 2008	Self-sustaining execution phase (2008–2012): targeted to produce sufficient biodiesel for 20% blending by end of 11th Five-Year Plan (2008–2012)	Conventional low-yielding *Jatropha* cultivars and lack of large-scale plantations, seed collection and extraction infrastructure, buy-back arrangement, capacity- and confidence-building measures among farmers impeded the progress of this phase
Calendar year 2010	An estimated 0.5 million hectares has been covered under *Jatropha* cultivation of which two thirds of plant populations are believed to be new plantation and would take 2–3 years to mature	Assuming 80% biodiesel requirement is met through *Jatropha* oilseeds; the biodiesel thus obtained will just meet 0.01% of total biodiesel required for 5% blending by 2011
Calendar year 2011	No additional wastelands have been brought under *Jatropha* cultivation except for few captive plantations managed by OMCs	–

(continued)

Table 9.1 (continued)

Date	Action	Comments
Calendar year 2012	The production of biodiesel from *Jatropha* seeds remained commercially insignificant	According to the MoP&NG, no biodiesel (from *Jatropha*) has been procured by oil marketing companies for blending with diesel in the last 3–4 years
Calendar year 2013	Biodiesel production from multiple feedstocks (crude oil, used cooking oils, animal fats, etc.) was an economically viable option left with the producers	Industry sources claimed that the average purchase price of biodiesel in India then was around Rs 45–48 per litre and seems viable for blending as regular diesel was selling at a price premium of 18–20% over biodiesel
Calendar year 2014	Industries' engagement with tree-borne oilseeds as alternative to *Jatropha* for biodiesel production gets due attention	Seed yield from *Jatropha* plantation (on pilot scale) were observed to be significantly lower than stipulated. Consequently, cost of production of biodiesel from Jatropha seed is too high providing little incentive for producers to go full throttle
Calendar year 2015	The cabinet has decided to suitably amend Para 5.11 and Para 5.12 of the national biofuel policy for facilitating consumers of diesel in procuring biodiesel directly from private biodiesel manufacturers, their authorized dealers and JVs of OMCs authorized by the MoP&NG. On August 10, GoI had issued notification to allow the sale of biodiesel (B100) by private manufacturers to bulk consumers like railways, state transport corporations and other bulk consumers	The amendment will allow private biodiesel manufacturers, their authorized dealers and JVs of OMCs authorized by the MoP&NG as dealers and give marketing and distribution functions to them for the limited purpose of supply of biodiesel to consumers
		As the price of diesel is already deregulated, private biodiesel manufacturers are encouraged to sell biodiesel directly to consumers subject to their product meeting prescribed BIS standards
		On August 11, 2015, Minister of State (I/C), Petroleum and Natural Gas, launched sale of B-5 diesel. As part of the initial run, B-5 will be sold to customers at some retail outlets in New Delhi, Vijayawada, Haldia and Vishakhapatnam
Calendar year 2016	Biodiesel development is still in nascent stage. Commercial availability of biodiesel and its availability across major retail centres will take its own time	Few bulk users such as road transport companies, state transport corporations (plying public buses) and railway depots (diesel locomotives) claim to have utilized biodiesel for transporting goods and people

Table 9.2 Statistics about biodiesel in India

Calendar year	2010	2011	2012	2013	2014	2015
Beginning stocks (million litres)	45	38	42	45	45	50
Production (million litres)	45	64	73	75	85	85
Imports (million litres)	0	0	0	0	0	0
Exports (million litres)	0	0	0	0	0	0
Consumption (million litres)	52	60	70	75	80	90
Ending stocks (million litres)	38	42	45	45	50	45
Production capacity						
No. of biorefineries	5	5	5	6	6	6
Name plate capacity (million litres)	450	450	460	465	480	480
Capacity use (%)	10	14.2	15.8	16.1	17.7	17.7
Feedstock use (in thousand tons)						
Used cooking oil	38	42	48	49	50	50
Animal fats and tallow	6	6	7	7	6	5
Other oils	50	58	65	70	75	85
Market penetration						
Biodiesel (on-road use) (million litres)	26	30	35	38	40	45
Diesel (on-road use) (million litres)	42,625	45,520	49,343	49,354	49,605	52,239
Blend rate (%)	0.06	0.07	0.07	0.08	0.08	0.08

The growth in the biodiesel industry has been driven by mandates and tax incentives for blending biodiesel with diesel for energy security and climate change mitigation reasons. There is a clear indication about the various initiatives to promote biodiesel blending although a lot of challenges are yet to be addressed. There also exist underutilized, locally available nonedible resources for biodiesel production in India. Table 9.3 describes the potential of underutilized resources for biodiesel production in India (Planning Commission 2003).

While the policy framework to promote the biodiesel in India is very encouraging, experience has shown that the government's initiatives have not translated into results on the production and commercialization fronts to meet the country's energy demand. This calls for a re-examination of the policy from various stages of the biodiesel supply chain.

9.2.1 Diesel Use Projections and Biodiesel Requirement

Annual diesel consumption is projected to grow at a rate of 50% between 2016 and 2026, requiring 28 billion litres of biodiesel for 20% blending target by 2016. This blending requirement may need huge capacity expansion for biodiesel production infrastructure. Table 9.4 gives brief description of the sector-wise annual diesel

Table 9.3 Potential availability of some feedstocks in India

Oil	Potential ('000 tons)	Utilization ('000 tons)	% Utilization	Current uses
Rice bran	474	101	21	Edible oil, wax mfg., industrial use
Sal	720	23	3	Soap manufacture, cooking, lighting
Neem	400	20	6	Soap making, insecticide and fungicide formulations
Karanja	135	8	6	Washing soap making, leather tanning, candle making, etc.
Niger seed	–	75	–	Paints and varnishes

Table 9.4 Annual diesel use projections and biodiesel blending requirement

Projections for sector-wise annual diesel use and biodiesel requirement for 20% blending (million litres)								
Calendar year	On-road	Agriculture	Construction and mining	Shipping and rail	Industry	Heating	Diesel total	20% blending requirement
2016	56,111	11,222	3741	4676	10,287	7481	93,518	18,704
2017	58,422	11,684	3895	4869	10,711	7790	97,371	19,474
2018	60,829	12,166	4055	5069	11,152	8111	1,01,382	20,276
2019	63,336	12,667	4222	5278	11,612	8445	1,05,560	21,112
2020	65,945	13,189	4396	5495	12,090	8793	1,09,908	21,982
2021	68,662	13,732	4577	5722	12,588	9155	1,14,436	22,887
2022	71,491	14,298	4766	5958	13,107	9532	1,19,152	23,830
2023	74,436	14,887	4962	6203	13,647	9925	1,24,060	24,812
2024	77,503	15,501	5167	6459	14,209	10,334	1,29,173	25,835
2025	80,696	16,139	5380	6725	14,794	10,760	1,34,494	26,899
2026	84,021	16,804	5601	7002	15,404	11,203	1,40,035	28,007

consumption projections from 2006 to 2026 and corresponding 20% blending requirements (Bandyopadhyay 2015). With this backdrop, India provides a potential market for biodiesel. But the market penetration of biodiesel is hindered by lack of assured supplies of feedstocks, sufficient production and distribution infrastructure.

9.3 Forward Linkages: Market Overview

For every finished product to be successful, it should have good market for its end use. As an alternative to petro-diesel, biodiesel has its end uses in transportation, electricity generation for lighting and irrigation. Although the market for biodiesel in India is at its infancy, it is assumed to grow at par with the energy demand of the country. The infancy stage for the biodiesel market is due to the lack of awareness

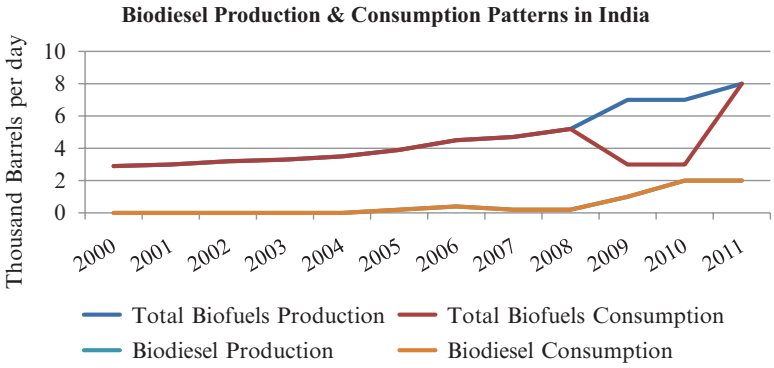

Fig. 9.7 Biodiesel production and consumption patterns in India

about climate change among the consumers, unsteady and seasonal availability of the feedstocks for biodiesel making, less research and development inputs to the agronomy and development of nonedible energy crops.

In India, transportation sector is the large consumer of the diesel. Sections of transport sector that consume the most diesel include road transport and railways. Although air transport and water transport do consume diesel in appreciable amounts, that amount is considered small when compared with the inland transport. Strategic analysis of the Indian biofuel market provides an overview of the current and future markets for biodiesels in India. It also provides feedstock analysis, market drivers, restraints and future strategies for the industry. Figure 9.7 explains the biodiesel production and consumption patterns in India. Large gap in biofuel utilization is due to gap between fuel ethanol production and consumption over the period from 2008 to 2011. It is also evident that the total biodiesel produced was used either in stationary applications or blending requirements. The Government of India deferred the ethanol blending petrol (EBP) programme due to short supplies of sugarcane and sugar molasses in 2008–2009.

Since there was no official notification released, oil marketing companies have not started 10% ethanol blending. The Expert Committee in March 2011 had recommended that ethanol be priced 20% lower than gasoline. There is no consensus yet on pricing policy of ethanol. When ethanol supply runs short, government proposes to reduce import duty on alcohol and molasses. All these caveats led to the large dip in the consumption of fuel ethanol produced in the country for blending requirements.

According to the "National Policy on Biofuels", the goal is to ensure that minimum level of biofuels become readily available in the market to meet the demand at any given time. An indicative target of 20% blending for both biodiesel and bioethanol by 2017 is proposed (MNRE 2016). Figure 9.8 describes the blending requirements at a rate of 5% in diesel and realized magnitudes of biodiesel production and consumption patterns over the period from 2005 to 2012. The observed blending rate for the biodiesel in India is far below the pre-decided target of 20%.

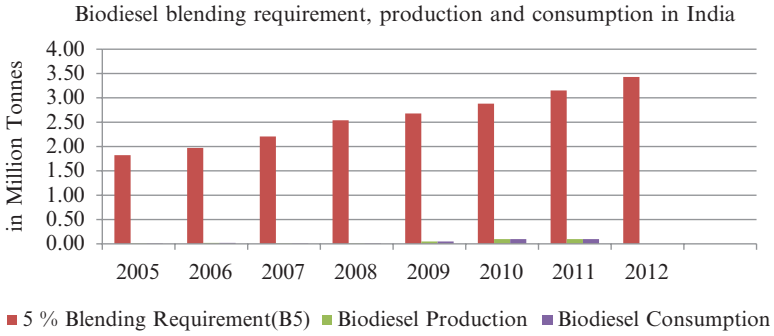

Fig. 9.8 Biodiesel blending requirement, production and consumption patterns in India

9.3.1 Commercial Biodiesel Production in India

The Ministry of Petroleum and Natural Gas is under the auspices of the National Common Minimum Programme (NCMP) of the Government of India, stretching across various focus areas identified therein, such as enhancing energy security by shifting towards alternative sources of energy for transportation and power generation. The ministry is already implementing 5% ethanol blending petrol programme, which is designed to support the agriculture and the rural sector.

India launched the National Biodiesel Mission (NBM) after identifying *Jatropha* (*Jatropha curcas* L.) as the most suitable nonedible oilseed for biodiesel production. The Planning Commission of India had planned an ambitious target of planting 11.2–13.4 million hectares of land to jatropha by the end of the 11th Five-Year Plan (2011–2012). The central government and several state governments provide fiscal incentives in support of planting jatropha and other nonedible oilseeds (NBM 2013).

India is having limited production capacity for commercial biodiesel manufacture since biodiesel has to compete with fossil fuels for its commercial success. Administered prices for crude oil products were heavily subsidized, whereas biodiesel purchase price is far below the cost of production in most of the cases. There are limited production facilities for the commercial biodiesel production in India.

Most of these commercial biodiesel production facilities are dependent on imports of feedstocks and exports of the final product as there is no commercial viability of the sale of biodiesel in the domestic market. Most of the production facilities use palm oil imported from Malaysia and Singapore.

9.3.2 Market Linkages

Although there exists negligible amount of commercial production of biodiesel in India, the lack of established distribution and marketing channels has forced commercial production facilities to withdraw from their regular operations. Most of the final product is exported due to lack of existence of market demand. Most of the

commercial production facilities are grappling with the problems such as lack of assured supplies of feedstock, technological challenges such as low conversion efficiencies, etc.

Greater government involvement is needed to improve feedstock production in the Indian biofuel market. Held back by the lack of large-scale availability of feedstock, the Indian biodiesel market trails its global counterparts by a long way. It is likely to take a while for biodiesel to be established as an effective biofuel, since *Jatropha* plantations in the country are still in the initial stages of development. Three to 4 years later, the country may have the feedstock necessary for the large-scale production of *Jatropha* oil for use in biodiesel. The absence of a clear government policy on *Jatropha* oil production has inhibited several biofuel manufacturers from entering this market. Hence, Indian manufacturers are considering importing palm oil to produce biodiesel.

9.3.2.1 Indian Railways

Indian Railways (IR), one of the world's largest rail networks, is planning to reduce their dependence on expensive fossil fuels and move towards cheaper alternatives like biodiesel and used cooking oil to power locomotives across the country by 2015. The International Union of Railways (UIC), a body that promotes rail transportation, has selected a UK-based consulting firm to help the biodiesel project of Indian Railways in earning carbon credits. UIC has tied up with EcoSecurities to conduct a study for the Indian Railways' *Jatropha* plantation and biodiesel project. To be able to earn carbon credits, Indian Railways needs to prove to the United Nations (UN) that its biodiesel project has resulted in reduction of greenhouse gas emissions.

If proven, it can earn carbon credits, and Railways can sell these carbon credits to companies from developed countries that have to meet greenhouse gas emission reduction norms. If successful, this may end up becoming the first project from the railway sector to be registered as a Clean Development Mechanism (CDM) project at the UN. IR signed a memorandum of understanding (MoU) with Indian Oil Corporation Limited (IOCL) to prepare a biodiesel blend for the trains. Laboratory tests at Indian Railways had proved the viability of biodiesel for commercial use and trial runs had been carried out successfully. The railway, which runs approximately 7200 locomotives, intends to source 15% of its diesel consumption through biodiesel by 2015.

The railways consume about two billion litres of diesel every year, which costs them about Rs 8000 crore. The Railway Ministry has put together a task force that will help reduce dependence on imported fossil fuels and increase reliance on homegrown fuels like biofuels derived from both used cooking oil and nonedible vegetable oils extracted from plants like *Jatropha curcas* (ratanjot) and *Pongamia pinnata* (karanja).

IR is planning to invest Rs 70 crore initially to set up biodiesel extraction and processing plants with a capacity to generate 200,000 litres per annum. The IR has proposed to set up biodiesel plants at Tughlakabad under Northern Railway, Raipur under South East Central Railway, Itarsi in West Central Railway and Erode under

Southern Railway. Southern Railway has already pioneered the use of biodiesel in locomotives. While it is currently using 10% biofuel-blended diesel to run its fleet on the suburban Trichy, Karaikudi–Thanjavur and Chennai-Tondiarpet routes, it is running its train engines on used cooking oil collected from hotels like Taj Coromandel and ITC's Park and Chola Sheraton in Chennai.

On an average, between 2006 and 2008, Southern Railway paid Rs 29.36 per litre for buying HSD, while the cost of biodiesel (used cooking oil and nonedible vegetable oil combined) averaged Rs 20.70 per litre. Thus, the cost of biodiesel over this three-year period was 42% less than HSD. However, Southern Railway currently pays Rs 32.52 per litre for HSD and Rs 42 per litre for *Jatropha*/*Pongamia* oil. Southern Railway increased biofuel blending in diesel from 5 to 10% in 2007. It proposes to increase this to 20%.

9.3.2.2 Brihanmumbai Electricity Supply and Transport (BEST), Mumbai

BEST which is also known as Brihanmumbai Electricity Supply and Transport undertaking will soon be using biodiesel in their buses which ply in Mumbai taking hundreds of passengers to their respective destinations. This decision to use biodiesel in the buses was taken by the administration and the BEST Committee to reduce the pollution in the city. For this purpose BEST is going to buy 6000 litres of fuel per day which will give them an extra earning of Rs 1.36 crore per annum. In addition to this, BEST will also make an extra profit of Rs 2 crore carbon credit in the international market. This decision was taken by the administration and the BEST Committee for using biodiesel in buses in Mumbai because this will not only reduce the emission of gases in the atmosphere but at the same time will help in reducing global warming.

BEST has been using biodiesel since the last 2 years in buses plying from the Vikhroli Depot on experimental basis. BEST has found that mixing of 10–20% biodiesel not only saves energy but also reduces smoke emission greatly. And at the same time, it also does not affect the functioning of the engine of the buses. The biodiesel supplied to BEST is cheaper compared to regular diesel by around Rs 2 a litre. Around 80 buses from the corporation's 3400-strong fleet are being run on the blended fuel as a pilot project.

With the price of vegetable oil soaring, the price of biofuels is unlikely to fall anytime soon. This is probably the point at which public policy must intervene. City authorities must decide whether cleaning up the city's air is worth the extra cost, and pass the cost on to bus or boat passengers, or alternatively, the government should switch the subsidy it provides on regular diesel (by some estimates, this is currently as high as 11%) to blended diesel to encourage its use.

9.4 Backward Linkages: Feedstock Issues

The success of biofuel industry in India is mainly driven by cheaper raw material supply and R&D inputs given for the process optimization and cost minimization. As biodiesels do consume edible oil for its manufacture, there exists a controversy

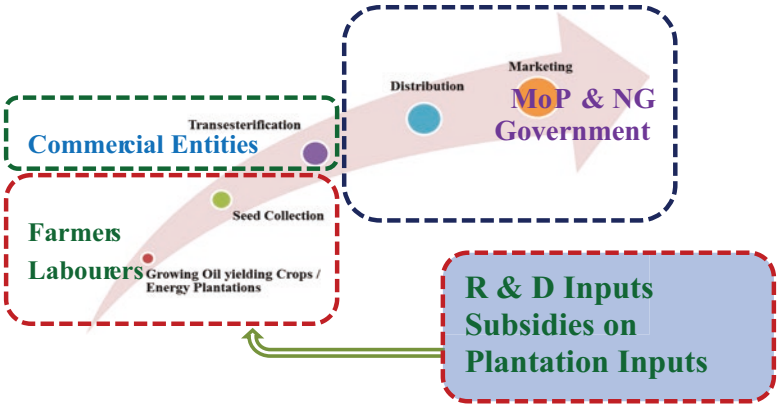

Fig. 9.9 Supply chain for commercial biodiesel production

of "food versus fuel"/"crime against humanity". Figure 9.9 describes the processes and operations involved in the supply chain of biodiesel production and associated stakeholders with each process.

9.4.1 Food Versus Fuel Controversy/Crime Against Humanity Controversy

There is an urgent need to rethink before we rush to biofuels, which has done more harm by pushing up food prices than good by reducing greenhouse gases in the western world. The UN Food and Agriculture Organization (FAO) states that policies encouraging biofuel production and use in Europe and the United States created pressure on food prices but made little impact on weaning car users away from oil. According to its "Annual State of Food and Agriculture" report, biofuels will offset only a modest share of fossil energy use over the next decade and will have much bigger impacts on agriculture and food security. It also mentioned the rising of the prices of agricultural commodities in the next 10 years.

Anti-hunger campaigners have blamed biofuels, converting crops such as maize, sugar, oilseeds and palm oil into liquid fuel for use in cars, pushing up global food prices and contributing to soaring food bills in the last 2 years. The food versus fuel debate was stoked by the decisions of using arable land to make fuel which is considered as "crime against humanity." The FAO report does not quantify biofuels' contribution to commodity price spikes which were also due to poor harvests and demand for a richer diet in places like China and India. But it does say the rise in biofuels has put more people at risk of hunger, requiring food aid and other assistance.

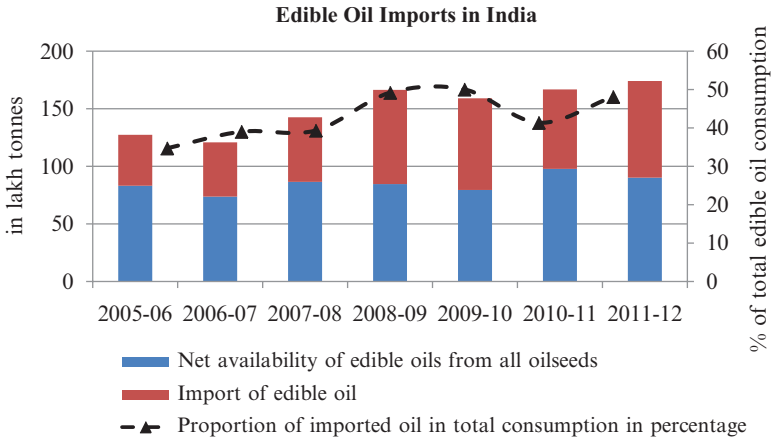

Fig. 9.10 Edible oil import trends in India

9.4.2 Raw Material Supply Potential in India

India has rich and abundant forest resources with a wide range of plants and oilseeds. The production of these oilseeds can be stepped up manyfold if the government takes the decision to use them for producing diesel fuels. Economical feasibility of biodiesel depends on the price of the raw material and their supply and the cost of transporting biodiesel to long distances to remote markets in India. Further, the strict regulations on the aromatics and sulphur contents in diesel fuels will result in higher cost of production of conventional diesel fuels.

9.4.2.1 Edible Oils

Until the mining of petroleum was developed during the nineteenth century, vegetable oils, especially the nonedible varieties, formed an important source of heat and light. Usually the seeds, which store much higher concentration of fatty oil than any other part of the plant, are used for extracting the oil. Most of the common oilseeds contain 20–30% of oil by weight and in some cases up to 42%. India depends on imports for its edible oil and the import fraction is as high as 50% over the past 5 years. Also the rising prices for edible oils make biodiesel production infeasible and create a lot of challenges in commercial biodiesel production. Figures 9.10 and 9.11 describe the edible oil import trends and edible oil price trends in India (MoAg 2013).

In pursuance of the policy of liberalization of the government, there have been progressive changes in the import policy in respect of edible oils during the past few years. Edible oil, which was in the negative list of imports, was first de-canalized partially in April 1994 when import of edible vegetable palm olein was placed under Open General Licence (OGL) subject to 65% of basic custom duty. Subsequently import of other edible oils was also placed under OGL, except coconut oil. In order to harmonize the interests of farmers, processors and consumers and, at the same

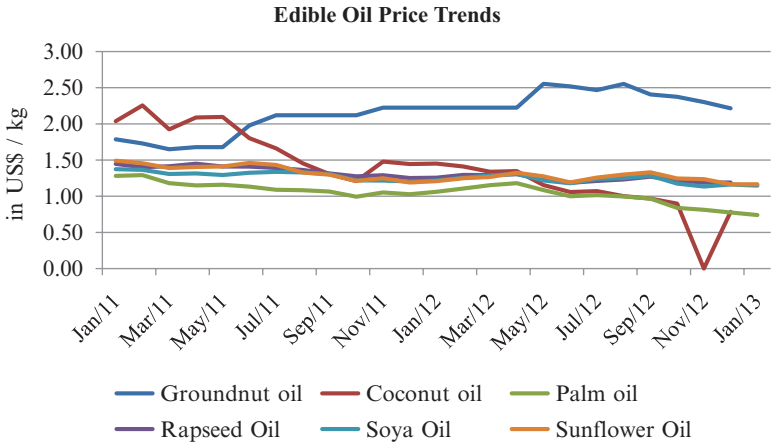

Fig. 9.11 Edible oil price trends in India

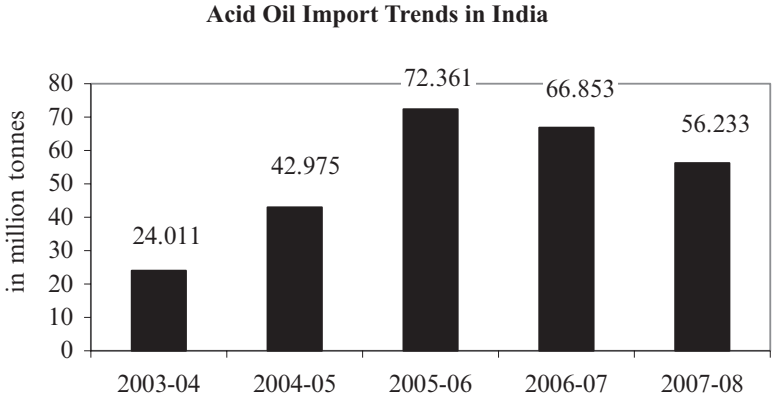

Fig. 9.12 Acid oil import trend in India

time, regulate large import of edible oils to the extent possible, import duty structure on edible oils is reviewed from time to time.

9.4.2.2 Acid Oil

Acid oil is the byproduct obtained from the vegetable oil refineries. As its FFA content ranges between 60 and 80%, it is a good candidate for considering it as a cheaper raw material for biodiesel making. Current uses of acid oil include soap making and food-grade ester production. Even though India is having abundant sources of acid oil within the country, it is not able to suit the current needs. India is importing acid oil from Malaysia and Singapore. The acid oil import trend is shown in Fig. 9.12.

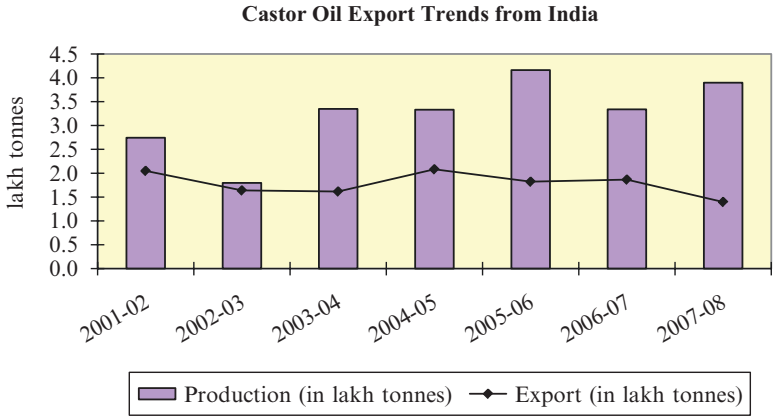

Fig. 9.13 Castor oil export trend from India

9.4.2.3 Castor Production in India

Castor (*Ricinus communis*) is cultivated around the world because of the commercial importance of its oil. India is the world's largest producer of castor seed and meets most of the global demand for castor oil. India produces 8–8.5 lakh tons of castor seed annually, accounting for more than 60% of the entire global production. Because of its unlimited industrial applications, castor oil enjoys tremendous demand worldwide. The current consumption of castor oil and its derivatives in the domestic market is estimated at about 300,000 tons. India is also the biggest exporter of castor oil and its derivatives at 87% share of the international trade in this commodity. Castor oil is used as a raw material in the manufacture of a number of chemicals used in the manufacture of surfactants, specialty soaps, biodiesel, surface coatings, cosmetics and personal care products, pharmaceuticals, perfumes, plasticizers, greases and lubricants, specialty rubber, etc.

The annual domestic consumption of castor oil in India is only about 80,000–1,000,000 tons. Of this, the soap industry consumes about 25,000 tons, the paint and allied industries 35,000 tons and the lubricant and derivatives industry 20,000 tons (MoAg 2013). India annually exports around 2.0–2.4 lakh tons of commercial castor oil (Fig. 9.13). From India castor oil is exported in two forms, viz. special grade castor oil and commercial castor oil. There is a large scope for improving India's earning from castor by converting the castor oil to various derivatives. A considerable quantity of the castor oil is also used in adulteration of edible oils like groundnut oil due to price differential.

9.4.2.4 Jatropha Plantations in India

Under the Rs 1286 crore demonstration project for cultivation of nonedible oil-bearing plants and tree for production of biodiesel, the Ministry of Rural Development (MoRD) has released an amount of Rs 4900 crore to the nine states

Table 9.5 Wastelands in India

Details of wastelands in India (category wise)				
S. no	Category	Area (in ha)	% of total geographical area covered	Suitability for energy farming
1	Gullied and/or ravenous land	2,055,335	0.65	Suitable
2	Land with or without scrub	19,401,429	6.13	Suitable
3	Waterlogged and marshy land	1,656,845	0.52	Not suitable
4	Land affected by salinity/ alkalinity	2,047,738	0.65	Suitable
5	Shifting cultivation area	3,514,220	1.11	Suitable
6	Underutilized/degraded notified forest land	14,065,231	4.44	Suitable
7	Degraded pastures/grazing land	2,597,891	0.82	Suitable
8	Degraded land under plantation crop	582,809	0.18	Suitable
9	Sands – inland/coastal	5,002,165	1.58	Not suitable
10	Mining/industrial wastelands	125,213	0.04	Suitable
11	Barren rocky/stony waste/ sheet rock area	6,458,477	2.04	Not suitable
12	Steep sloping area	765,629	0.24	Not suitable
13	Snow covered and/or glacial area	5,578,849	1.76	Not suitable
Total wasteland area		635,851,831	20.17	

identified under the project. These states are Chhattisgarh, Gujarat, Andhra Pradesh, Himachal Pradesh, Tamil Nadu, Rajasthan, Sikkim, Tripura and Assam.

Non-forest areas proposed for *Jatropha curcas* plantation in these nine potential states have been identified on the basis of availability of wasteland, rural poverty ratio, below poverty line (BPL) census and agroclimatic conditions suitable for jatropha cultivation. Each district will be treated as a block, and under each block, 15,000 ha of jatropha plantation will be undertaken through farmers. Proposal is to provide green coverage to about 3 million hectares of wasteland through plantation of jatropha in 200 identified districts over a period of 3 years. In tribal belts of Chhattisgarh and Rajasthan, *Jatropha* oil is used for lighting, heating and other conventional energy use (Table 9.5).

There is about 63.58 million hectares of wasteland in the country (Table 4.1), out of which 6.98% _amounting to 4.43 million hectares is suitable for cultivation of jatropha. On the basis of the above analysis, it should be reasonable to assume that with proper extension, research, availability of planting material and funds, plantation of *Jatropha curcas* on 13.4 million hectares of land is feasible in the immediate future. Institutional finance for private plantation and governmental allocation for public lands will have to be provided. A significant proportion of such lands can also be brought under *Jatropha curcas* plantation in an economically feasible manner. It will result in rehabilitation of degraded lands. But only 14% of the reported wasteland is actually available for jatropha cultivation in India (MoEF 2008). But

Table 9.6 Comparison between *Pongamia* and *Jatropha* farming

	Pongamia pinnata	*Jatropha curcas*
Crop type	Nitrogen-fixing tree	Shrub
Agroclimatic conditions	Tropical and subtropical	Tropical and subtropical
Oil content (%)	25–35	25–35
Maturation phase (years)	5	3
Expected useful life(years)	40–50	20–25
Planting density (no.s/ha)	100–500	1670–3330
Potential yield (kg/ha)	Up to 5000	Up to 1000
Characteristics of oil cake	More valuable	Less valuable

according to the evaluated methods of energy farming strategies, *Pongamia pinnata* has much potential to yield high amount of biodiesel than *Jatropha curcas*. The comparison is shown in Table 9.6.

9.4.2.5 Algae Oil as a Biodiesel Feedstock

Algae are photosynthetic organisms that occur in most habitats. They vary from small, single-celled forms to complex multicellular forms, such as the giant kelps that grow to 65 m in length. The US Algal Collection is represented by almost 300,000 accessioned and inventoried herbarium specimens. According to a report by the US Department of Energy, algae are one of the more promising feedstocks owing to their widespread availability and higher oil yields (a theoretical yield of 7660 l oil/ha per annum in an algae pond).

While a number of feedstocks are currently being experimented for biodiesel production, algae have emerged as one of the most promising sources for biodiesel production. Though research into algae as a source for biodiesel is not new, the current oil crises and fast depleting fossil oil reserves have made it more imperative for organizations and countries to invest more time and efforts into research on suitable renewable feedstock such as algae. Table 9.7 shows various species of algae suitable for oil production (DoE-USA 1998).

Just by way of history, petroleum is widely believed to have had its origins in kerogen, which is easily converted to an oily substance under conditions of high pressure and temperature. Kerogen is formed from algae, biodegraded organic compounds, plankton, bacteria, plant material, etc., by biochemical and/or chemical reactions such as diagenesis and catagenesis. Several studies have been conducted to simulate petroleum formation by pyrolysis. On the basis of these findings, it can be inferred that algae grown in CO_2-enriched air can be converted to oily substances. Such an approach can contribute to solving two major problems such as air pollution resulting from CO_2 evolution and future crises due to a shortage of energy sources.

Like plants, algae require primarily three components to grow: sunlight, CO_2 and water. Photosynthesis is an important biochemical process in which plants, algae, and some bacteria convert the energy of sunlight to chemical energy. This chemical

Table 9.7 Algae species suitable for oil production

Chemical composition of algae expressed on dry matter basis (%)				
Strain	Proteins	Carbohydrates	Lipids	Nucleic acids
Scenedesmus obliquus	50–56	10–17	12–14	3–6
Scenedesmus quadricaude	47	–	1.9	–
Scenedesmus dimorphus	8–18	21–52	16–40	–
Chlamydomonas reinhardtii	48	17	21	–
Chlorella vulgaris	51–58	12–17	14–22	4–5
Chlorella pyrenoidosa	57	26	2	–
Spirogyra sp.	6–20	33–64	11–21	–
Dunaliella bioculata	49	4	8	–
Dunaliella salina	57	32	6	–
Euglena gracilis	39–61	14–18	14–20	–
Prymnesium parvum	28–45	25–33	22–38	1–2
Tetraselmis maculata	52	15	3	–
Porphyridium cruentum	28–39	40–57	9–14	–
Spirulina platensis	46–63	8–14	4–9	2–5
Spirulina maxima	60–71	13–16	6–7	3-4.5
Synechococcus sp.	63	15	11	5
Anabaena cylindrica	43–56	25–30	4–7	–

energy is used to drive chemical reactions such as the formation of sugars or the fixation of nitrogen into amino acids, the building blocks for protein synthesis. Algae capture light energy through photosynthesis and convert inorganic substances into simple sugars using the captured energy.

Since algae need sunlight, CO_2 and water for their growth, they can be cultivated in open ponds and lakes. Due to the fact that these systems are “open”, they are much more vulnerable to being contaminated by other algal species and bacteria. The real challenge with open-air bioreactors (like a pond) is that the species of algae that have the highest oil content are not necessarily the quickest to reproduce. This creates a problem where other species take over the pond. Undesirable algal species taking over specific strains is one of the more significant problems in algaculture with the possible exception of *Spirulina* which itself is extremely aggressive and also grows at a pH that is extremely high, thereby eliminating the possibility of contamination to some extent. For this reason, the number of species that have been successfully cultivated for a given purpose in an open system is relatively small. In addition, in open systems there is relatively less control over water temperature, CO_2 concentration and lighting conditions. These imply that the growing season is largely dependent on location and climate. While the above are the disadvantages with “open systems”, some of the benefits of this type of system are that it is one of the cheaper ones to produce and easy to close it off to make it a closed system by a greenhouse.

9.5 Discussion

There exist underutilized resources, which will become potential feedstocks for biodiesel making. The reasons for underutilization might be the higher cost of production, higher cost of collection or lack of technology. We have to make use of these underutilized resources so that the potential for making biodiesel can be improved. Although several factors affect the cost of biodiesel fuel, its average cost exceeds that of petroleum-based diesel fuel. The relative cost of converting an existing fleet to biodiesel blends, however, is much lower than the cost of converting to other alternative fuels. This makes the biodiesel as a fuel of immense importance for achieving energy security and reduction in greenhouse gas emissions.

Jatropha and *Pongamia* are well-established species for biodiesel production, but *Jatropha* has wider adaptability and multiple benefits. Other oilseeds may be considered, if suitable to specific agroclimatic conditions. The cost of *Jatropha* cultivation could be reduced by agroforestry interventions. The seed price and byproduct sale price are the major factors affecting the price of biodiesel. Management of byproducts will be the most important factor for the economic viability of biodiesel production. The concept of biorefinery is suggested to make use of every byproduct after some value addition.

Biofuel is not the ultimate solution to the energy challenges facing India or the world. But it is part of the solution, especially when it not only stretches finite supplies of conventional fuel, but restores the land it grows on, does not displace more viable agricultural land and requires minimal water inputs. Lack of assured supplies of feedstock has hampered efforts of private sector to set up biodiesel plants in India. In the long run, a focused research programme is necessary to create a sustainable market for biodiesel. Long-term research activities need to be directed towards four major areas:

- Helping biodiesel become more cost competitive
- Improving the quality of biodiesel by optimizing its fuel properties
- Quantifying and fully understanding the nature of biodiesel emissions from diesel engines
- Estimating the economic effects of developing a biodiesel industry on Indian agriculture

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Biofuel Sector in Malaysia: Challenges and Future Prospects

10

Kalaivani K. Nadarajah

Abstract

The energy demands of the world and the eventual depletion of fossil fuel have urged the search for alternative fuel sources. The R&D on biofuels in Malaysia first commenced in 1980s, and since then it's commercialization has progressed at a very slow pace. with many uncertainties. In the last two decades, researchers and scientists have looked into identifying resources with immense potential for production of biofuel as a renewable, sustainable energy resource of the future, and palm oil and palm oil waste have remained the main sources for production of renewable energy for the last decades. Various policies and acts were put in place to assist Malaysia with the agenda of developing renewable energy sources as an environmentally friendly and sustainable alternative to fossil fuels. This chapter attempts to compile the state of the renewable energy scenario in Malaysia, its potential, governmental involvement, challenges, and future prospects of the biofuel industry in Malaysia.

Keywords

Biodiesel • Palm oil • Malaysia • Renewable energy

10.1 Introduction

The world's current energy needs are met by fossil fuels that are slowly approaching depletion. Fossil fuel continues to dominate the energy requirements to date even while its continued use has had some damaging consequences to the environment.

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The demand for energy has increased exponentially over the past century with the increase in population and the growing needs of the population. Oil, natural gas, coal, hydropower, and biomass are the primary energy resources of energy supply in Malaysia. In the past decade, Malaysia's energy consumption has increased by 14.3 Mtoe from 2001-2010 averaging an increase of 3.6% per annum. In this duration, oil production dropped by 0.8 Mtoe while in this corresponding time the consumption increased from 22 Mtoe to 25.3 Mtoe in 2010. However while oil production dropped, natural gas increased from 42.2 Mtoe to 59.8 Mtoe within the same time frame as a consequence of corresponding increase in consumption. The final energy demand in Malaysia is expected to grow considerably from 33.9 Mtoe in 2003 to 83.5 Mtoe in 2020, at a rate of 5.4% per annum with the anticipated increase in the Malaysian population (Department of Statistics Malaysia 2010; 2015). With the exception of hydroelectricity and nuclear fusion energy, all current major energy sources are finite. Hence, the finite nature of energy source has caused the government to look into biofuels as a potential source of security in energy, economic development, and poverty reduction and preservation of environment from pollution and to reduce greenhouse gas (GHG). Renewable energy sources are indigenous, and therefore it is a sure way of reducing dependency on oil imports and increasing security of a constant supply of energy.

Biomass is the most practical and suitable sources toward the production of renewable fuels for the future (Bull 1999; Sanderson 2011). The interest in renewable energy is mostly attributed to its potential to produce environmentally friendly and sustainable alternatives to fossil fuels (Gui et al. 2008). Malaysia is strategically positioning as a significant player in the global dynamics of biodiesel production due to it being the second largest producer of palm oil in the world, accounting for 40% of total global demand for crude palm oil (CPO). Over the past decades, CPO production in Malaysia has increased significantly from 2.6 M/tonnes in 1960 to 19.7 M/tonnes in 2013 (Lam et al. 2009). The rapid expansion of palm oil and CPO production has resulted in large-scale deforestation, loss of biodiversity, and pollution (Lam et al. 2009). This therefore brings into question the sustainability of the palm oil industry as there is only so much land that may be allocated toward the expansion of this industry.

Within this period, Malaysia recorded increased dependency on petroleum causing concerns on greenhouse gas (GHGs) and their effects and threats to global warming. This therefore has led the government into looking at policies and acts that may be put in place to encourage the development of the renewable energy industry in the country, thus reducing Malaysia's dependency on fossil fuel. Formulation of policies such as NBP (National Biofuel Policy) and the BIA (Biofuel Industry Act) encouraged renewable energy utilization through sustainable production of palm oil based biodiesel in Malaysia (Lopez and Laan 2008; National Biofuel Policy 2006).

The NBP was launched in 2006 where a series of measures were enabled to promote and develop biofuel usage in transport and other energy-dependent industries.

This was looked upon as a new economic opportunity intended to reduce dependence on imported fuel and promote economic development and poverty reduction, especially in rural areas. However, promotion of biofuels could turn out to be counterproductive if the initiative was not sustainable. Although biofuel production provides economic opportunities for producer countries, its development creates controversies, where cultivation of biofuel feedstocks results in deforestation, biodiversity loss, land conflicts, competition with land for food, and an increase in carbon emissions from land-use change (Engelhaupt 2007; Kismurtono and Naiola 2008; Pearce 2005; Searchinger et al. 2008).

While the production of biofuels is in line with addressing the climate change issues, it runs the risk of conflicting with sustainable development goals. To support an industry like biofuels in Malaysia, it would require substantial involvement by the government to support the economic viability of this venture through subsidies, tariffs, fuel mandates, or other government support. The cost of implementing biofuels by the government and consumers would require a very clear balance between sustainability of forest and agriculture and the production cost of biofuels. For example, it would be tragic if money spent to promote biofuels ultimately financed rainforest destruction or worsened the living conditions of the poor. However, if the implementation of biofuels in Malaysia reflects positively on the environment and the socioeconomics of the country, there is hope for the industry to flourish and create opportunities for both the people and country. This would require that the biofuel promotion policies are focused on the potential for energy security, economic development, and short-term economic benefits. Often, environmental obstacles and potential implications of land-use change and effects on food security, were not adequately taken into account (Kismurtono and Naiola 2008).

10.1.1 What Are Biofuels?

Biofuels can be defined as solid, liquid, or gaseous fuels that are predominantly extracted from biomass that provides an alternative source of energy that is both sustainable and without serious environmental impact (Demirbas 2007a, b). As an ultimate replacement to fossil fuels, research and deployment have been intensified in many countries to create efficient production and utilization of biofuels (Demirbas 2009). Currently, there are the first- to the third-generation biofuels available. First-generation biofuels are biodiesel and/or bioethanol that are derived from bioresources such as sugar, starch, corn, vegetable oil, or animal fats using conventional technology. First-generation biofuels has been a source of debate on food vs. fuel, and thus, there is a need to address issues like food security vs. energy security, food shortage, and food price rises. In the case of second-generation biofuels, the source is derived from non-food biomass and non-food crops. Second-generation biofuels can also be defined based on the type of feedstock or technologies used. Finally, third-generation biofuels have now arrived where the source of feedstock to fuel this industry comes mainly from algae and microbes (advanced biofuels).

10.1.2 Benefits of Biofuel Industry in Malaysia

The oil crises in the 1970s resulted in biofuel development as a means to abate issues such as oil price rise, over-dependency on fossil fuels, and the GHG issue arising from over utilization of fossil fuels. Various fuel policies, acts, and projects were initiated in Malaysia to pave the way for the development of the biofuel industry in Malaysia (Energy Information Bureau (EIB) 2010). As in various other countries, governmental support towards the development of domestic biofuel industry is based upon the benefits to society and environment.

Among the benefits of biofuel industry are:

1. *Oil palm industry expansion:* In Malaysia, where palm oil and palm oil waste products are largely available, initiatives towards the development of biofuel industry seems set on aspects of feedstock availability and pricing. Palm oil is cheaper with a higher oil yield per hectare compared to other vegetable oil feedstock utilized in biodiesel production. Currently, biodiesel is produced primarily from edible oils such as rapeseed oil, sunflower oil, soybean oil, tallow, and palm oil (Borugadda and Goud 2012; Gui et al. 2008).
2. *Societal implications:* Biofuel production would create employment, increase in incomes, increase in export earnings, increase in value of local biodiesel feedstock, infrastructure development, enhanced energy security, and reduced dependency on fossil fuels.
3. *Properties of biofuels:* The properties of biodiesel are similar to petroleum-based diesel; however, biodiesel is a biodegradable, non explosive, and non-toxic fuel which significantly reduces toxic emissions when burned (Liang et al. 2006; Sarin et al. 2009).
4. *Environmental effects:* Biodiesel is thus considered an environmental friendly and sustainable alternative to fossil fuels. The combustion of biodiesel can lead to approximately 90% reduction in total unburned hydrocarbons and polycyclic aromatic compounds (Demirbas 2007a). Furthermore, the overall life cycle analysis of biodiesel is considered to be carbon-neutral (Lin et al. 2011).
5. *Ease in application:* Biodiesel produced from palm oil can either be used directly without major modification in diesel engines or blended with petroleum diesel. Examples of typical biodiesel blends are B2, B5, B7, B20, and B100 where the number denotes the percentage of biodiesel in the blend, e.g., B2 indicates 2% biodiesel and 98% petro-diesel (Demirbas 2007a, b; Mofijur et al. 2012).

Hence, the global significance of biodiesel as a clean and sustainable biofuel for the future is set to increase over the years. This has resulted in the increase in utilization of oils for the production of biodiesel. In Malaysia, for example, the fraction of palm oil used for biodiesel production has increased significantly over the years (Gui et al. 2008; Borugadda and Goud 2012). This has resulted in the food vs. fuel debate where proper planning and consideration is required in advancing the biofuel industry without sacrificing food supply. In addition to the above, one major

disadvantage of palm biodiesel is its solidification behavior at low temperatures, which may restrict its use in cold countries. However, this deficiency can be partially eliminated by adding additive and winterizing agents to lower its melting point. Sarin et al. (2007) have made an attempt to blend jatropha and palm biodiesel to study their physicochemical properties and to get an optimum mix to achieve better low temperature properties.

On the whole, the production of biofuels would benefit the farmers, plantation owners, and exporters by producing par value to other vegetable oils, such as soybean and rapeseed oil. This increase in income was reported by Quirke et al. (2008) who noted that the conversion of vegetable oil to produce the world's requirement of biodiesel has contributed to the dramatic rise in palm oil prices. In addition, the production of biofuels is expected to benefit by paving the way for new industries such as nutraceuticals (vitamin supplements), detergents, glycerine, and fine oleochemicals.

In view of these advantages, the government had involved two of our main palm oil entities, MPOB (Malaysian Palm Oil Board) and the PORIM (Palm Oil Research Institute of Malaysia), in discussing, establishing, and implementing the nation's biofuel strategy. As a consequence, comprehensive biofuel development programs were established. Among the landmark policies are the NBP and the BIA. The primary objective of NBP is to position Malaysia as a key player in the global biodiesel industry. In addition, it promotes stakeholders participation in agro-based trade and the development of biofuels and biorefineries in Malaysia.

10.1.3 Policy Implementations

As a country with more than 30 million people (Department of Statistics 2013) and gross domestic product (GDP) estimate of about US 250 billion, Malaysia is heavily dependent on fossil fuels as source of energy to support the various energy requirements of the country. As a large portion of the fossil fuel is used to run and execute the transport and the industrial sectors in Malaysia, biofuels will certainly be a welcomed alternative to lighten the demand on fossil fuel. The major sources of energy production have predominantly been derived from coal, petroleum, and natural gas, with nominal contribution by renewable energy (Malek 2010).

Generally, any blueprints on the development of energy policies will exploit the resources and supply to fulfill the energy needs to ensure sustainability. The utilization objectives target energy conservation and efficiency promotion whilst eliminating wasteful, nonproductive energy applications. In addition, the environmental objectives are designed to try and lessen the environmental issues while taking advantage of energy sources. The National Depletion Policy was launched a year after the National Energy policy with the intention to conserve the main energy resources (mainly oil and gas) of the country. This eventually led to the Four-Fuel Diversification Policy in 1981, which emphasized the utilization and diversification of the four main sources (oil, gas, coal, and hydro).

Table 10.1 Malaysian RE studies, policies, and programs

No	Date	Description of studies, policies, or programs
1	1980s	Stand-alone solar photovoltaic systems for rural communities and remote areas in Malaysia
2	1999	Study on Strategy for Renewable Energy as the Fifth Fuel by KTKM (with the support of DANCED). Study on RE potential in Malaysia and recommendation on the legal, regulatory, and financial framework for utilization
3	1999	RE is the national fifth fuel
4	April 2001	RE utilization as fifth fuel incorporated into the Eighth Malaysia Plan
5	May 2001	Small Renewable Energy Power (SREP) Program announced. Production of RE by small power plants to be sold to national grid such as Tenaga National Berhad (TNB)
6	2001	SCORE set up – with the following members: EPU, KPPK, KPKT, MPOB, SIRIM, JAS, TNB, SESB, BCSDM, ST, and PTM
7	2001	The SCORE secretariat, a one-stop center for the SREP programs
8	2001	Biomass-based power-generating companies given various fiscal incentives by the government
9	2002	Biogen Full Scale Model (Biogen FSM) Demonstration Project
10	April 2002	SREP guidelines issued
11	2002	Prices of RE negotiated to benefit stakeholders
12	July 2005	MBIPV program launched to promote the use of PV technology in buildings
13	March 2006	Diversification of fuel through greater utilization of RE under the Ninth Malaysia Plan. Efficient allocation of resources and emphasis on reduced dependency on petroleum
14	2006	Study on SREP Program and RE development in Malaysia undertaken by DANIDA
15	July 2006	Increase of RE price to RM0.19/kWh for biomass and biogas
16	August 2007	Increase of RE price to RM0.21.kWh for biomass and biogas

Ref: (Ahmad et al. 2009)

Since these policies, various other policies ensued encouraging the use of renewable energy. Below are some of the renewable energy studies, policies, and programs (Table 10.1). The section below will highlight some of these policies and their implications to the renewable energy industry in Malaysia.

10.1.3.1 Five-Fuel Diversification Policy

Under the 8th Malaysia Plan, the Five-Fuel Diversification Policy was implemented aimed at generating 500 MW of electricity to the national grid. This target was not achieved as only 12 MW was obtained from the Small Renewable Energy Power (SREP) Program (UEP 2001). At this point, the development pace of renewable

energy in Malaysia was in the infant stage and progress was rather slow. There were various factors impeding the establishment of renewable energy from the slow buy-in by the consumers to the lack of infrastructure. Ten years after the announcement of the fifth energy policy, the renewable energy contribution is 1% of the total energy mix (Islam et al. 2009).

10.1.3.2 SREP Program

The SREP Program was launched on May 11, 2001, in conjunction with the country's Fifth Fuel Policy. In accordance with this policy, 5% of the total national energy generating capacity is to be obtained from renewable energy sources. Under the Ninth Malaysia Plan (2006–2010), the target electricity to be generated by West Malaysia was 300 MW, while it was set at 50 MW for East Malaysia. For the coordination and implementation of the SREP projects, a Special Committee on Renewable Energy (SCORE) was established at the Ministry of Energy, Water and Communications (now known as the Ministry of Energy, Green Technology and Water), with the Energy Commission acting as the secretariat. The Ministry was responsible for ensuring that the objectives stipulated under the 9th Plan were achieved. The main objective of this policy was to allow small power producers which utilize renewable energy sources to sell their electricity to national grids such as TNB. The maximum power export by these power producers was limited to 10 MW. The source of renewable energy for these producers was from palm oil mills, in particular empty fruit bunch (EFB) and palm oil mill effluent (POME) (SREP 2011).

10.1.3.3 The UNDP-GEF Biomass Power Generation and Demonstration (BioGen) Project

Following SREP, another project was implemented in 2002 for energy cogeneration from oil palm plants. This project was initiated by the UNDP and the GEF and was technologically and financially supported by the government. The project aimed to reduce GHG emission and to utilize palm oil waste such as EFB to generate electricity for sale to the public grid. Under this project, two small-scale projects were initiated. They are FELDA Besout POME Biogas Project and Bandar Baru Serting Biomass Project (Jalal et al. 2009).

(i) FELDA Besout POME Biogas Project

This project was involved in operating anaerobic ponds in Jengka where the methane gas released from these ponds was captured, while the excess biogas is used to generate electricity. The efficiency of the operations in the FELDA Besout POME Biogas Project was around 16%, which made it a stand-alone low-efficiency project.

(ii) Bandar Baru Serting Biomass Project

This is a government initiative under the Fifth Fuel Policy which encouraged developing biomass and biogas facilities to generate electricity from palm waste. This too was a stand-alone, low-efficiency project that was eventually abandoned due to financial issues.

10.1.3.4 National Biofuel Policy

The initial indication of governmental support for the utilization of renewable energy in Malaysia is clearly seen under the 8th Malaysia Plan where the Fifth Fuel Policy was imposed. The Fifth Fuel Policy was continued in the 9th Malaysia Plan between 2006 and 2010 with the hope that since a considerable investment had been made into the development of renewable energy, the energy contribution by RE would be higher within this period (UEP 2006). However in the 11th Malaysia Plan,the energy mix has not improved significantly from the ~2% since the 9th MP. In August 2005, the Malaysian government launched its NBP. This policy was aimed to expand the market for palm oil, improve energy security, and create new export industry. Petroleum prices in Malaysia for transportation is subsidized (Lopez and Laan 2008). Hence, the replacement of a portion of petro-diesel with biodiesel would help reduce the subsidy burden of the government. In addition to the reduction in the monetary burdens of the country, the utilization of biofuels will positively impact the environmental consideration where it would fit into reducing ambient air quality and greenhouse gas emissions. The five-fuel diversification strategy adopted by the NBP or policy thrusts is as follows:

1. Biofuel for transport: Here the priority was to subsidize and promote diesel blends (such as B5) for land and sea transport nationwide.
2. Biofuel for industry: Biodiesel blends such as B5 were supplied as industrial fuel for manufacturing and construction machinery.
3. Biofuel technologies: Promote public-private funding and commercialization of biofuel R&D technologies and increase biofuel utilization.
4. Biofuel for export: Establish biorefineries for producing export grade biofuels to facilitate sustainable development and increase Malaysia's position in the global biofuel market.
5. Biofuel for cleaner environment: Promote biofuel utilization and minimize GHGs, reduce overdependence on fossil fuels, and maintain environmental sustainability (National Biofuel Policy 2006)

Under the biofuel for transport trust, the Envo Diesel was introduced in 2006. The Envo Diesel is a combination of 5% refined palm oil and 95% petroleum-derived diesel (Jayed et al. 2011). Although Envo Diesel has its environmental and economic advantages, this fuel encountered several obstacles particularly from diesel engine makers who were concerned by the diesel clogging up the filters, corrosion of fuel systems, and material inconsistency (Lim and Teong 2010).

Eventually, Envo Diesel was replaced by B5 (5% palm methyl ester and 95% petroleum diesel) that was tested in selected government departments and agencies such as the Armed Forces and Kuala Lumpur City Council (Abdullah et al. 2009; Chua and Oh 2010; Zuraimi 2009). The B5 blend was also sold as biodiesel at all petrol stations in the central and southern regions of the country. To ensure adequate supply, biodiesel refining depots and blending facilities were established in Port Klang, Selangor, Tangga Batu in Melaka and the Valley Distribution Terminal in

Selangor, and Negeri Sembilan (Ong et al. 2011). These blending facilities later facilitated the production of other oil blends for the local and international market.

10.1.3.5 Biofuel Industry Act

The BIA was established in 2007 to regulate the Malaysian Biofuel Industry and to compliment the NBP. Under this Act, the EU standards were adopted to ensure that the biodiesel produced by Malaysia met international standards and export requirements and specifications. The BIA's responsibility includes blending specifications and the licensing process (MPOB 2009). Currently, the biodiesel produced from crude palm oil through alkaline transesterification complies with EN 14214 and ASTM D 6751 standards for biodiesel (Basri et al. 2008; MPOB 2008, 2009; Ramli et al. 2007; Yee et al. 2009).

In August 2006, the first commercial biodiesel plant started its operations. The following year, the Malaysian government had approved around 92 licenses for biodiesel projects to produce 10.2 M tonnes per year (Lopez and Laan 2008). Between 2006 and 2007, the MPOB statistics recorded that Malaysia had exported 47,790 and 95,010 tonnes of biodiesel, respectively (MPOB 2008). The shortfall in the production of biodiesel in this period is mainly attributed to the delay in the construction of the biodiesel plants itself (MPOB 2009). Though 14 biodiesel plants were established within this period, only 8 remained operational, while the rest succumb to the pressures of production costs. Even with the operation of the eight biodiesel plants, the production of biodiesel was only 2.12 M tonnes which is lower than the anticipated 10 M tonnes projected under the policy. In addition, the targets for biodiesel exports and domestic utilization are also short of the expected projections of the government.

The Malaysian biodiesel production declined significantly from 2009 to 2012 from the record highs of 246,749 tonnes in 2009 to 31,523 tonnes. This is tied up with the closure of a number of biodiesel plants during the same period (Chin 2011). However, in 2012, the production and export of biodiesel picked up again due to increased investments and global demand for biodiesel which brought about the significant increase in the overall output of the Malaysian biodiesel industry from 2009 to 2014. This could be attributed to the favorable environment created by the government through investments in infrastructure, tax breaks, and incentives over the period (Chua and Oh 2010). The financial industry has provided funds for refinancing existing investments in biodiesel companies especially during high feedstock costs (Lim and Teong 2010).

In the 10th MP, it is anticipated that a large portion of this renewable energy will be attained from biomasses mainly palm oil biomasses and biogases derived from palm oil mill effluent (POME). However, in the recent years, other sources of locally produced indigenous resources that have been exploited are forestry residue (wood waste), municipal solid waste (MSW), agriculture residues (rice husk, rice straw, rubber by-product, sugarcane bagasse, animal manure), energy crops (jatropha, algae), and other non-biomass-based renewable energy such as hydro, solar, wind, and geothermal. The fact remains, however, that while the sources of biomass used in the production of renewable energy are varied, a large portion of the energy is

derived from palm oil biomass and biogas from POME. Based on the estimate of palm oil waste per annum and assuming that the entire waste is utilized in the production of energy, it is assumed that this will only contribute towards 1500 MW of energy which does not achieve the intended 10% national grid electricity (National Energy Balance 2011; SEDA 2011).

10.2 Challenges to Biofuel Development

10.2.1 Feedstock

Suitable and sustainable availability of feedstock will influence the success of the RE industry. In Malaysia one largely available feedstock is oil palm. However the use of crude palm oil (CPO) has met with controversies where there is the constant check on the availbility of CPO to meet both the food as well as the energy industry needs (Balat and Balat 2010; Yee et al. 2009). This can only be achieved through the increase in the land area utilized for the cultivation of palm oil to benefit both industries. In addition, analysts believe that the global food market dynamics will be disrupted by the continued production of biodiesel from edible oils (Atabani et al. 2012). Further, the governmental and industrial push to use CPO as the primary feedstock has hampered the search for alternative source of feedstock for this industry. This has created a "perceived lack of options," thereby hindering the exploration of alternative feedstock for biodiesel production.

10.2.2 Costing

The increasing demand for CPO over the years has resulted in an increase in CPO price which inadvertently resulted in diseconomies of scale for biodiesel companies in Malaysia. The increase in CPO pricing resulted in the increase of operating costs, low capacity utilization, and low profit margins which created uncertainties in the biodiesel market and dampened investor confidence in the industry. Therefore, even though many biofuel plants were opened in line with the countries agenda to promote the biofuel industry, many of these plants were forced to shut down or decrease output due to pricing. This downward trend in biodiesel production is clearly correlated to the price of crude oil and will therefore result in a sharp drop in production. The effect of price ratios reveals that biodiesel production will remain less competitive as long as the production profits remain less lucrative. For the production of biodiesel to achieve profit, CPO prices need to plummet or crude oil prices should soar, thereby lowering the palm oil-crude oil price ratio (Lopez and Laan 2008).

10.2.3 Fossil Fuel Subsidies vs. Biofuel Incentives

To induce the biofuel industry, the government of Malaysia intervened through subsidies in order to bolster confidence, stimulate exports, and support the development of the domestic biodiesel mandate in the industry. This initial intervention process involved commitment in cash and incentives to support the biodiesel program that was aimed at increasing the competitiveness of the CPO-based biodiesel industry as a good alternative and renewable energy source for Malaysia. However, these measures were not successful as these efforts were muted by the already existing fossil fuel subsidies which took the edge of the competitiveness of biodiesel and other novel fuel technologies in the pipeline. In addition, there are also subsidies to establish fossil fuel technologies which already enjoy economics of scale, and this results in the dampened development and competitiveness of biodiesel. In effect, fossil fuel subsidies implemented by the government are hampering the biodiesel development in Malaysia. Further, the carbon lock-in effect, i.e., the cumulative returns enjoyed by fossil fuels, has indirectly resulted in the impediment of the biodiesel industry (Unruh 2000). Hence, as long as the returns on the industry are not comparable to that of fossil fuels, there will be a lag despite socioeconomic and environmental benefits (The Star Online 2013).

10.2.4 Compatibility Issues

In addition to the above factors, the compatibility of the fuel with the engines whether in cars or in factories will determine the success of this product. Hence, to make the biofuel amenable to various engine applications, the source of feedstock, viscosity, calorific value, flash point, cold flow, and cetane number should significantly influence engine compatibility. One major problem with biofuel/biodiesel produced from palm oil is the high-saturated fatty acid content that contributes toward poor cold flow. In addition to cold flow, there are other technical challenges for palm oil-based biodiesel such as high fuel consumption, excessive carbon deposition, gum deposition, and erosion of engines. However, R&D has been directed toward creating blends and modifications in processing techniques that are able to weather the cold and reduce detrimental effects to the engine (Bouaid et al. 2007; Yusuf et al. 2011).

10.3 Future Prospects

Biofuels have been promoted largely as non-polluting renewable energy sources which provide a solution in addressing GHG problems and also with the promise of socioeconomic improvement. Here, we address the future prospects of palm oil-based biodiesel and the prospect of this industry in Malaysia.

10.3.1 Environmental Effects

The utilization of biodiesel was the thrust of the NBP in Malaysia which was established with the intention of reducing Malaysia's dependence on fossil fuels and to also curb the nation's GHG emissions (World Bank Data Indicators for Malaysia 2013). The transport and the industrial sectors are the main culprits in contributing toward the increase in GHG, and the emission levels are likely to increase significantly in developing countries which account for 60% of the increase by 2020 due to the booming industrialization in these nations. The WEO Special Report (2013) postulates that in order to reduce the global energy-based GHG emissions, strategies need to be implemented to encourage technologies and policies that implement efficient energy measures in motor transport and industrial production. Based on studies conducted on fossil fuels compared with biofuels, it is evident that this renewable energy source has lower carbon intensities and would reduce GHG.

Biofuels are environmentally friendly and cause insignificant effects toward the environment compared with fossil-based fuels such as diesel, gasoline, and natural gas. In addition, the palm diesel produced in Malaysia is greener compared to biodiesels produced by other oil seed as its CO_2 emissions during conversion is considerably lower. Lam et al. (2009) in their report investigating the life cycle analysis of biodiesel production from palm oil corroborated this finding and list the superiority and sustainability of palm oil as a biodiesel feedstock based on its CO_2 sequestration potential which is 20 times higher than jatropha biodiesel (Lam et al. 2009). As a consequence of this finding, the RSPO (Roundtable on Sustainable Palm Oil) has encouraged companies operating in the Malaysian palm oil industry to observe the social and environmental criteria. The RSPO in return certifies the palm oil produced by the companies as a strategy to reduce the negative impacts of palm oil cultivation in CPO-producing communities in Malaysia (Roundtable on Sustainable Palm Oil).

It is hoped that through the biodiesel production activity and the ability of farmers to obtain income from palm oil as well as the biodiesel from CPO and palm oil waste products, this will reduce the deforestation to increase cultivation of land for increased income. It has been reported that Malaysia has one of the highest deforestation statistics which is resulting in a large number of our endemic species being affected. Furthermore, the extensive deforestation also has contributed towards anthropogenic GHG emission that disrupts the climate, water cycle, and biodiversity of species (Foley et al. 2005). Sustainable forest management is crucial in protecting the environment and the smallholders who not only account for a significant proportion of palm oil production but also subsist on the land for their livelihood.

10.3.2 Contribution to Socio-economics

The production of palm oil-based biodiesel is expected to result in socioeconomic growth and development that will result in job opportunities, social development, and better living standards for Malaysians. To encourage this, the government has established modalities to ensure the development of biofuels as a major supplier of

the energy industry. The government invested in grants to encourage the development of the biodiesel industry. This allocation was primarily towards infrastructural development of biorefining facilities for blending and upgrading biodiesel at existing petroleum diesel depots around the country. The upgrading of the biorefinery standards and capabilities will definitely enhance the ability of these stations to produce blended oils for various industries. The investment by the government into this project will also increase investors' confidence in the biodiesel industry, create job opportunities, improve the countries' infrastructure, as well as open opportunities for other biodiesel-based industries.

In addition to the funds allocated for the infrastructure development, the government also allocated funds toward subsidizing biodiesel to cater to the ancillary cost of blending fuels in plants and keeping the pricing competitive with fossil fuels. Higher oil prices would create a favorable situation for biofuels and would eliminate the need for subsidies in Malaysia. Eventually with the phasing out of subsidies, biofuels would be more competitive. Further, the government's initiative in lowering the palm oil import tax only induced trade flows and catalyzed growth in the downstream sector. It is expected that with the newly revamped biodiesel mandate, the overall biodiesel consumption would be raised significantly per year (CIMB Regional Sector Navigator 2013; CIMB Regional Sector Navigator 2014).

10.3.3 Biofuel Technologies

For enhanced utilization of biodiesel in Malaysia, the industry would need to be supported by technology innovation that will address the issues arising from the implementation of biodiesel in transport, logistic, equipment, and engine industries (CIMB Regional Sector Navigator 2013). Hence, further blending of the oils was initiated to reduce issues such as oil dilution, filter clogging, injector deposits in engines, and after treatment systems. This resulted in more blending-refining facilities being set up nationwide to generate various biodiesel combinations that would improve its application. (CIMB Regional Sector Navigator 2013).

Some of the innovative development in biodiesel includes MCCT (Menlo Clean Carbon Technology), BNT (Benefuel ENSEL Technology), and JSP (jatrodiesel super process). MCCT is a green, continuous flow, multi-feedstock biodiesel process that has the lowest carbon footprint of any biodiesel production process since it operates at ambient temperature, atmospheric pressure, and short residence times. BNT on the other hand employs catalysts to esterify and transesterify low-cost feedstock, while JSP is produced without the need for catalysts (MPOB 2008).

10.4 Conclusion

This chapter has examined the advancement in renewable energy scenario in Malaysia taking into account government involvement, challenges, and future prospects towards this industry. In general, the outlook for the biofuel industry in

Malaysia is largely dependent on governmental support such as subsidies and incentives. Without continuous support, it is likely that it will dwindle away. With the current fuel price situation facing us today, the biofuel industry is vulnerable to the fluctuating palm oil and petroleum prices and restrictive biofuel policies in key consumer markets. However, the palm oil industry which is the key contributor to the biofuel production will not be affected by the volatility of the biodiesel industry. The demand for palm oil as food is expected to increase with the population increase and will remain independent of how well the biofuel industry thrives.

However at this point, the Malaysian government will need to revisit the role of biofuel industry in the government's economic plans. The direction it decides to take the industry whether for local consumption or for export will determine its partner for engagement (EU, USA, or local authorities). Malaysia will also need to be sensitive to the environmental and socioeconomic effects of this industry, taking great care to put appropriate policy safeguards in place to regulate land-use changes and promote methane capture technologies. In addition, it is expected that biofuels will not serve as a significant new source of energy for Malaysia. Hence, it would be difficult to justify the introduction of a separate subsidy for this industry. Therefore, it would be beneficial and financially sound for the biofuel sector to diversify the choice of feedstocks to reduce dependency on palm oil. Technology development to support this sector will also serve positively toward the advancement of this industry.

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Assessment of Non-plantation Biomass Resources Potential for Energy in India

11

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Abstract

The objective of the present study is to prepare an inventory on non-plantation biomass resources and to create a database of existing information within India. The information is gathered from existing databases in India. The authentic sources for information collected include several national- and state-level departments, universities, and institutes and several national- and state-level agencies. The set of information collected and compiled includes non-plantation biomass resources from following three major sectors: forestry, agriculture, and municipal solid waste (MSW) in India. The results of the current study deliver a general overview on the total non-plantation biomass resources present and available surplus for energy applications in India. The outcome of the study will be helpful for future policy formulations for achieving a bio-based economy.

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Keywords
Non-plantation biomass • Forestry • Agriculture residues • Municipal solid waste • Bioenergy

11.1 Introduction

Energy is considered the basic for the progress and prosperity of nations and societies. Availability and resourceful consumption levels of energy are the best indicators of sustainable development. Given the rising demand for energy and declining non-renewable resources within the region, most of the developing economies are concerned over energy supply security and are looking for alternative options. Most of the countries have identified biomass-based energy as one option for diversifying sources of energy for consumption and pressed into service to decrease the energy deficit.

Biomass-based energy systems remained as reliable sources of renewable energy in most of the agrarian economies. The utilization of biomass resources assumes importance due to the soaring crude price and depleting reserves of fossil fuels coupled with the rising environmental concern. Biomass resources tend to be more spatially concentrated and show seasonal variability due to constraints such as climate and crop-harvesting schedule. Biomass can be used in a variety of energy-conversion processes to yield heat and/or power and fuel. Biomass energy sources include:

- Agricultural crops and residues
- Dedicated energy crops/plantations
- Forestry products and residues
- Residues and by-products from food, feed, fiber, wood, and material-processing plants
- Post-consumer residues and wastes, such as fats, greases, oils, construction and demolition wood debris, and other urban wood waste, municipal solid wastes, and wastewater

India being an agriculture-dominant country produces more than 600 million tonnes of biomass residues annually, and out of which about 150–200 million tonnes is surplus (UNDP 2011). These residues are used as animal feed, for thatching of homes, and as a source of domestic and industrial fuel. Almost 85 million tonnes of unused crop residues are burnt in the fields primarily to clear the leftover straw and stubbles after the harvest (Pathak et al. 2010).

According to Energy Alternatives India (2010), the biomass potential for energy applications is huge and can be exploited with technological developments in harvesting, post harvest processing, and supply chain management of biomass. The total potential is estimated to be around 35 GW_{eq}, out of which crop residues and

cattle manure share is approximately 80%. The following figure depicts the potential of biomass for energy applications.

Biomass continues to be the world's major source of food, fodder, and fiber as well as a renewable resource of hydrocarbons for use as a source of energy and chemicals. Various biomass sources are used as feedstocks to produce energy carriers in the form of solid fuels (chips, pellets, and briquettes), liquid fuels (methanol, ethanol, butanol, and biodiesel), gaseous fuels (synthesis gas, biogas, and hydrogen), electricity, and heat.

Bioenergy is the traditional source of energy with renewed interest due to its carbon mitigation potential assuming CO_2 neutrality, need for diversification of energy sources, and the renewable nature of feedstocks. A bioenergy system or bioenergy chain/route consists of a series of conversion steps by which raw biomass feedstock is transformed into a final energy product (heat, electricity, or transport biofuel). There are many bioenergy chains as a result of the wide range of raw biomass feedstocks (wood, grass, oil, starch, fat, etc.), a broad spectrum of conversion technologies, and a variety of possible end uses.

A typical bioenergy system comprises of a series of activities such as growing biomass for energy applications, harvesting biomass, pre-processing, and transportation of processed biomass to conversion facility, biomass conversion to biofuel/bioenergy carrier, transportation of biofuels, conversion of biofuel into energy by using energy technology. Many of these steps are energy intensive. Biomass-based energy generation is the major focus area among renewable energy programs in India. But, these systems compete for natural resources (such as land and water). They also include supply chain with a large number of stakeholders and energy-intensive transportation of biomass or biofuels. The biomass feedstocks also have competing alternative uses for food and fodder production. Keeping view of all these factors and evaluating the performance of these systems in the event of resource crunch will be crucial. There is a need to deepen our understanding about efficient allocation of precious natural resources toward bioenergy systems deployment.

There exist many variants of conversion routes to suit the different physical and chemical composition of the feedstocks. While some routes are simple (e.g., direct combustion of forest wood for heat production), others necessitate several pretreatment, upgrading, and conversion steps, such as those required for the production of liquid biofuels that can be used in an internal combustion engine. Bioenergy pathways can be categorized as:

- *Thermochemical conversion*, by which biomass undergoes chemical transformation induced by high temperature. The listed pathways in this category are combustion, gasification, pyrolysis, and torrefaction, which differ mainly due to the temperature and pressure profiles of the process, heating rate, and amount of oxygen present in the reaction.
- *Physicochemical conversion* is used to produce liquid biofuels (biodiesel or vegetable oil) from oil crop by oil extraction possibly followed by transesterification process.

- *Physical processes* like size reduction, mixing, and compression are used to produce pellets and briquettes.
- *Biochemical conversion* uses living microorganisms (bacteria, enzymes) that convert the feedstock to produce liquid and gaseous fuels. Biological pathways are numerous, key mechanisms being fermentation of sugars and anaerobic digestion of organic waste to produce ethanol and methane, respectively. Biophotochemical pathways (e.g., hydrogen production using algae) require the action of sunlight.

The current annual availability of biomass in India is estimated at about 500 million tonnes per year (MNRE 2011). It is estimated that around 61 million tonnes of crop residue is being used as fuel and around 242 million tonnes of crop residue is being used as fodder for animals in India (TIFAC 2008). The Biomass Resource Atlas has also estimated the power generation potential using the surplus biomass which amounts to nearly 176 million tonnes (165 million tonnes in 2008 representing 26.5% of total crop residue generation, according to TIFAC), which on efficient utilization would produce 23,250 MW of electricity (IISc 2013). As per MNRE statistics, bioenergy cumulative installed capacity is around 4953 MW as on 31 May 2014. Grid interactive bioenergy share in the total installed capacity is 83% with a capacity of 4120 MW, including biomass power generation, biomass gasification, bagasse cogeneration, and waste to energy. Off-grid bioenergy installed capacity is 833 MW reflecting in a very low share of 17%.

11.2 Review of Biomass Resources in India

Bioenergy is the energy made from biomass through physical, thermochemical, physicochemical, biochemical, and chemical approaches. The final energy carrier produced can be used for heat, electricity, or liquid biofuels for transport. Biomass includes residues from forestry and agriculture and dedicated purpose-grown crops. Biomass resources are relatively cheaper and widely available in India. India is spread across the geographical area of about 328.73 million hectares (Ha) – out of which, 141.58 million Ha is the net area sown in 2011, according to the agricultural statistics 2013 released by the Ministry of Agriculture (MoAg). Approximately 63.60 million Ha of net area cropped is irrigated with a cropping intensity of 140.54% in 2011.

Strength of Indian biomass resource lies in the agricultural sector. Hiloidhari et al. (2014) estimated bioenergy potential from crop residue biomass in India, covering 39 different crop residues from 26 crops in 28 states. It was estimated that around 686 million tonnes of crop residues were generated in 2008 and 34% total gross generation was estimated to be surplus. The estimated bioenergy (thermal) potential from surplus biomass is around 4.15 exajoule (EJ), which is equivalent to 17% of the total primary energy consumption in India (Hiloidhari et al. 2014). Estimates of the biomass consumption remain highly variable since most of the biomass is not transacted on the market (Ravindranath and Hall 1995). Table 11.1 lists the summary of literature review on crop residue potential in India since 1990.

Table 11.1 Summary of literature reviews on crop residue potentials in India since 1990

Author(s) (year of publication)	Number of crop residues investigated	Competing uses accounted for	Year	Gross availability (in million tonnes)	Surplus availability for energy applications (in million tonnes)
Singh and Gu (2010)	22	–	2009	850	Not estimated
Sukumaran et al. (2010)	14	Animal fodder, manure, paper industry, and cooking energy needs	2009	600.8	164.6
Hiloidhari et al. (2014)	26	Animal fodder, thatching homes, and cooking energy needs	2008	686	234
Buragohain et al. (2010)	9	Animal fodder, cooking energy needs	2007	500	150–160
Ravindranath et al. (2005)	20	Animal fodder, cooking energy needs, thatching homes, composting, and mulching	1997 (estimated for 2010)	626.5 (1113)	325.3 (450.7)
Shyam (2002)	15	Animal fodder, cooking energy needs	1997	544.5	Not estimated
Prasad et al. (2007)	20	–	1997 (estimated for 2010)	626.5 (840.6)	325.3 (450.7)
Jorapur and Rajvanshi (1997)	–	–	1995	320	100 (30% of the gross availability)
Kishore et al. (2004)	Non-fodder residues	–	1995	160	160
Ravindranath and Balachandra (2009)	9	Residues burning on the field	–	450.7	225.3 (50% of the gross availability)

Ravindranath and Balachandra (2009) studied the avenues for bioenergy applications for power, heat, and transport in India. According to the study, 255 million tonnes of crop residues are available for biomass-based power generation. Bioenergy for heat includes applications such as cooking, water heating, and operation of kilns. There are three bioenergy options, namely, shifting to efficient cooking stoves, biogas, and methanol, available for meeting cooking energy needs. India has the highest bovine population of around 177 million (MoAg 2013) and produces recoverable dung of 458 million tonnes per annum (Ravindranath and Balachandra 2009). A study also advocated the promotion of dedicated energy crops for transportation biofuels, mainly biodiesel and bioethanol.

Potentials of various bioenergy technologies based on the availability of surplus biomass and their level of achievement in India are discussed by Ravindranath and Balachandra (2009). The bioenergy technologies discussed include cookstoves with improved efficiency, biomass gasifier stoves and biogas for cooking and heating energy needs, and biomass gasification, biomass combustion, and biomethanation for biomass-based power. The study also includes the estimates of abatement costs of different bioenergy technologies for a particular end use.

Biomass alone currently meets cooking energy needs of the 67% of the total households in India (MoSPI 2013). Biomass energy contributes about 26% of total energy consumption in India in 2010 (Singh and Setiawan 2013). In a country like India, where 69.9% of the population inhabit rural areas (World Bank 2013), biomass-based energy systems will have a key role in achieving energy independence and energy security. According to 2011 census, almost 85% of the rural households are dependent on traditional biomass fuels for their cooking energy needs, partly because non-biofuels tend to be expensive (MoSPI 2013). The National Sample Survey 2009–2010 reveals the continued dependence on firewood in rural areas for cooking, with percentage households depending on firewood remaining at 76.3% in 2009–2010 in comparison to 78.3% in 1993–1994. National Sample Survey results also show that in the year 2009–2010, almost 40% households in rural India were using kerosene as a primary source of energy for lighting, representing the dependence on fossil fuels for lighting and lack of access to grid electricity. Thus, a transition to cleaner forms of energy would have implications not only on energy security but also lead to emission reductions.

11.2.1 Objectives

Biomass resource assessment is essential in evaluating the bioenergy potential of a given region, as well as the social, environmental, and economic impacts associated with resource production and use. Biomass resource assessments guide industry development strategies and support decision-making processes.

There are competing uses for biomass resources because of their economic and environmental value for a variety of purposes. As mentioned earlier, biomass material is used to generate power and heat and produce transportation fuels. Biomass is

also used by the food processing industry, animal feed industry, and wood product industry, which includes construction and fiber products (paper and derivatives).

The objective of the present study includes:

- To prepare an inventory on non-plantation biomass resources
- To list competing uses of non-plantation biomass resources
- To estimate surplus non-plantation biomass resources for energy applications

11.3 Assessment Methodology

The inventory prepared consists of the following three main sections (forestry, agriculture, and municipal solid wastes). In each section, breakups of the residues available are collected according to their subtype (such as stalks, husk, straw, pod, shell, cobs, branches, bark, twigs, leaves, etc.).

11.3.1 Biomass from Forestry

The availability of biomass from forestry includes sources of biomass from open forests, dense forests, and scrubs. The subtype of biomass sources includes branches, bark, twigs, leaves, etc. The total forest cover in India is around 3089 million hectares, generating biomass amounting to 155 million tonnes. The surplus biomass available from forestry is estimated to be 105 million tonnes, which is equivalent to a power generation potential of 14.5 GW_{eq} (IISc 2004).

11.3.2 Biomass from Agriculture

Agricultural crop residues form a significant portion of the non-plantation biomass resources available in India. Availability of surplus agricultural crop residues for energy applications needs better understanding of trends in area under agricultural crops, cropping patterns and existing utilization patterns of crop residues. After examining the crop production trends in India, it is found that sugarcane, rice, wheat, and cereals are the most grown crops in India.

11.3.2.1 Crop Residue Generation

The total area under agriculture in India is approximately 204 million hectares, amounting to an annual crop production of about 432.5 million tonnes/year (MoAg 2012). The respective non-plantation biomass generation from crop residues is around 415 million tonnes/year (IISc 2004). The surplus biomass available from crop residues is approximately 102 million tonnes/year, which is equivalent to a power generation potential of 12.5 GW_{eq} (IISc 2004). It is found that sugarcane, *rice*, and *wheat* are the most grown crops in India. Even a small percent of surplus residue generated from these crops results in a substantial figure.

11.3.2.2 Crop Residue Consumption

Many crop residues have competing uses such as fodder, domestic fuel, and fuel for commercial applications. The residues generated from rice, wheat, maize, jowar, ragi, and bajra are mainly used as animal feed. The residues from cotton, pulses, and oilseeds are mainly used as fuel for household cooking needs. Rice husk is mainly used in boilers as fuel and bagasse mainly for power or paper production. The consumption of crop residues as fodder is estimated by examining the animal population trends in India and respective fodder requirements. Table 11.2 describes the fodder requirement for animal population in India.

According to TIFAC, a potential *61.1 million tonnes* of fuel crop residue and *241.7 million tonnes* of fodder crop residue are being consumed by farmers themselves (TIFAC 2009). By considering the average fodder requirements for animal species, the total fodder requirement is estimated to be around 1270 million tonnes. But, the annual dry fodder requirement of about 465 million tonnes is higher than the total annual dry fodder generation. The fodder requirement is assumed to be partly met by reserves and open grazing.

11.3.2.3 Crop Residue Surplus

Sugarcane tops are the most available surplus residue as it is mostly burnt in the agricultural field itself. Crop residues from cotton, cereals, and oilseeds generate surplus because they do not have much other use apart from fuel. These residues are typically burnt in the fields or used to meet household energy needs by farmers. As India makes further economic progress, farmers are likely to shift to modern fuels such as kerosene. This will further increase availability of such crop residues. An additional 4 million tonnes of **bamboo plant** would be available as potential biomass (TIFAC 2009). There is very little surplus from fodder crops as it is consumed by cattle.

11.3.2.4 Collection of Crop Residues

A sustainable biomass supply chain is a necessity for any biofuel manufacturing facility. However, the sources of biomass are highly dispersed in rural areas. Even existing biomass power plants face the challenge of collecting vast amounts of crop residue for sustained power production. Given this scenario, it is advisable to have certain "anchor suppliers" of biomass for any biofuel manufacturing facility. These anchor suppliers could be existing concentrated sources of biomass like sugar mills and rice mills. For other types of crop residues, cooperatives or other local bodies could be encouraged to collect and supply a fixed amount of crop residues over a sustained period, the way milk is collected by large cooperatives in many states of India.

11.3.3 Biomass from Livestock

There exists a huge potential from livestock in terms of cattle dung. A theoretical potential of about 1750 million tonnes of annual cattle dung availability exists in

Table 11.2 Fodder requirement for animal population in India

Animal category	Green fodder (kg/day/animal)	Dry fodder (kg/day/animal)	Conc. feed (kg/day/animal)	Population in millions (2003)	Annual green fodder requirement (million tonnes)	Annual dry fodder requirement (million tonnes)	Annual conc. feed requirement (million tonnes)	Total annual feed requirement (million tonnes)
Buffalo in milk	8.9	6.34	1.05	33.3	108.2	77.1	12.8	198.0
Dry buffalo	9.72	4.95	0.52	17.6	62.4	31.8	3.3	97.6
Adult male buffalo	7.11	7.47	0.36	6.7	17.4	18.3	0.9	36.5
Young heifer/calve	6.1	2.22	0.19	40.3	89.7	32.7	2.8	125.2
Cattle in milk	5.92	5.5	0.64	35.8	77.4	71.9	8.4	157.6
Dry cattle	4.66	4.02	0.4	28.7	48.8	42.1	4.2	95.1
Adult male cattle	7.12	6.03	0.33	57.6	149.7	126.8	6.9	283.4
Young stock	3.95	2.13	0.18	63.1	91.0	49.1	4.1	144.2
Goat	1.5	0.2	0.06	124.4	68.1	9.1	2.7	79.9
Sheep	1.65	0.19	0.04	61.5	37.0	4.3	0.9	42.2
Others*	15.65	6.72	0.49	1.2	6.9	2.9	0.2	10.0
Total					756.6	465.9	47.3	1269.7

*Others include horse, donkey, camel, and mule

India (Livestock Census 2012). But, the collection efficiencies for cattle dung are very low since most of the fodder requirement is met by open grazing. By assuming a 50% collection efficiency, India can have around 800 million tonnes of cattle dung availability per year. The power generation potential of cattle dung depends mainly on the conversion route adopted and physicochemical characteristics of the dung. Assuming 13.61 MJ/kg lower heating value for the cattle dung, the total theoretical power generation potential is estimated to be 345 GW_{eq} (ECN Phyllis 2013).

11.3.4 Biomass from Municipal Solid Waste

The biomass from municipal solid waste is estimated using census data 2011. The total population multiplied by the per capita waste generation gives us the total MSW generation in India. As per the estimates, the total MSW generation is 178 million tonnes per year. The biodegradable portion of the total waste is around 107 million tonnes. The power generation potential of the biodegradable portion of the waste is approximately 62.8 GW_{eq}, considering its lower heating value of 18.56 MJ/kg.

11.4 Discussion

In India, a great part of the available biomass from forestry, agriculture, and MSW remains unexploited. It can create a lot of socioeconomic impact if harnessed properly with scientific practices. Total theoretical potential of non-plantation biomass resources for energy application is estimated to be approximately 434 GW_{eq}. Table 11.3 explains the theoretical power generation potential of various surplus biomass resources.

Table 11.3 Theoretical power generation potential of surplus biomass

Biomass type	Surplus (million tonnes)	Power generation potential (GW_{eq})
Forestry	104	14.5
Crop residues	145	12.5
Cattle dung	800	345
MSW	107	62.8
Total	1156	434.8

11.4.1 Uses of Non-plantation Biomass Resources Inventory

The inventory of non-plantation biomass resources can be used:

- To estimate total available non-plantation biomass, i.e., technological biomass potential, the fraction of theoretical potential which is available under the current technological possibilities such as harvesting techniques, infrastructure and accessibility, processing techniques
- To estimate used biomass (biomass utilized for energy, chemical, and material production)
- To estimate net biomass potential (total available biomass – used biomass)
- To estimate energy content of total available biomass by using the low heating value of a specific biomass reported in Phyllis database (ECN Phyllis 2013)
- To identify geographical location (main geographical locations where biomass was produced/collected)

11.4.2 Pitfalls of the Biomass Assessments in India

- It is not always clear which type of biomass potential is used for the single biomass categories.
- Apparently, in some cases, availability of different biomass has been calculated taking into account different types of biomass potential.
- A lot of discrepancies in the available data and only few estimates exist regarding the biomass database in India.
- Statistical reports are not always complete or contain mainly highly aggregated data.

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Agrotechnology, Production, and Demonstration of High-Quality Planting Material for Biofuels in Arid and Semiarid Regions

12

Ashwani Kumar and Shikha Roy

Abstract

Several plant families widely growing in Rajasthan have great potential as renewable source of energy. Euphorbiaceae (*Euphorbia antisyphilitica*, *E. tithymaloides*, *E. caducifolia*, *E. lathyris*, *E. neriifolia*, *Jatropha curcas*, etc.), Asclepiadaceae (*Calotropis gigantea* and *C. procera*), Asteraceae, and Apocynaceae have a large number of valuable plants. These plants are able to grow well in arid and semiarid conditions of Rajasthan. We developed agrotechnology for some of these plants and suggested a three-tier system for optimum production of biomass. Influence of growth regulators, edaphic factors, cropping pattern, and nutritional factors have been optimized. The biofuel yield could be increased by employing technologies developed at University of Rajasthan, Jaipur. Production of high-quality plant material of *Jatropha curcas* was carried out at 35 ha Energy Plantation Demonstration Project Center, University of Rajasthan, under the Department of Biotechnology, Government of India project sanctioned to authors. The selected plant materials were characterized for their hydrocarbon and oil contents. High-yielding accessions of *Jatropha curcas* have been deposited at the National Bureau of Plant Genetic Resources, New Delhi. Accession IC565601 and IC565602 have been planted at Viratnagar near Jaipur. Elite strains have given excellent performance depending on edaphic, climatic, and nutritional conditions. This paper will try to review mainly our own work carried during last 30 years giving citations.

Keywords

Biodiesel • Bioenergy • Biofuel • Biological conversion • Biomass

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12.1 Introduction

According to the Energy Information Administration (http://www.eia.doe.gov/emeu/international/reserves.html), current estimates of worldwide recoverable reserves of petroleum and natural gas are estimated to be 1.33 trillion barrels and 6186 trillion cubic feet, respectively. The International Energy Agency reports that global demand for oil will rise from 84.7 million barrels per day in 2008 to 105 million barrels per day in 2030 (International Energy Outlook 2009). The majority of this increase will take place in China and India, with China expected to overtake the United States as the world's largest importer of oil and gas by 2025. Currently, 85% of global energy demand is met by fossil fuels (International Energy Outlook 2009). At current consumption levels, worldwide reserves of oil will be exhausted in 40 years and reserves of natural gas in 60 years (Vasudevan and Fu 2010).

Renewable biofuels provide a pathway to reduce reliance on fossil fuels, reduce greenhouse gas (GHG) emissions, and enhance rural economies (McLaughlin et al. 2002). Biomass represents one of the most promising sources of energy and chemicals for future generations on the basis that biomass can be utilized without depleting reserves; therefore, it represents a renewable energy source. Reports state that global biofuel supply from biomass can increase from a current value of 30–140 EJ primary energy to 130–400 EJ by 2070 (Deng et al. 2015). In order to use biomass effectively for CO_2 reduction, the efficacy of biomass use has to be increased. This can be achieved by focusing on a "cascade utilization of biomass"; the use of biomass as raw material and as energy carrier should be optimized in an integrated manner.

12.2 Nonconventional Plants for Biofuels

As land and water resources are limited in developing countries and food crops cannot be used as fuel crops in these regions, we have undertaken investigations over the last 30 years on plants growing in arid and semiarid regions. This review paper summarizes some of our important work carried out on these plants. Agrotechnology for biofuel crops which are able to grow on wastelands has been developed in order to avoid food vs. fuel competition (Kumar 1987, 2013; Nagesh 2012; Zhang 2013; Deng et al. 2015; Chen and Zhang 2016).

Several families widely growing in Rajasthan have great potential as renewable source of energy. Euphorbiaceae (*Euphorbia antisyphilitica*, *E. tithymaloides*, *E. caducifolia*, *E. lathyris*, *E. neriifolia*, etc.), Asclepiadaceae (*Calotropis gigantea* and *C. procera*), Asteraceae, and Apocynaceae have a large number of valuable plants (Calvin 1976; Buchanan et al. 1978; Hall 1980, 1982; Hall and Rosillo-Calle 1998; Bhatia and Shrivastava 1983; Bhatia et al.1983, 1984, 1986; Garg and Kumar 2012a,b). The potential plants could be characterized under the following categories: (i) hydrocarbon-yielding plants, (ii) high molecular weight hydrocarbon-yielding plants, (iii) nonedible oil-yielding plants, (iv) short-rotation fast-growing energy plants, and (vi) hill plants growing on Aravalli (see also Chap. 4 this volume).

Hydrocarbon-yielding plants

Family Euphorbiaceae:

1. *Euphorbia lathyris* Linn.
2. *Euphorbia tirucalli* Linn.
3. *Euphorbia caducifolia* Haines.
4. *Euphorbia neriifolia* Linn.
5. *Edilanthus tithymalides* Linn.
6. *Euphorbia antisyphilitica* Zucc.

Family Asclepiadaceae:

7. *Calotropis procera* (Ait.). R. Br.
8. *Calotropis gigantea* (Linn.) R. Br.

High molecular weight hydrocarbon-yielding plants

Parthenium argentatum Linn.

Nonedible oil-yielding plants

Jatropha curcas L., Simmondsia chinensis C.K. Schneid, *Schleichera oleosa* (Lour) Oken, *Madhuca longifolia* var *latifolia* (Koening) Macbr, *Shorea robusta* Roxb ex Gaernf, *Pongamia pinnata* (L) Pierre, *Azadirachta indica* A. Juss, *Simarouba glauca* DC.

Short-rotation energy plants (Roy and Kumar 1990; Kotia and Kumar 2001; Bender and Kumar 2001)

Tecomella undulata (Sm.) Seem
Prosopis juliflora (Sw.) DC.
Pithecellobium dulce (Roxb.) Benth.
Azadirachta indica A. Juss.
Dalbergia sissoo Roxb.
Acacia tortilis (Forssk.) Hayne
Holoptelea integrifolia (Roxb.) Planch.
Parkinsonia aculeata L.
Cassia siamea Lam.
Albizia lebbeck (L.) Benth.
Acacia nilotica (L.) Willd. ex Delile

Hydrocarbon-yielding plants grow wild in arid and semiarid conditions across the globe. Some of them were used for experimentation and their agrotechnology developed (Kumar 1984a, b, 1987, 1994, 1995, 1996, 1998; Kumar 2000, 2001, 2004, 2007, 2008, 2011, 2013; Kumari and Kumar 2005: Kumari et al. 2005; Kumar and Joshi 1982; Kumar and Kumar 1985, 2002; Kumar and Vijay 2004; Kumar and Garg 1995; Kumar and Roy 1996; Roy and Kumar 1998a, b, 1990; Garg and Kumar 2013; Kumar et al. 1995; Kumar 2004). *Jatropha curcas* (Roy and Kumar 1990) and *Simmondsia chinensis* and wood energy plants (Roy and Kumar 1990)

12.3 Experimental Region

Experiments were performed in Rajasthan. Rajasthan state is situated between 23°3′ and 30°12′ N latitude and 69°30′ and 78°17′ E longitude. Total land area of the state is about 3,24,239 km^2, out of which about 1,98,100 km^2 is arid and the rest semiarid. It has different agroclimatic zones as given in Fig. 12.1. Out of the total area, forests cover only about 37,638 km^2 (Chand et al. 2003). Sand dunes occupy a greater part of western Rajasthan (1,20,983 km^2). The soils of the desert plains are loamy sand to loam. The eastern part of Rajasthan has alluvial soil which supports good forests and agricultural crop. Occurrence of saline soils with pH up to 9.0 is a common feature in the sandy areas of Rajasthan (Figs. 12.2, 12.3, 12.4, and 12.5).

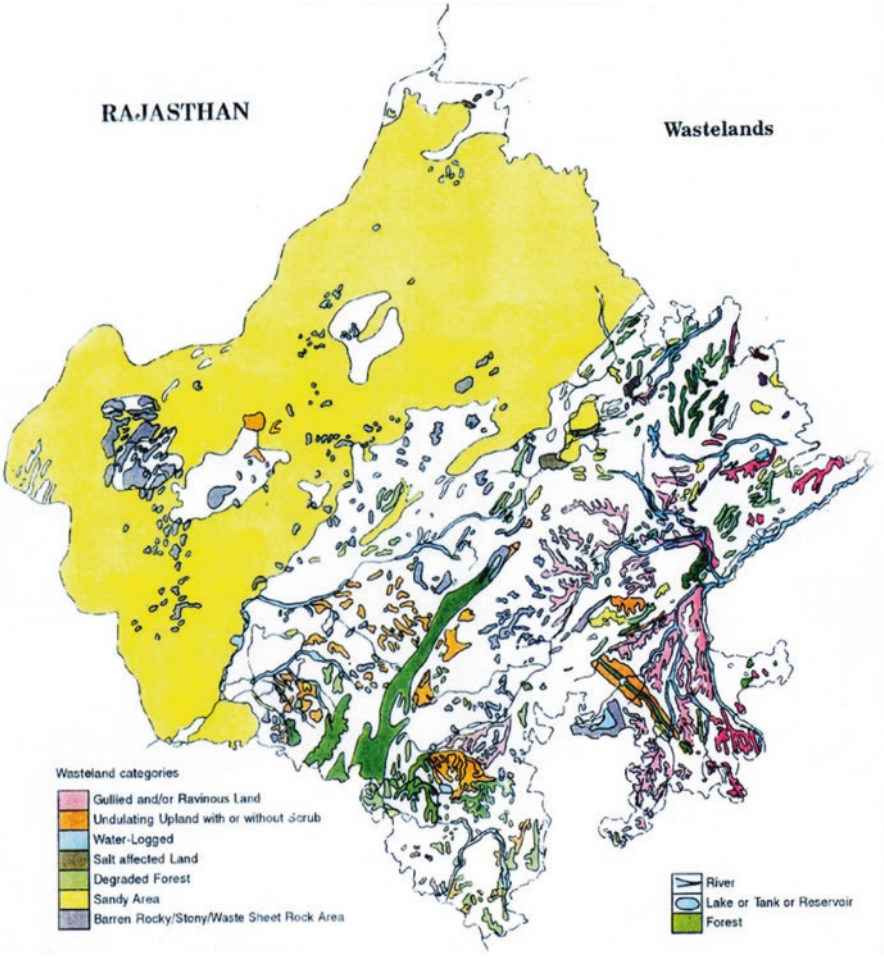

Fig. 12.1 Aravalli hills divide Rajasthan into hot and dry northwest region and southeast region

Fig. 12.2 Energy Plantation Demonstration Project Center (EPDPC), University of Rajasthan, Jaipur, 1984. Barren land with only one tree (*Holoptelea integrifolia* (Roxb.) Planch)

Fig. 12.3 The soils of Rajasthan are sandy and support poor vegetation

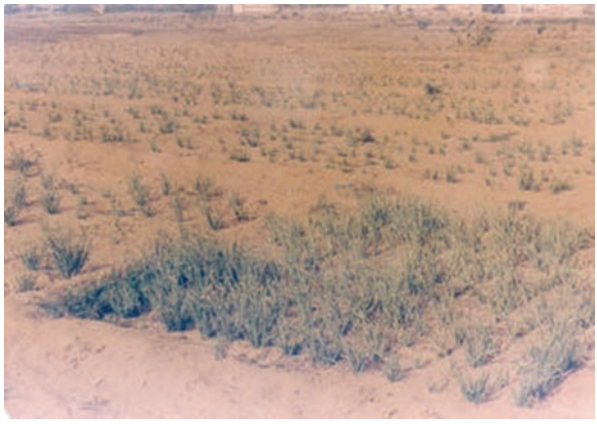

Fig. 12.4 Initial experimentation with *Euphorbia antisyphilitica* nursery stage, with close spacing

Fig. 12.5 Initial experimentation with *Calotropis procera*, a hydrocarbon plant used to colonize

12.3.1 *Euphorbia antisyphilitica* Zucc. (Euphorbiaceae)

Euphorbia antisyphilitica Zucc., commonly called candelilla, is native to Mexican desert. It is a source of commercial candelilla wax found as thin film on stem surface giving it whitish look. Plant contains latex which is rich in hydrocarbons. It can be easily multiplied in arid and semiarid regions for wax. Refined wax can be used for polishes, creams, leatherware, furniture, sealing waxes, and chewing gums. It has been introduced from Mexico and is successfully established in arid parts of Rajasthan. The wax content varies from 2 to 5% and can be harvested any time during the year (Paroda et al. 1986). *Euphorbia antisyphilitica* hydrocarbons can be cracked using catalytic cracking to obtain petroleum. Agrotechnology for the plant *Euphorbia antisyphilitica* was developed (Johari et al. 1990a, 1991; Johari and Kumar 1994a,b, 2013) (Fig. 12.6). However this plant also suffers from charcoal rot during rainy season (Johari and Kumar 1993).

12.3.2 *Euphorbia lathyris* Linn. (Euphorbiaceae)

It is commonly called as caper spurge, gopher plant, and mole plant. This plant grows throughout the medium-temperature areas of the world, preferring open, relatively mesic habitats. In California, it grows along the coast. In Australia, it has naturalized in the vicinity of Sydney and Melbourne, in the humid southeast, but occurs in the arid southwest. Plant by-products have some commercial and medicinal importance. Investigations on several plant species have been carried out at our center including *Euphorbia lathyris* (Kumar and Kumar 1985,1986a; Garg and Kumar 1987a, b, c, 1989a, b, 1990; Kumar and Garg 1995; Garg and Kumar 2011a, b, 2012a,b, 2013).

Fig. 12.6 *Euphorbia antisyphilitica* and *Jatropha curcas* in background

Fig. 12.7 *E. neriifolia* growing as hedge plant

12.3.3 *Euphorbia neriifolia* Linn. (Synonym: *E. ligularia*, *E. pentagona*) (Euphorbiaceae)

It is commonly called as Indian sugar, suda-suda (Philippines), and susura (Indonesia). It grows throughout the Philippines in wastelands and open grasslands and is usually very abundant. It is also abundant in Sri Lanka, Thailand, Indonesia, Malaysia, Nepal, and India. In India, it grows throughout and extends to Malaya. Occasionally, it is cultivated for ornamental purposes. It is very common on rocky places of Rajasthan, Konkan, and Deccan peninsula, in the Siwalik tract of northwestern Himalayas and Gujarat, and in Ahmadnagar and Bijapur districts in western peninsula. Poor-looking plants also occur in dry barren soils in Bengal (Srivastava 1986). It can thrive well in semiarid regions (Kumar 1990, 1994). It also has medicinal importance (Fig. 12.7).

12.3.4 *Euphorbia nivulia* Buch.-Ham. (Euphorbiaceae)

It is found in Northwest Himalayas and on dry rocky hills of Gujarat and Deccan peninsula. It occurs in barren and rocky places of Rajasthan, Bihar, UP, Gujarat, and southern states of Mysore, Madras, and Kerala.

Fig. 12.8 *Euphorbia tirucalli* from Udaipur division

12.3.5 *Euphorbia tirucalli* Linn. (Euphorbiaceae)

Its common names are stick plant, African milkbush (English), consuelda (Spanish), suerda and pobreng kahoy (Philippines), kayu urip (Indonesia), paya-raibia (Thailand), and sehund and konpal (India). As a native of Africa, this species is now planted in most tropical countries. It is common in Brazil, Africa, Israel, some semiarid lands, and the drier western parts of Bengal, Bihar, Punjab, Puri, and South India (Srivastava 1986). It also does not require good soil and grows well in uncultivated areas which are not suitable for food crops. It is vegetatively propagated through cuttings. It can grow in semiarid regions where rainfall is about 25–50 cm per year. Agrotechnology for *Euphorbia tirucalli* was developed (Kumar and Kumar 1985, 1986a). It yields many components which have higher values as pharmaceuticals (Upadhyay et al. 2010) (Fig. 12.8).

12.3.6 *Pedilanthus tithymaloides* (Linn.) Poit. (Euphorbiaceae)

It is commonly called as zigzag plant (English), patah (Indonesia), and solsoldong (Philippines). The plant, a native of Mexico, is now cultivated for ornamental purposes in most tropical and subtropical countries. In India, it is also known as redbird cactus or slipper flower. They are adaptable to wide variety of soils and tolerant to various degrees of water application (Srivastava et al.1985). It does require good soil and grows well in uncultivated areas and dry locations. It is vegetatively propagated. In India, seven different varieties of *Pedilanthus* are cultivated as ornamental or hedge plants. Experiments were conducted on its growth and improvement (Rani et al. 1990, 1991, 1996; Rani and Kumar 1994a,b) (Fig. 12.9).

Fig. 12.9 *Pedilanthus tithymaloides*

Fig. 12.10 *Calotropis gigantea*

12.3.7 *Calotropis spp.*

We carried out investigations on *Calotropis* spp., Family: Asclepiadaceae (*Calotropis gigantea* and *C. procera*) (Rani et al. 1990, 1991; Kumar and Kumar 2002; and Kumar 2005) (Figs. 12.10 and 12.11).

Fig. 12.11 Flowering twings of *Calotropis gigantea* (Kumar 2004; Kumar and Vijay 2004; Kumari and Kumar 2005)

12.4 Agrotechnology for Laticiferous Plants

Growth and productivity of different laticifers was studied. Maximum growth was observed during March to October. Increase in hexane extractable was recorded up to 6–7 months; thereafter percent hexane extractable (HE) did not increase significantly in *E. lathyris*, *E. antisyphilitica*, and *P. tithymaloides*. Higher levels of HE were recorded in leaves as compared to the stem in *E. lathyris and P. tithymaloides*. *E antisyphilitica* can be easily propagated through cuttings. The optimum period for raising cuttings is in June–July and March–April. Cuttings from apical and middle portions of *E. antisyphilitica* exhibit 100% survival rate, while none of the cuttings from the basal portions survived. Regarding environmental variations, March to October period was best suitable for *E. antisyphilitica* because linear increase in growth was recorded in this period (Kumar 1990). Overall growth and productivity were lowest in the winter months from November to February. Higher accumulation of hexane extractable corresponded with higher temperatures of summer season (Johari and Kumar 1992). Soil types influenced growth of laticifers. Among different soil types, sand was best for the growth of *E. lathyris* (Garg and Kumar 1990) and *P. tithymaloides* (Shrivastava 1985; Shrivastava and Bhatia 1986; Rani et al. 1990, 1991, 1996; Rani and Kumar, 1992, 1994a, b, Rana and Kumar 2012), while red loamy soil was best for *E. antisyphilitica*. A combination of different soil types (red + sand + gravel) yielded maximum growth of *E. antisyphilitica* (Johari et al. 1990a). A mixture of gravel + sand favored maximum increase in height, fresh weight, and dry weight in *E. lathyris* (Garg and Kumar 1990; Kumar and Garg 1995). Environmental factors influenced the growth and yield of *Calotropis procera* (Rani et al. 1990).

Considerable differences were recorded in percent dry weight and hydrocarbon contents in various plant species investigated. The percent dry weights ranged from 8.8% (*E. tirucalli*) to 22.63% (*E. lathyris*). In others the yield was *Calotropis gigantea* (22.0%), *Euphorbia hirta* (20.0%), *Calotropis procera* (16.8%), *Pedilanthus*

tithymaloides var. *cuculatus* (15.7%), *P. tithymaloides* var. *variegatus* (15.5%), *P. tithymaloides* var. *green* (14.7%), *Euphorbia neriifolia* (11.59%), *E. nivulia* (11.3%), and *E. antisyphilitica* (10.57%) (Kumar et al. 2002).

12.4.1 Nonedible Oils

Several nonedible oils are utilized to generate fatty acid methyl ester (**FAME**). They include *Azadirachta indica* A. Juss., *Pongamia pinnata* (L.) Pierre., *Ricinus communis* Linn., *Schleichera oleosa* (Lour) Oken, *Madhuca longifolia* var *latifolia* (Koening) Macbr, *Shorea robusta* Roxb ex Gaernf, *Simarouba glauca* DC. and *Jatropha curcas* (*wild castor*, *ratanjot*) (Kumar and Roy 2004; Kumari and Kumar 2005; Kumar 2011) (Figs. 12.12, 12.13, 12.14, and 12.15).

12.4.2 Several Grasses

Several perennial forage grasses in particular are salt-tolerant growing in Rajasthan (*Panicum antidotale, Brachalari mutica, Panicum maximum, Diplochne fusca*) (Kumar 2011) and are easy to manage (Corwin et al. 2008) (see also Chap. 4 and 6 this volume). Among the grasses, switchgrass(*Panicum virgatum*) is most commonly mentioned in the United States (Schmer et al. 2008). Switchgrass does not

Fig. 12.12 *Pongamia pinnata* (*Karanja*)

Fig. 12.13 *Azadirachta indica* (*neem*)

Fig. 12.14 *Simarouba glauca* (Simaruba)

Fig. 12.15 *Madhuca longifolia var latifolia*

require annual tillage and planting and is grown on conservation reserve lands that have uneconomic yields of annual crops or are too erosive. *Miscanthes* spp. are another example of plants as possible source of bioufel.

12.4.3 Woody Plants

Wood obtained from forests legally or illegally is used for burning directly into poorly devised "chulhas" or fireplace resulting in loss of energy. Karve (Chap. 6 this volume) described better methods to use wood and light biomass for gaining maximum bioenergy.

- The estimated oil yield per hectare for *Jatropha* is highest among tree-borne oilseeds.
- With an average seed production of 3.75 tons/ha, oil content of 30–35%, and oil yield of 1200 kg/ha estimated compared to 375 kg/ha for soybeans in the United States and 1000 kg/ha for rapeseed in Europe.

A three-tier planting system for arid and semiarid regions for maximum productivity has been developed (Kumar 2013). *Acacia tortilis* at the top and *Jatropha curcas* at the middle and *E. antisyphilitica* at the lowest level yield maximum biomass per square meter on wasteland with minimal irrigation and rainfall range from 35 to 400 mm (Kumar 2013) (Fig. 12.16).

Fig. 12.16 Three-tier system with *E. antisyphilitica* in foreground *Jatropha curcas* in the middle and *Acacia tortilis* in upper tier

Jatropha curcas has been extensively grown in India under the Department of Biotechnology supported micro mission projects with an object to identify, characterize, and multiply high-yielding strains and study their growth and productivity under different agroclimatic conditions.

12.4.4 Cultivation of *Jatropha curcas*

12.4.4.1 Background

Jatropha curcas, a nonedible oilseed crop, has a potential to yield up to 41% oil (Kumar unpublished data). *Jatropha curcas* grows wild in different parts of Rajasthan particularly in hilly areas as local people call it plants of "Dungar" (hills).

Udaipur division is a natural habitat of *Jatropha curcas*. Wild *Jatropha curcas* grows on hilly tracts near Kumbhalgarh fort and in entire Udaipur division (as seen in the picture). Tribals harvest seeds and use them for soap making which they call habu in local language. They also sell *Jatropha* seeds in the local market of Udaipur division. *Jatropha* yield varies in this region from 3 kg to 15 kg per tree per season.

Fig. 12.17 Kumbhalgarh fort, Udaipur

12.4.5 Summary of Experimentations

Plant material was collected from Udaipur division in Rajasthan at Udaipur, Bhilwara, Banswara, Pali, Chittorgarh, and Rajsamand districts which have native populations of *Jatropha curcas*. The climate of Udaipur is tropical. The summer season is hot, with an average temperature hovering around 38.3 °C (max) to 28.8 °C (min). The climate of Udaipur, Rajasthan, is quite pleasant in winters. The average temperature falls in the range of 28.3 °C (max) to 11.6 °C (min). Jaipur has hot and scorching summers and cool winters, which are pleasanter. The mercury rises to as high as 45 °C in summers, when the minimum temperature is 25.8 °C. In winters the maximum temperature restricts itself to about 22 °C. However, nights can be cold, and temperature can be as low as 8.3 °C. The criteria used to determine the elite plant accessions included plant height, canopy girth, stem diameter, seed yield, number and bearing of seeds, seed weight, and oil contents. One hundred ten accessions were collected. Selection of superior material based on established criteria of oil and yield was carried out. Standardization of agrotechnology was carried out. Samples collected were analyzed at TERI, New Delhi, and all the accessions have been deposited at the National Bureau of Plant Genetic Resources, New Delhi. Influence of inorganic and organic fertilizers, growth regulators, and physicochemical factors was determined, and proper agrotechnology has been developed. Out of this four accessions have been included among the 100 accessions used for all India trials in different agroclimatic zones. University of Rajasthan was recognized as one of the centers in the north zone of the country.

12.4.6 Nursery Multiplication of Elite Strains, Cultivation and Extraction.

Details of experiments are described earlier (Kumar 2013). The seed oil contents were RU I (35.53%), RU II (36.41%), RU III (36.36%), and RU VIII (35.25%). Only accessions (RU II, RU III, and RU VIII, RU XIII, RU XIV, RU XVII, RU XVIII) with over 35% oil content were selected for plantations. Sample P107 from RU II yielded 36.674, and P108 from RU III yielded 36.714 during the third year of fruiting (Figs. 12.18, 12.19, 12.20, 12.21, 12.22, and 12.23).

The elite material has been deposited at the National Bureau of Plant Genetic Resources (NBPGR), New Delhi (Table 12.1).

12.4.7 Biotechnological Studies and *Jatropha* Cultivation

Council of Scientific and Industrial Research – Central Salt and Marine Chemicals Research Institute (CSIR-CSMCRI) based on its experiences, extensive survey and studies on growth, and yield attributes has identified a few germplasm as elites through recurrent selection, which are consistent in yield. CSIR-CSMCRI had demonstrated successful establishment and reasonable production of *J. curcas* on wastelands/marginal land (Mastan et al. 2014, 2016; Rathore et al. 2015). Rathore et al.

Fig. 12.18 High-yielding cultivar RU II being multiplied at University of Rajasthan, Jaipur

Fig. 12.19 Large-scale multiplication of *Jatropha curcas* at Thanagazi near Sariska Alwar

(2015) reported the micropropagation of elite genotype of *Jatropha curcas* L. through enhanced axillary bud proliferation and *ex vitro* rooting (*Biomass and Bioenergy*, *83*, 501–510). The composition of fatty acids of some of the samples is given in Table 12.2.

12.4.8 Jatropha a Dream

Ywe Jan Franken, an expert on biofuels for FACT Foundation, a research group in the Netherlands, says this plant grows all over the tropics, including Indonesia, the Philippines, Cambodia, India, and Latin America (http://www.npr.org/sections/the-salt/2012/08/22/159391553/how-a-biofuel-dream-called-jatropha-came-crashing-down). Around 2007 and 2008, they spent money for *Jatropha* projects, including huge plantations covering tens of thousands of acres, all over the tropics. Mozambique, in southern Africa, was among the most active new centers of *Jatropha* cultivation. Ywe Jan Franken, from FACT Foundation, says much of the enthusiasm about *Jatropha* was based on a misunderstanding. If you actually want a good harvest of oil, he says, the plant "needs nutrients and water, just like any other crop." But Franken also believes that the *Jatropha* story isn't quite finished. Meanwhile, on the high-tech side, scientists are studying this tree for the first time. They're selecting plants that produce more seeds, breeding high-yielding varieties,

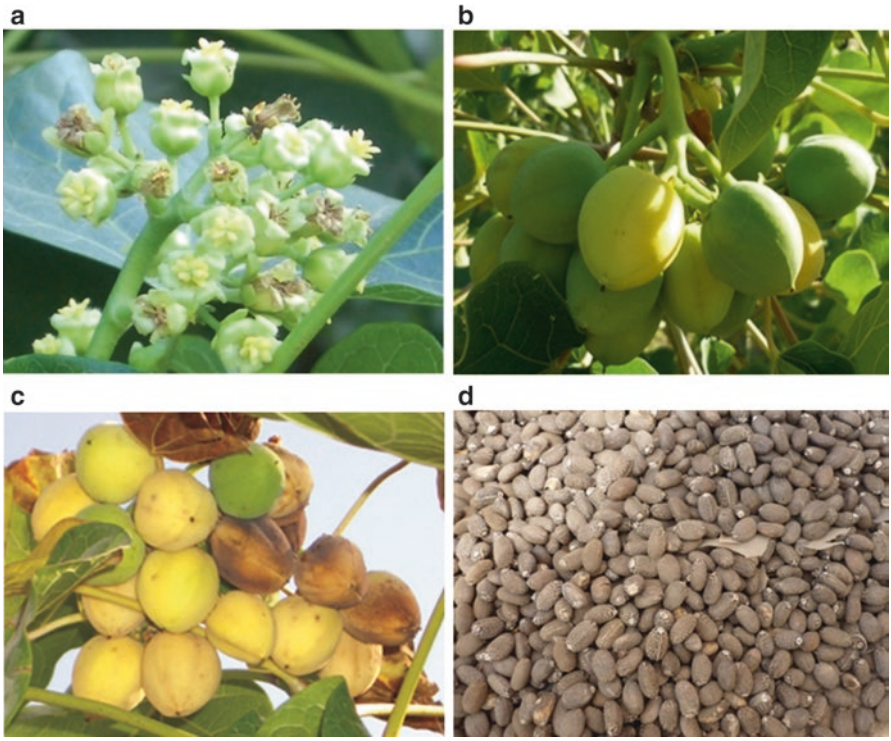

Fig.12.20 (**a**) Flowering in the September season. (**b**) Unripen fruits of *Jatropha curcas*. (**c**) Ripen fruits for harvesting. (**d**) Seeds of *Jatropha curcas*

turning it from a semi-wild plant into a real crop. It is highly susceptible to frost and cannot grow well in areas with rainfall below 400 mm per annum or in other words "It's true, he says, that the tree can survive droughts, and poor soil. But under those conditions, it won't produce many seeds." Perhaps, after many years of such breeding, it will become as productive as corn or palm trees. To this we wish to add that *Jatropha* requires a typical hilly conditions and can be cultivated on hedges of crop fields successfully but is difficult to grow as a normal crop due to its specific requirements (Kumar unpublished data).

Fig. 12.21 *Jatropha* fences for fuel. Its cultivation on fences provided reduction in leaf curl virus on the chili crop grown in Jaipur district

Fig. 12.22 Decentralized production of biodiesel in cold process

Fig. 12.23 Villagers being demonstrated the use of *Jatropha curcas*

Table 12.1 University of Rajasthan Accession number of samples deposited in National Bureau of Plant Genetic Resources (NBPGR, New Delhi, India) and their IC numbers

Accession No.	NBPGR Accession	HE% DW
RU III	IC565603	36.40
RU I	IC565601	35.50
RU II	IC565602	36.40

Hexane Extractable of samples deposited in terms of percent dry weight of sample

Table 12.2 Composition of fatty acids varied depending on various factors. Sample with seed oil >35%

S. no.	Sample name	% of seed oil content	Palmitic acid	Oleic acid	Linoleic acid
1.	RU XV	35.055	21.31	38.59	35.26
2.	RU XIII	35.285	18.41	35.49	41.48
3.	RU XVII	35.590	17.66	34.40	43.71
4.	RU XVIII	35.899	19.12	34.82	41.68
5.	RU XX	36.012	21.51	37.59	35.39
6.	P107	36.674	16.46	29.71	43.95
7.	P109	36.714	17.19	36.76	42.15

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Alternative Biomass from Saline and Semiarid and Arid Conditions as a Source of Biofuels: *Salicornia*

13

Ashwani Kumar, Ebin Abraham, and Arti Gupta

Abstract

The world population is increasing, and there is a limited amount of freshwater in the world. Global efforts are being made for turning saltwater into an alternative to freshwater for agriculture. The Gujarat State Fertilizers & Chemicals Ltd. (GSFC), Baroda, plans to promote the cultivation of *Salicornia*, a special saltwater or wasteland plant that has export potential and yields several value-added products. Gujarat with its 1600-km-long coastline has a vast potential for such farming. Some of the plants in this region are environment friendly. Besides potential as biomass, *Salicornia* provides value-added products: its seeds yield edible oil that is low in cholesterol and contains antioxidants; its succulent tips are used widely in Europe and the USA in green salad dressings; the plant itself can be an excellent fodder. This fodder has increased milk yield by 15% in addition to making it protein rich. The dry biomass is used to prepare particleboard for use in furniture. *Salicornia* cultivation can provide cheap, locally available energy to catalyze the all-round socioeconomic development process. This can also offer an effective low-cost strategy for reclaiming barren wastelands into productive areas.

Keywords

Salicornia • Coastal areas • Saline water • Biomass

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13.1 Introduction

Cultivating biofuel crops, trees, and grasses for biofuels may compete with agriculture especially in developing countries as there can be unfavorable ramifications of the "food versus fuel" use of plant products. Corn ethanol and soybean diesel are falling out of favor as energy crops made from algae, *Jatropha*, and other non-food woody plants are garnering international attention and investment. Global food needs are expected to roughly double by 2050, putting expanding demand for space to grow food crops and biofuel feedstocks on a collision course (Negash 2012; Shaik and Kumar 2014). Alternative biomass-producing plants like halophytes and desert vegetation or plants of wasteland can provide an answer. Out of Indian coastline of 7517 km Gujrat alone has a 1600-km coastal stretch which can yield an area of 2500 km^2 for saltwater farming (Fig. 13.1a, b, c). Several halophytic plants are able to grow in saline soils of which *Salicornia* is one major plant in the coastal areas of Gujarat. *Salicornia* species can generally tolerate immersion in saltwater. They use the C_4 pathway to take in carbon dioxide from the surrounding.

13.2 Alternative Crops

Biofuels from saline water or seawater-grown crops (see also Chaps. 14 and 17 this volume): plants growing in saline habitats (halophytes) easily adapt themselves to a high salt content of the soil in the course of ontogeny. Among the six obligatory halophytes (*Salicornia europaea*, *Spergularia marina*, *Atriplex hastata* var. *salina*, *Aster tripolium*, *Glaux maritima*, *Triglochin maritima*), and other halophytes like *Haloxylon recurvum, Salsola bryosma, Sueda nudiflora,* studies were conducted on the salinity tolerance and bioremediation using some of them under saline irrigation in Rajasthan (Shekahwat et al. 2005, 2006 and Shekhawat and Kumar 2006). Sediment salt concentration appears to be an important determinant of the distribution of *S. bigelovii*. Once established, *S. bigelovii* can tolerate very high sediment salt concentrations. Plants from the coastal areas could provide enough material for biofuel production without competing with water or agricultural soils. *Rhizophora* sp also supports marine ecosytem of the coastal areas (Fig. 13.1d, e). *Salicornia bigelovii* is one such example. Marine algae can be another example (see also Chap. 17 this volume.) for providing the biomass. The "holy grail" of biofuel development is source plants that do not compete with food crops and do not require large amounts of water or energy to cultivate.

13.3 Integrated Production

Those involved in shrimp farming too can grow *Salicornia*. The seawater can be collected in fishponds used for shrimp farming. Marine algae are one more possible source of biofuel (see Chap. 17 this volume.). Once the fish are harvested and removed, *Salicornia* can be grown in the same water, which is rich in nutrients after the fish have been harvested thereby leaving behind excreta. The plant helps in the

absorption of carbon dioxide and reduces global warming and helps in controlling the greenhouse effect (http://www.baroda-online.com/spectoms/Trends.html). *Salicornia* species are used as food plants by the larvae of some Lepidoptera species including the *Coleophora* case-bearers *C. atriplicis* and *C. salicorniae* (the latter feeds exclusively on *Salicornia* spp.).

13.3.1 Habit and Habitat

Mangrove forests grow in creeks, estuaries, bays and lagoons and in inter-tidal areas – area between the high tide and the low tide. The unique mangroves located along India's 7516-km coastline are in Gujarat, Maharashtra, Goa, Karnataka, Kerala, Odisha, Tamil Nadu, Andhra Pradesh and Andaman and Nicobar Islands. *Rhizophora* spp. are found along coastal areas of Gujarat intermixed with other halophytes (Fig. 13.1d–e).

13.3.2 The Plant

Coastal areas have large number of salt tolerant plants like *Suaeda* sp (Fig. 13.2a) and halophytes (Fig. 13.2b) along with *Salicornia* sp. (Fig. 13.2c, d). The *Salicornia* is a plant of which 250 species exist and is full of seeds that are used for the oil production, cosmetics, and food for cattle, among others. The *Salicornia* species are small, usually less than 30 cm tall, succulent herbs with a jointed horizontal main stem and erect lateral branches (Fig. 13.2c). The leaves are small and scalelike and as such the plant may appear leafless. Many species are green, but their foliage turns red in autumn (Fig. 13.2d). The hermaphrodite flowers are wind pollinated, and the fruit is small and succulent and contains a single seed (Fig. 13.4).

Salicornia spp. are worldwide in distribution including coastal India, North America, South America, Eastern Europe, Southeast Asia, Africa, Oceania, the Middle East, Eastern Asia, and Western Europe. *Salicornia* species are native to North America, Europe, South Africa, and South Asia. Common names for the genus include **glasswort**, **pickleweed**, and **marsh** samphire: these common names are also used for some species not in *Salicornia*. *Salicornia europaea* L. is a halophyte that often occupies the lowest and most saline (>3.5% total salt) areas of salt marshes. *Atriplex prostrata* Boucher is less salt tolerant than *S. europaea* and often grows in a less saline (<2.0% total salts) zone adjacent to *S. europaea* (Egan and Ungar 2001).

Salicornia can grow in a wide range of salt concentrations (0.1–2.0 M) and can accumulate up to 40% salt of its dry weight. The oil content of the plant's seeds is about 30 % of its total weight compared with 17–20 % for the soybean, according to tests by the University of Arizona's Environmental Research Laboratory (ERL) in Tucson, which has spearheaded *Salicornia* development. *Salicornia* oil also contains around 72% linoleic acid—a healthy polyunsaturated fat (PUFA). This is close to the level found in safflower oil and more than twice that of oil from soybeans.

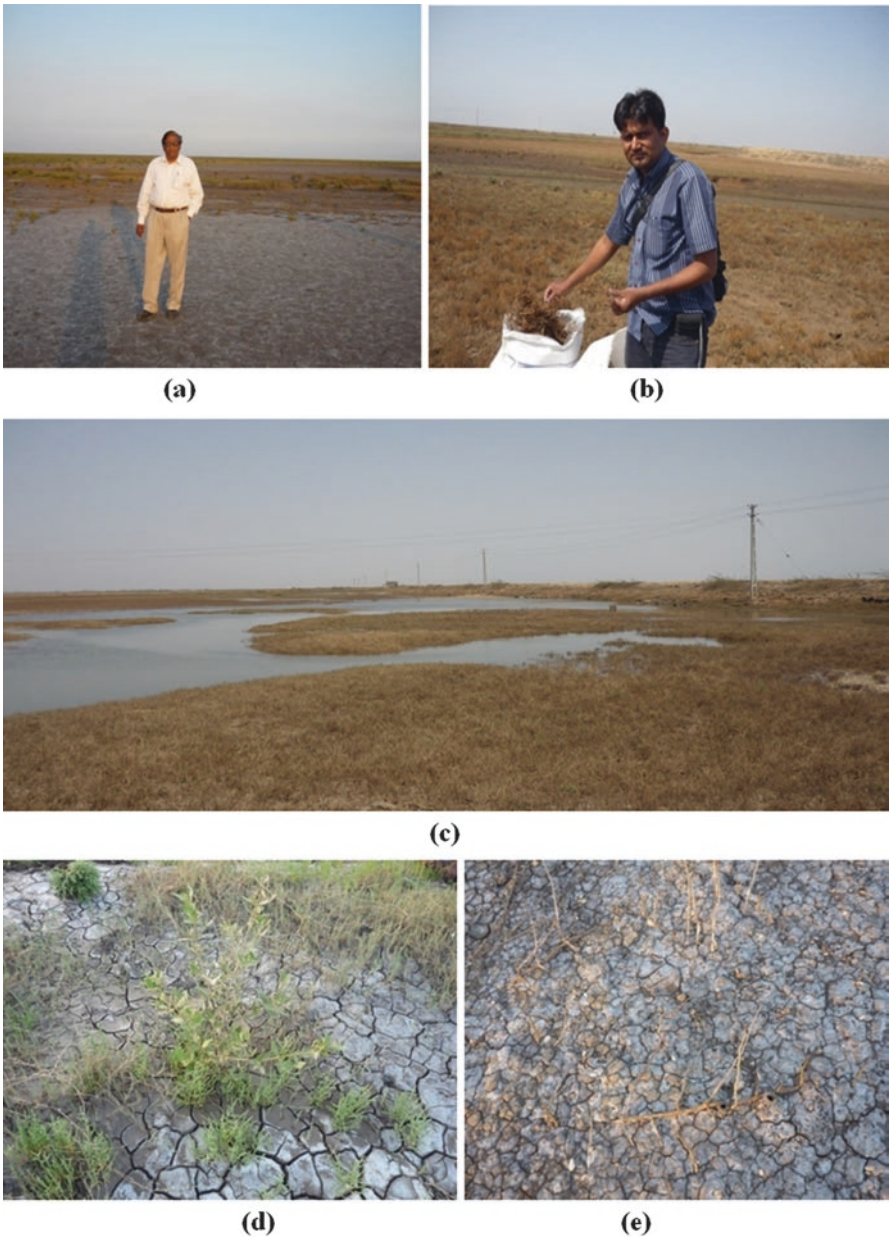

Fig. 13.1 (**a**) Costal area rich in *Salicornia*, (Photo by AK) (**b**) *Salicornia* sp. collection, (Photo by EA) (**c**) vast inland areas, (**d**) *Rhizophora* (in centre), (**e**) *Rhizophora* roots

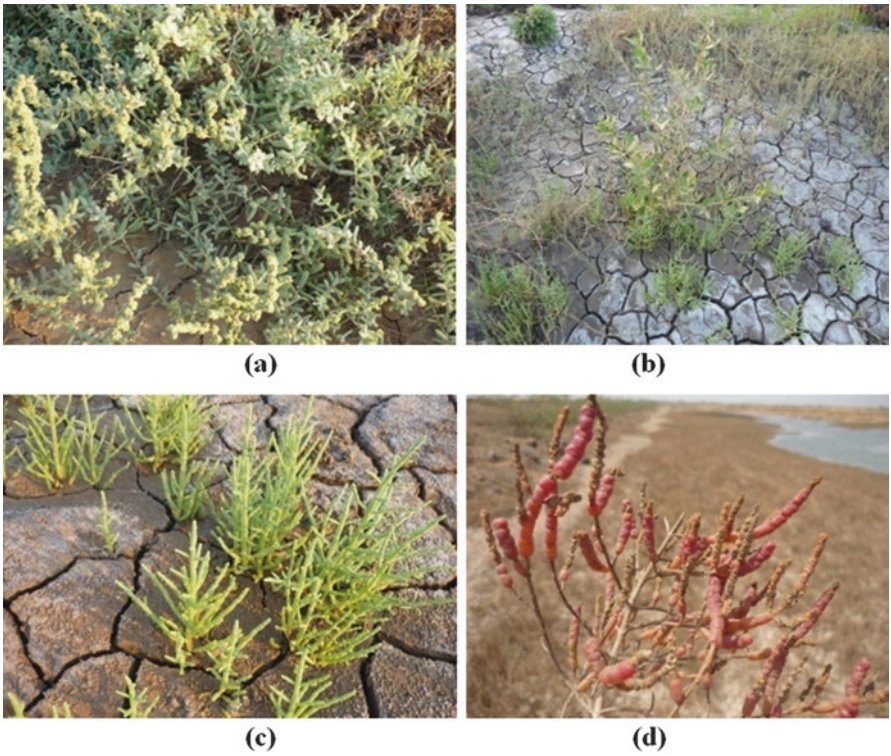

Fig. 13.2 (**a**) *Suaeda* sp., (**b**) halophytes, (**c**) Young *Salicornia*, (**d**) mature *Salicornia* sp.

Saltwater energy farms could be raised in coastal areas. *Salicornia* has very high economic value, including a 30% of oil, more than it is possible to obtain from the soybean seeds. The oil also provides raw material for a series of cosmetic and pharmaceutical products. Thus *Salicornia* in culture is better than the soybean with major yields and minor production costs, notwithstanding that it captures CO_2, reducing therefore the contamination. Besides this its ability to grow in nonfertile earth with saline irrigation apparently has an economic potential and high productivity under adverse conditions. The biomass of the *Salicornia* provides proteins of high quality; near the 40%, their uses can be balanced and/or forage for the cattle, pulp from the straw can be used for the production of heavy paper, and leaves are laminated as water cover in the construction of houses and for the weavers of the mattresses. *Salicornia brachiata* Roxb. (Amaranthaceae), a leafless succulent annual halophyte, has a unique genetic makeup which allows them to grow and survive under stress conditions. It is found commonly growing on the Gujarat coast in India. Another species, *Salicornia europaea*, a salt-accumulating halophyte, is a vegetable succulent plant, completely edible for humans and capable of accumulating up to 50% NaCl of dry matter (Kong and Zheng 2014). *Salicornia rubra* Nels. is one of the most salt-tolerant species in the western half of the USA and Canada

Fig. 13.3 (**a**) Harvested *Salicornia* sp., (**b**) seed extraction from *Salicornia*, (**c**) seed separated from chaff

(Khan et al. 2000). The halophyte *Salicornia bigelovii* Torr. is a valuable new high-yielding oilseed crop for direct seawater irrigation in coastal areas of the arid regions (Glenn et al. 1991).

Jefferies et al. (1981) examined the biology of two cleistogamous diploid populations of *Salicornia europaea* from the upper (landward) and lower (seaward) levels on the north Norfolk coast. Although seeds of both populations of this annual germinated in spring, upper-marsh seedlings grew little until July, whereas continuous growth characterized individuals from the lower marsh. The upper-marsh plants most closely resembled the description of *Salicornia ramosissima* J. Woods, whereas plants from the lower marsh appeared to be *Salicornia europaea* L. (sensu stricto). The specific limits of these closely related species within the *S. europaea* group, however, are obscured by considerable phenotypic plasticity and by local differentiation of populations in Northwest Europe; populations of *S. ramosissima* are widespread in the upper parts of salt marshes (Ball and Tutin 1959). In contrast, populations of *S. europaea* (s.s.) are present in open or sandy habitats, which are common in the lower levels of marshes (Jefferies and Gottlieb 1982).

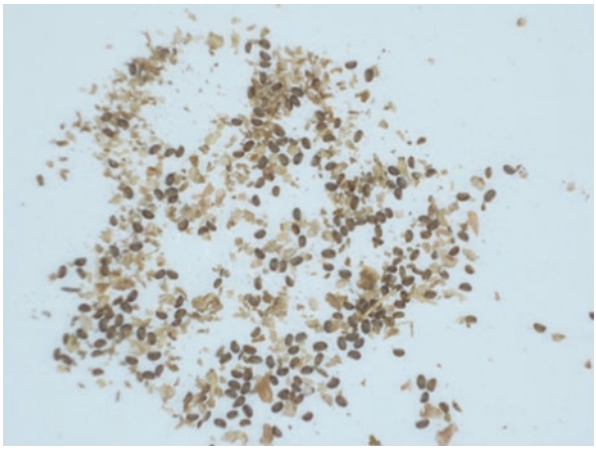

Fig. 13.4 Seeds of *Salicornia* sp.

13.3.3 Seed Extraction

Seeds of *Salicornia* spp. are collected by harvesting the complete plants and thrashing the inflorescences manually through different grades of sieves (Figs. 13.3a,b,c and 13.4).

13.3.4 Seed Germination

Halophyte seeds have the ability to maintain seed viability for extended periods of time during exposure to hypersaline conditions and then to commence germination when salinity stress is reduced (Ungar 1982; Woodell 1985; Keiffer and Ungar 1995; Khan and Ungar 1996, 1997; Gul and Weber 1999). Annual *Salicornia* sp. are highly salt tolerant but vary in their response to salinity (Langlois 1966; Ungar 1977; Philipupallai and Ungar 1984). *Salicornia europaea* showed a 10% germination at 5% (860 mM) NaCl (Ungar 1967a), *Salicornia bigelovii* had 63% germination at 8% (1376 mM) NaCl (Rivers and Weber 1971), and *Salicornia stricta* was reported to have 10% germination at 10% (1720 mM) NaCl (Chapman 1974). Salinity alone may not be the only critical environmental factor in the germination of annual halophytes (Khan and Ungar 1998a). Interactions between salinity and temperature occur, and they determine optimal conditions for seed germination in halophytes (Hogan 1968; Rivers and Weber 1971; Philipupallai and Ungar 1984; Badger and Ungar 1989; Khan and Ungar 1996, 1998b).

Salicornia seeds (*Salicornia bigelovii* Torr. seeds) can be sown in March in acid-washed quartz coarse sand in each flat trays pH 6.9. The plant requires distilled water or rainwater for seed germination. They can also be grown under long cool white fluorescent lights, which provide a 14-h photoperiod under 650 UE m^{-2} s^{-1} photosynthetically active radiation (PAR; 425–700 nm). The pots are flooded to saturation with distilled water, and within 24 h, abundant germination can be

observed. Plants were regularly watered as per requirement to avoid drought. Approximately 1/2-strength Hoagland's nutrient solution was used to water seedlings after 9 days. The seedlings can also be raised in hydroponics (Fig. 13.5a). Seedlings can be transplanted to the pots and then to the field individually after 60 days (Fig. 13.5b).

Optimal germination of *Salicornia rubra* in 12/12 hr light/dark conditions occurred in distilled water at temperatures of 20–30 °C and 25–35 °C. Seed germination decreased with an increase in NaCl concentrations at all temperatures. A delay in germination was more obvious at 5–15 °C than with other temperatures regimes. Change in temperature regimes significantly affected the germination of *S. rubra* seeds. At the higher temperature regime, 25–35 °C, seeds in non-saline controls had about 98% germination compared to less than 50% germination at the lower temperature regime 5–15 °C. Few seeds germinated in the 1000 mM NaCl treatment.

Salinity must be moderated by seasonal inundation of bay water in the fall, perhaps augmented by high precipitation during or immediately after inundation for colonization to occur. Germination and early seedling establishment will fail with too short a period of inundation or with water that is too saline (Fig. 13.1c) (Panuccio et al. 2014).

Seed germination is sensitive to salinity, and hence seed germination takes place during the rainy season with rainwater. However, plants are tolerant to salinity and are able to grow in coastal saline water. Based on the observation that the seeds of most salt marsh plants germinate in spring or during seasons of high precipitation and relatively low evaporation when salt stress was not as great, Chapman (1974) concluded that a reduction in soil salinity was a prerequisite for successful germination. In fact, seeds of many halophytic species germinate best under freshwater conditions and at salinities below 0.5% NaCl (Ungar 1991). Together, sediment salt and water relations appear to be the primary determinants of Salicornia distribution on Horse Island Flats.

Fig. 13.5 (**a**) Young *Salicornia* in hydroponics, (**b**) *Salicornia* in pot

Salicornia rubra seeds were able to germinate in high salinity (1000 mM NaCl) under laboratory conditions and may be included among those halophytes which have the ability to withstand high salinity stress during germination. *Salicornia rubra* seeds had maximum germination at the 25–35 °C temperature regime for all NaCl concentrations tested. This is consistent with the results of other Great Basin Desert species indicating that a higher temperature is better suited for their germination. In early spring, lower temperatures would delay germination. The ability of *S. rubra* to germinate quickly at the higher temperatures confers an ecological advantage in maintaining the fitness of the population. Seeds are highly tolerant to salinity when stored in the soil; however, when salinity of the playa is reduced due to snowmelt, they do not germinate quickly but can remain dormant until higher temperatures occur.

13.3.5 Genetics of *Salicornia* spp.

Since these local populations are strongly cleistogamous, it is important to examine their genetic variability. The second objective was to determine whether consistent genetic differences could be detected between them which would reinforce the taxonomic criteria on which the recognition of the two species was based. Accordingly gel electrophoresis was used to examine enzymic variation in individuals from different populations. Several factors (water, temperature, light, and salinity) that regulate seed germination interact in the soil interface (Ungar 1996). Other variables may coact with the seasonal variation in temperature to determine the temporal pattern of germination (Khan and Gul 1998). Osmotic and matric potential of soils narrow the range of temperature that is effective for the germination of seeds (Hegarty 1978).

The purpose of this work was to characterize the *Salicornia* using genetic markers and to investigate the possibility of growing *Salicornia* under saline irrigation conditions in different regions of Rajasthan and Gujrat and study its growth and productivity and bioremediation potential. The project aims at the development of the cultivation of *Salicornia* and its possible agro-industrial and agro-energy uses in the Gujrat and Rajasthan. This will contribute to the development of bio-combustibles of third generation like the biodiesel, bioethanol from biomass, and biogas to generate electricity in the zones non-communicated from the wastelands. The use of saltwater represents new alternatives of economic and social development for the uncultivated areas of the coastal region as well as Western Rajasthan. The selection of the definitive seeds as well as its improvement in yield by hectare will be one of the challenges of the project, generating at the end of this replicable economic model in all the regions.

MAPK cascade is an important intracellular signaling module and functions as a convergent point for cross talk during abiotic stress signaling. In this study SbMAPKK gene has been isolated from *Salicornia brachiata*, a highly salt-tolerant plant growing in costal marshes of Gujarat, India (Agarwal et al. 2010). The antioxidative defense mechanism to salinity was assessed by monitoring the activities of

some antioxidative enzymes and levels of antioxidants in an obligate halophyte, *Salicornia brachiata*, subjected to varying levels of NaCl (0, 200, 400, and 600 mM) under hydroponic culture. In the shoots of *S. brachiata*, salt treatment preferentially enhanced the activities of ascorbate peroxidase (APX), guaiacol peroxidase (POX), glutathione reductase (GR), and superoxide dismutase (SOD), whereas it induced the decrease of catalase (CAT) activity (Parida and Jha 2009). To obtain an insight into the comprehensive molecular characteristics of the salt tolerance mechanism, studies have been performed for screening of salt-inducible genes in a halophytic plant, *Salicornia herbacea*, using mRNA differential display. A comparative analysis of gene expression in *Salicornia* grown in control and salt-stressed conditions led to the detection of a gene that was induced by salt (Jha et al. 2011).

13.3.6 Commercial Cultivation in Middle East

Salicornia has been cultivated in large areas in the Middle East using seawater. *Salicornia* is a member of the halophyte family Amaranthaceae, and Halophyte Enterprises Inc. (HEI), based in Phoenix, Arizona, is managing the project for Behar. Previously, *Salicornia* crops had been grown successfully in trial plots in the United Arab Emirates, Egypt, and Kuwait, as well as Jubail.

Behar's optimal cultivation target for Ras al-Zawr is 4500 ha (11,120 acres), consisting of 90 pivot-irrigation circles, each 800 m (half a mile) across. If the plant takes firm root, however, circles of *Salicornia* could one day cover up to 200,000 ha (494,200 acres) along both coasts of Saudi Arabia, providing up to 120 million kilograms (34 million US gallons) of vegetable oil a year, according to a project feasibility study. Biodis (Spain) has also carried out studies on *Salicornia* spp in different parts of the world and author (AK) participated in some of them.

13.3.7 Carbon Credits

It is impossible for individual farmers to generate carbon credits as individuals. They must be part of a complex *regional biofuels program* that includes training in energy agricultural activity related to land improvement, harvest protection, and collection as part of the value chain that delivers biomass for refinery operations required to produce advanced liquid fuels and power.

13.4 Conclusion

Salicornia cultivation in coastal areas of the world in general and Gujrat in particular in integrated farming involving young of shrimps and tilapia and the beginning of mangrove swamp could yield valuable biomass and biofuel. Gujarat owns exceptional conditions of climate, of grounds, and necessary resources for the production of fish by means of aquaculture. Several are the species of fish that can be bred, but

Tilapia nilótica stands out for being one of the best businesses for the region. All these collectively can contribute to the development of bio-combustibles of third generation like the biodiesel, bioethanol from biomass, and biogas to generate electricity.

The *Salicornia* has very high economic value, including 30% of oil, more than it is possible to obtain from soybean seeds. The oil also provides raw material for a series of cosmetic and pharmaceutical products. Therefore, it is possible to be affirmed that the *Salicornia* in culture is better than the soybean with major yields and minor production costs, notwithstanding that it captures CO_2, reducing therefore the contamination, and the power to grow in nonfertile earth apparently has an economic potential and high productivity under adverse conditions. Besides these contents, the plantations of *Salicornia* will also have an ecological impact, since it absorbs carbon dioxide.

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Alternative Biomass from Semiarid and Arid Conditions as a Biofuel Source: *Calotropis procera* and Its Genomic Characterization

14

Ashwani Kumar

Abstract

Rajasthan has the largest area of wasteland in India measuring up to 60% of the total wasteland of India. It also occupies overgrazed pastures, beachfront, roadsides, and denuded areas. *Calotropis* is the first colonizer on the sand dunes and wastelands. *Calotropis* prefers open habitat with little competition. *Calotropis* is distributed in western India and central India mainly in semiarid and arid regions. It contains latex which is 30% hydrocarbons mainly triterpenoids. These hydrocarbons can be converted into biofuels. Thus, this plant can be a potential biofuel source of the semiarid and arid regions of the world.

Keywords

Calotropis procera • *Calotropis gigantea* • Semiarid and arid • Hydrocarbons

14.1 Introduction

Petroleum reserves of the world may not last forever. There is an urgent need to develop alternative and renewable sources of petroleum. Biomass provides environmental-friendly biofuel. Liquid fuels produced from biomass contain no sulfur, thus avoiding SO_2 emissions and also reducing emission of $N0_x$. Petro-crops are renewable sources of petroleum hydrocarbons (Kumar 2013). Several families widely growing in Rajasthan have great potential as renewable source of energy. Euphorbiaceae (*Euphorbia antisyphilitica*, *E. tithymaloides*, *E. caducifolia*, *E. lathyris*, *E. neriifolia*, etc.), Asclepiadaceae (*Calotropis gigantea* and *C. procera*),

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Asteraceae, and Apocynaceae have a large number of valuable plants (Kumar 1995; Kumar and Kumar 2002; Kumar et al. 2002). Calvin (1978) has considered *Euphorbia lathyris* L. to be a kind of "energy farm" capable of producing a mixture of reduced terpenoids which can be converted to a gasoline-like substance.

It has been recorded that some higher plants can convert the initially produced carbohydrate into terpenes instead of fatty acids and glycerides (such as seed oil). *Calotropis procera*, a laticifer, arid plant which is rich in triterpenoid hydrocarbon compounds and sugars, is a potential petro-crop. Biodegradation of latex may afford a milder and less energy-intensive technique of latex degradation (Behera et al. 2000). The biocrude consists of mono-, sesqui-, di-, tri-, and polymeric forms of long-chain aliphatics (waxes, wax esters, triglycerides, and fatty acids) and phenolics (phenols, flavonoids, and polyphenolics) (Calvin 1978; Adams and McChesney 1982). Upgrading of biocrudes from potential laticiferous species belonging to Euphorbiaceae, Asclepiadaceae, Moraceae, and Apocynaceae families was reported by Bhatia et al. (1984) Kumar (2001) and Buchanan et al. (1978).

Biocrude obtained from the latex of the plants is subjected to hydrocracking in the presence of zeolite catalysts which results in the production of low molecular weight compounds (C_{18}) like biodiesel to high molecular weight hydrocarbons like paraffins and waxes (Sharma et al. 1994). The study indicated that these plant species might be suitable as alternative source of hydrocarbons and other phytochemicals (Kalita and Saikia 2004; Kumar 2013).

The possibility of utilizing part of naphtha, one of the products of the conversion process, for the recovery of biocrude, was also explored (Bhatia et al. 1993). Dried biomass of *C. procera* is reported to yield hexane extractables 3.8% of dry matter from stem and 5.1% from leaves. The stem yielded 18.5% of dry matter and leaves 12.2% in the methanol extract (Behera et al. 2000).

14.2 Material and Methods

Calotropis procera was collected in the months of March–May from different localities of Rajasthan as given in Table 14.1. The map of Rajasthan showing rainfall pattern (Figs. 14.1 and 14.2).

Table 14.1 Geographical locations, average annual rainfall and average temperatue of locations from where the samples were collected

	Latitude	Longitude	Average annual rainfall (mm)	Average temperature Min–max (°C)
Barmer	25.45 N	71.20 E	277	5–47
Jaipur	27.00 N	75.50 E	556	9–40
Jaisalmer	26.55 N	70.54 E	150	7–45
Sikar	27.33 N	75.10 E	460	8–41

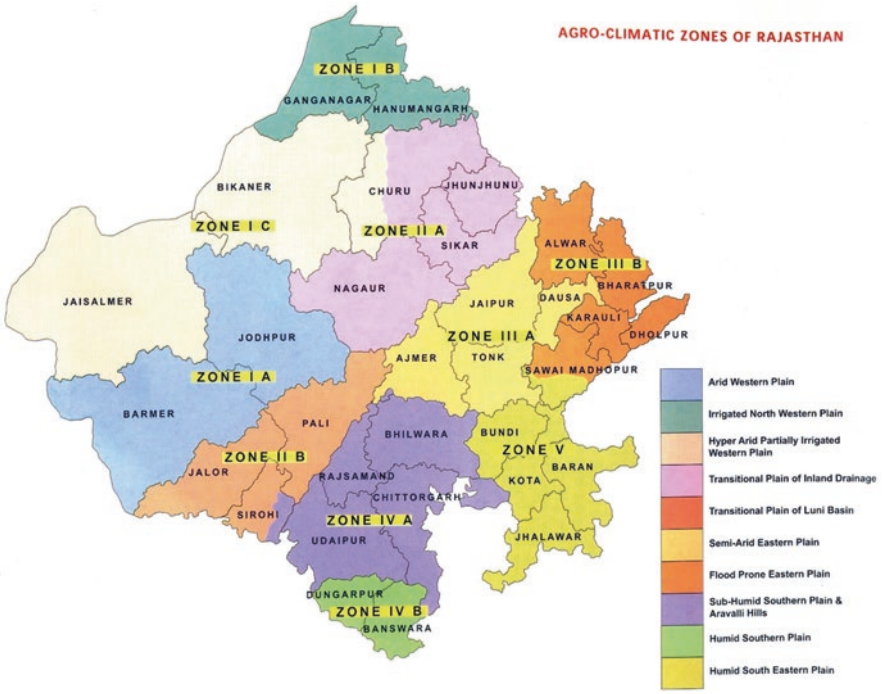

Fig. 14.1 Map of Rajasthan showing different agroclimatic zones

14.2.1 Collection of Plant Material

Plant material collected from Barmer (Fig. 14.3), Jaipur (Fig. 14.4), Jaisalmer (Figs. 14.5 and 14.6), and Sikar (Fig. 14.7) areas was identified with the help of standard local floras (Bhandari 1990) and deposited in Rajasthan University Botany Herbarium. Seeds from these elite plants were collected and grown at the EPDP Centre under uniform climatic and edaphic conditions. *Calotropis gigantea* was collected from Jaipur (Figs. 14.8 and 14.9).

Twelve accessions of *Calotropis procera* were analyzed, and their growth parameters were studied at the Energy Plantation Demonstration Centre, University of Rajasthan, Jaipur, under the Department of Biotechnology project.

The aboveground fresh weight and dry weight were determined from the representative plants. The plant parts were wrapped in blotting paper separately and dried in a SEW oven at 60 °C for 72 h or more till their weights became constant. Details of extraction procedure are given in Rana and Kumar (2012). Four replicates were used for each set of experiment and results are based on mean obtained from these replicates. Experiments were conducted to study the influence of growth regulators and nutrients on hydrocarbon contents. Detailed AFLP analysis was conducted on these high hydrocarbon-yielding plants.

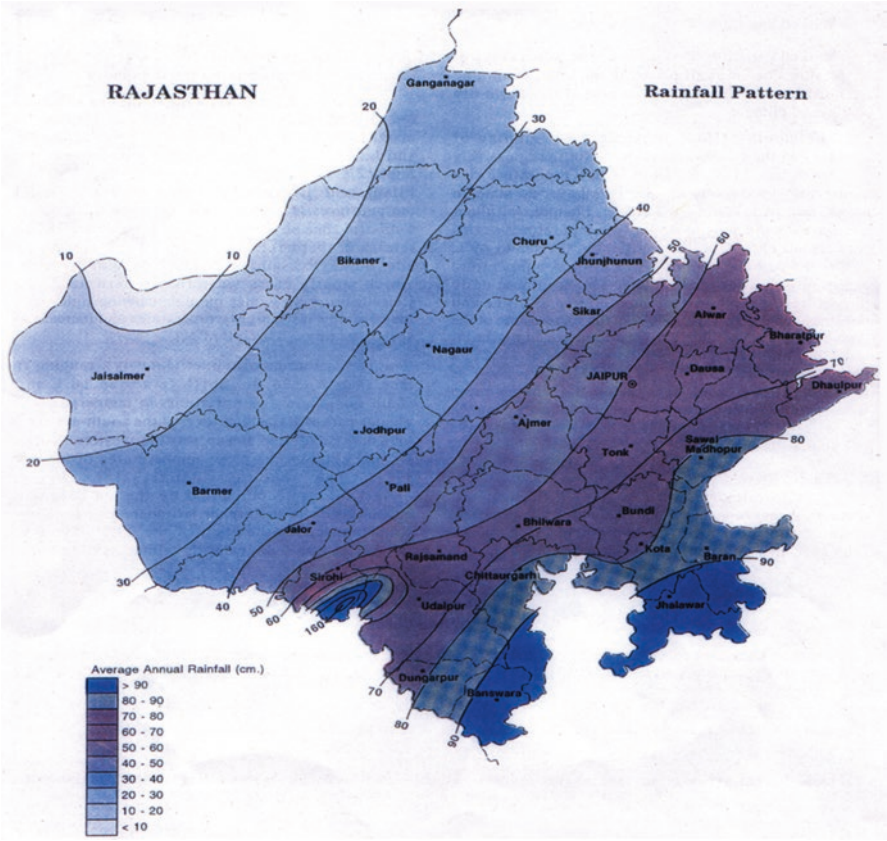

Fig. 14.2 Map of Rajasthan showing rainfall pattern

14.2.2 Extraction

The dried biomass of *C. procera* when subjected to nonpolar (*n*-heptane) solvent extraction yielded biocrude which was rich in source of triterpenoid type of hydrocarbons. The biocrude can be upgraded to useful liquid fuels using different conversion processes such as thermal and catalytic cracking (fluid catalytic cracking, FCC). High conversions (up to 92%) were obtained using FCC as compared to thermal process (57.7%).The fuel obtained by FCC was found to contain large proportions of aromatics and poly-aromatic hydrocarbons (PAH) (Kumari and Kumar 2005; Padmaja et al. 2009).

Fig. 14.3 *Calotropis procera* growing in Barmer, Rajasthan

Fig. 14.4 *Calotropis procera* growing in Jaipur, Rajasthan

Fig. 14.5 *Calotropis procera* from Jaisalmer, Rajasthan

Fig. 14.6 *Calotropis procera* assumes tree habit in arid regions of Jaisalmer, Rajasthan

Fig. 14.7 *Calotropis procera* growing in Banthala, Sikar, Rajasthan

Fig.14.8 *Calotropis gigantea* growing in Jaipur, Rajasthan

Fig. 14.9 *Calotropis gigantea* flowering branches and fruit

14.3 Experimental Area

Rajasthan state is situated between 23°3′ and 30°12′ N latitude and 69°30′ and 78°17′ E longitude. The total land area of the state is about 324,239 km^2, out of which about 198,100 km^2 is arid and the rest semiarid. It has different agroclimatic zones and rainfall zones as given in Figs. 14.1 and 14.2. Out of the total area, forests cover only about 37,638 km^2 (Kumar 2013). Sand dunes occupy a greater part of western Rajasthan (120,983 km^2). The soils of the desert plains are loamy sand to loam. The eastern part of Rajasthan has alluvial soil which supports good forests and agricultural crop. Occurrence of saline soils with pH up to 9.0 is a common feature in the sandy areas of Rajasthan.

Annual precipitation in different regions of Rajasthan varies from 15 to 1766 mm with an average annual rainfall in the state being 525–675 mm (Fig. 14.2). The climate of this state also shows extremes of temperatures. In hot and scorching summers, the mercury rises to as high as 52 °C in Jaisalmer. The minimum temperature is 0 °C in Churu region during winters. Frosts are generally severe during winter, particularly in the sandy areas. Tree saplings up to 2 m in height sometimes succumb to such frosts. The average humidity varies from 85% in July to 25% in April depending on the region.

The vegetation of the region is mostly dry tropical and thorny scrub forest types. Dust storms in the desert tracts may reach a velocity of 130 km/h and may deposit 50–75 mm of dust on the floors. The categorization of wasteland of Rajasthan through aerial estimation provides the following categories: salt-affected land, gullied or ravenous land, undulating upland with or without scrub, sandy area, barren hill ridge, or rock outcrop (Kumar 2013).

14.4 The Plant

14.4.1 Distribution

Calotropis procera (giant milkweed) is a potential plant for bioenergy and biofuel production in semiarid regions of the world (Rana and Kumar 2012). Giant milkweed is native to West Africa as far south as Angola, North and East Africa, Madagascar, the Arabian Peninsula, Southern Asia, and Indochina to Malaysia (Rahman and Wilcock 1991). The species is now naturalized in Australia, many Pacific islands, Mexico, Central and South America, and the Caribbean islands. The native range also covers southwest Asia (India, Pakistan, Afghanistan, Iran, Arabia, and Jordan) and Africa (Somalia, Egypt, Libya, South Algeria, Morocco, Mauritania, and Senegal). *Calotropis procera* is also native in Thailand and Vietnam. It has also been reported from the Caribbean islands and Central and South America and has been introduced to South Africa. It is also naturalized in Pacific islands, Mexico, and Hawaii and commonly harvested for its medicinal properties (Rana and Kumar 2012). *Calotropis procera* is recommended as a host plant for butterflies (Mikula 2001).

Susceptibility of *Calotropis* spp. to pests is high. *Calotropis* is a home to many insects like aphids, grasshoppers, and other insect pests which eat or suck the leaves despite of its contents of toxic latex. The caterpillar of the plain tiger butterfly, *Danaus chrysippus*, also feeds on *C. procera. Calotropis* plant contains cardiac toxins and these are transferred to the tissues of the adult butterfly. These toxins afford excellent protection against attack from vertebrate predators, such as birds and lizards (Neal 1965).

14.4.2 Habit and Habitat

Calotropis procera grows well on arid and semiarid lands which occupy one third of the earth's surface. The Indian arid zone occupies an area of about 0.3 million km^2. Ninety percent of which about 270,000 km^2 is confined to northwest India covering most of western Rajasthan, part of Gujarat, and small portions of Punjab and Haryana. India with its vast expanse of wasteland unsuitable for agricultural production (nearly 180 million ha) could be considered for economically viable production of biofuels. The *Calotropis procera* is widely distributed in western Rajasthan, while *Calotropis gigantea* is found in Jaipur, Bharatpur, Udaipur, Bhilwara, and Banswara division with relatively moderate climatic conditions (Kumar 2001; Kumar and Kumar 2002). The *Calotropis procera* has a bushy appearance and but attains tree forms under extreme conditions of Jaisalmer and Barmer where rainfall is less than 10 mm per annum and extremes of temperature are encountered from 0 to 49 °C (Figs. 14.3, 14.4, 14.5, 14.6 and 14.7). *Calotropis gigantea* has a gregarious habit (Figs. 14.8 and 14.9).

14.4.3 Morphology

Plants shed their leaves in winter and become leafless. Regenration in spring season is through sprouts coming out from the roots. Giant milkweed roots were found to have few branches and reach depths of 1.7–3.0 m in Indian sandy desert soils (Sharma 1968). *C. procera* has broadly ovate leaves while *C. gigantea* has oblong and elognated leaves with acute apex. *C. procera* has purplish white flowers as compared to white flowers of *C. gigantea* (Figs. 14.10, 14.11, 14.12, 14.13). Fruit is a pair of follicles (Figs. 14.14 and 14.15). The follicle dehisces by single suture to produce large number of seeds covered with silk tufts of white hairs (Fig. 14.16).

In Ayurvedic texts, the white-flowered variety called alarka is said to be of superior quality, though all the commentators are of the opinion that either of these can be used with equal effect, as both have the same properties. The white alarka could be presumably *Calotropis gigantea* which has generally white flowers. The description of the other two vetarka and arka probably represents *Calotropis procera* or its subdivisions. However, no subdivisions or subspecies of *Calotropis procera* have

Fig. 14.10 *C. procera* twigs with flowers

a. b.

Fig. 14.11 *C. procera* (**a**) flowers (**b**) a single flower

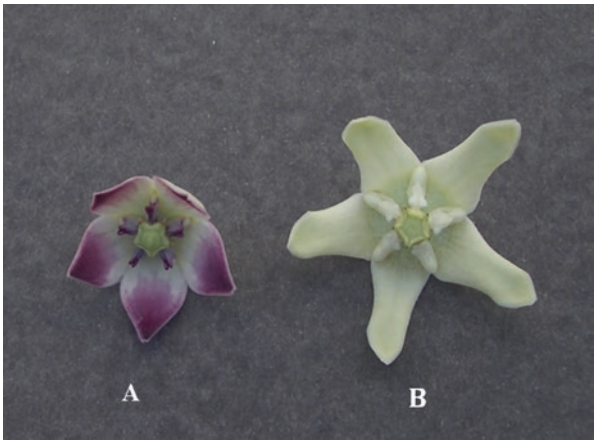

Fig. 14.12 Comparison of flowers (**a**) *C. proecra* (**b**) *C. gigantea*

Fig. 14.13 *Calotropis gigantea* flowers

been recognized in literature. During present investigation, certain differences in AFLP indicated differences in *C. gigantea* and *C. procera* and also among different samples collected from different places. This difference was also reflected in their contents of hexane extractables.

14.4.3.1 Anatomical Studies

Laticifers are some of the most peculiar elements of the plant body which secrete an endogenous milklike fluid in a network of laticifer cells in which subcellular organelles intensively synthesize proteins and secondary metabolites. Young stem showed poorly developed laticifers (Fig. 14.17). However mature stems have well developed laticifers containing latex (Fig. 14.18). The latex largely consists of triterpenoids which can be converted into petroleum products (see also Chap. 12).

Fig. 14.14 Fruiting in *Calotropis procera*

Fig. 14.15 Follicles of *Calotropis gigantea*

Fig. 14.16 An open follicle showing seeds with silk tufts of white hairs

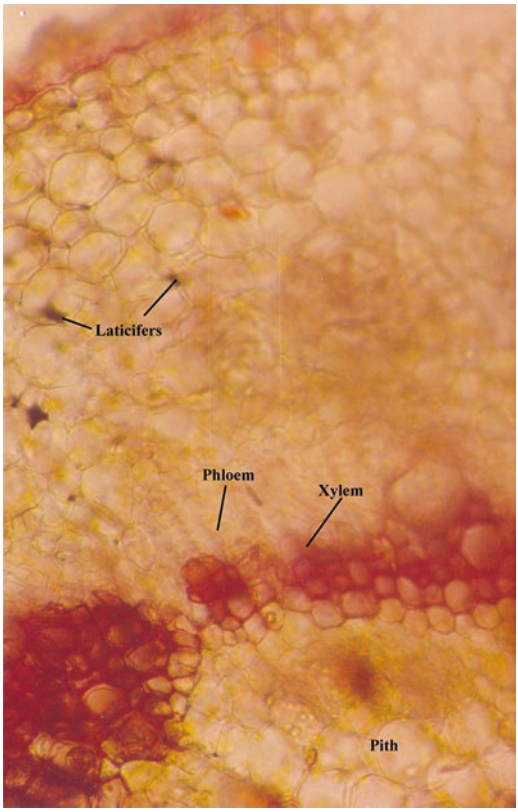

Fig. 14.17 T. S. of young stem of *Calotropis procera*

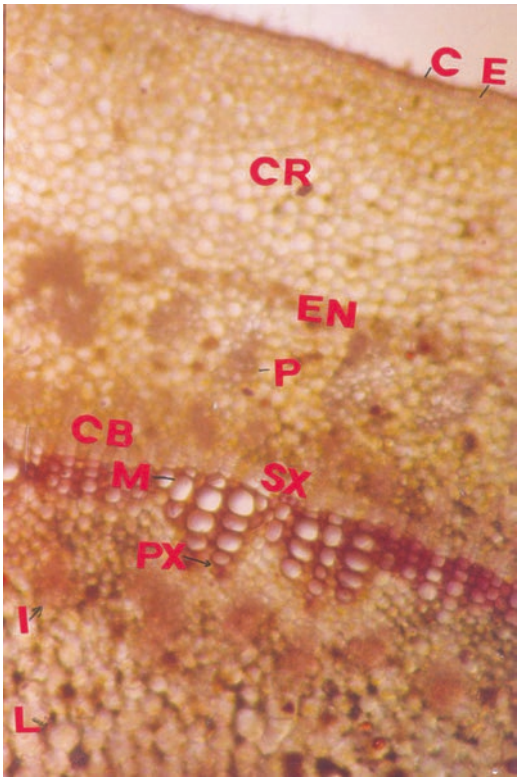

Fig. 14.18 T. S. of mature stem of *Calotropis procera*. *C* cuticle, *E* epidermis, *CR* cortex, *EN* endodermis, *P* pericycle, *CB* cambium, *SX* secondary xylem, *PX* protoxylem, Iintra xylary phloem, *L* latex vessel in pith

14.4.4 Production

Plants can be raised through seedling in polythene bags (Fig. 14.19). These seedlings are later transferred to field conditions. The plants can also be multiplied through cuttings (Fig. 14.20). The cuttings sprout to produce buds and these buds develop into shoots (Figs. 14.21 and 14.22). The roots developed in lower part of the cuttings. The plant has a growth potential of 2 dry tons to 40 dry tons per ha depending on the agroclimatic conditions of its growth. The plant has high level of regeneration potential and could be harvested up to four times a year. The plant yields valuable hydrocarbons which could be converted into diesel substitutes. The biodiesel derived from *Calotropis procera* is free from NO_x gases, SO_2, and suspended particulate matter (SPM) and has high cetane value. Due to its enormous potential for growth under adverse climatic conditions, *Calotropis procera* is suggested as potential plant for biodiesel production under semiarid and arid conditions (Kumar 1994; Kumar et al. 1994).

Fig. 14.19 Seedlings of *Calotropis procera* grew in polythene bags

Calotropis procera growing in Jaisalmer assumes gigantic proportions and people mistakenly identify it as *C. gigantea.* The occurrence of *Calotropis* species is related to the climatic conditions. It is generally not present on good soil except when raised at places of worship. *Calotropis gigantea* is a commonly worshiped plant and is found in Shiv temples. However, *C. procera* and *Calotropis gigantea* were studied on the basis of sample collections. The growth and productivity were determined.

14.5 Results and Discussion

Calotropis procera has shown great potential for biofuel production under semiarid conditions for production of biodiesel. Its genome characterization has revealed clear-cut differences in the *Calotropis gigantea* and *Calotropis procera*, but to establish differences within *Calotropis procera*, more studies are needed. A correlation between yield potential and plant characteristics has been carried out.

14.5.1 Studies on Seed Germination and Propagation of *Calotropis procera*

Germination studies have been carried out on seeds collected from Jaisalmer, Jaipur, Banthala, Nagaur, and Dausa. One hundred seed weight varied significantly in seeds collected from different places. Maximum 100 seed weight (0.84 g) was recorded for seeds collected from Jaisalmer which was followed by Nagaur (0.51 g); Lalsot, Dausa (0.450 g); Jaipur (0.49 g); and Banthala (0.49 g). Considerable variations were observed in percentage germination of seeds from different locations viz.: Jaisalmer (98%), Nagaur (96%), Jaipur (94%), Banthala (88%), and Lalsot (86%). Seeds were germinated in polythene (Fig. 14.19) and later transferred to the field. *C. procera* was also raised through stem cuttings which sprouted to produce buds and young shoots (Figs. 14.20, 14.21 and 14.22).

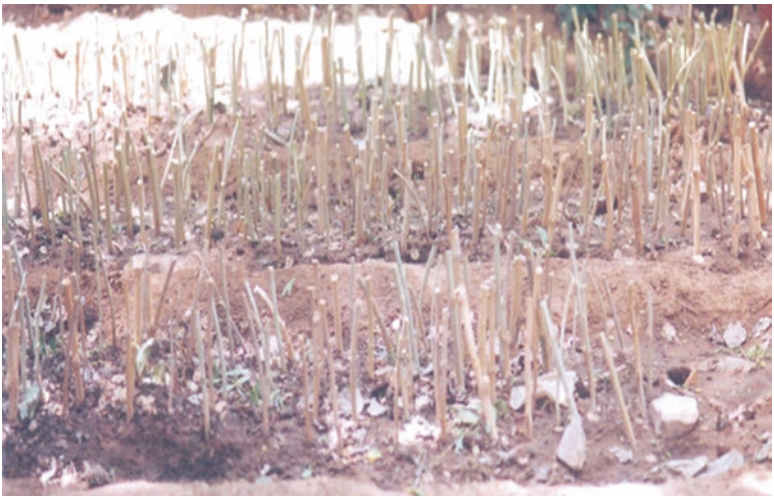

Fig. 14.20 *Calotropis procera* growing from stem cuttings

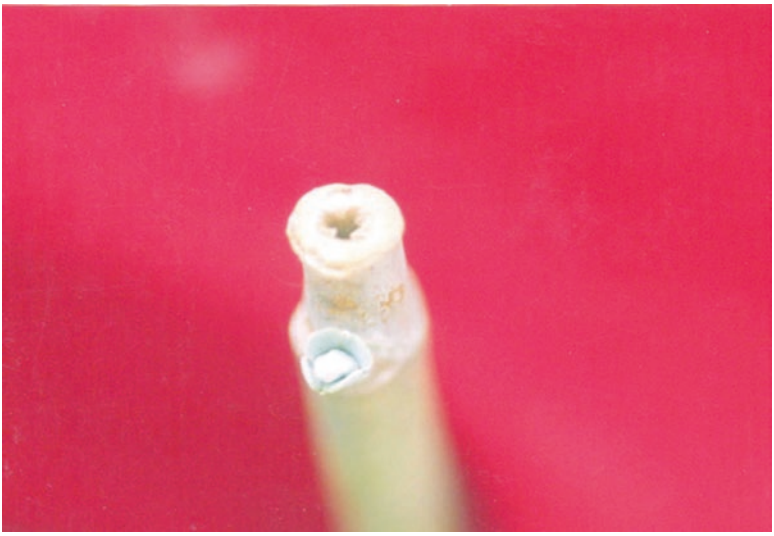

Fig. 14.21 Bud development on cutting on *C. procera*

14.5.2 Some Important Agronomic Aspects for *Calotropis procera* Cultivation

Although *Calotropis procera* can be germinated throughout the year, the suitable time for the sowing of *Calotropis procera* is in May–June period. *Calotropis* does not require deep tillage. Plants may be thinned to have suitable number of plant for per unit area for better plant growth.

Fig. 14.22 Bud proliferation on cutting of *C. procera*

Table 14.2 % HE extractable in plants of different regions

S. No.	Location/district	% HE/g DM
1.	Jaipur (Bassi)-J-1	6.87
2.	Jaipur (Sambhar)-J-2	8.81
3.	Jaipur (Chomu)-J-3	8.33
4.	Jaipur (Shivdaspura)-J-4	9.8
5.	Jaisalmer 1-J-11	6.2
6.	Jaisalmer 2-J-12	10.7
7.	Jaisalmer 3-J-13	8.08
8.	Jaisalmer 4-J-14	7.11
9.	Barmer (Gageria)-B-1	9.0
10.	Barmer (Gadra)-B-2	10.9
11.	Barmer (Chautan)-B-3	6.1
12.	Barmer-B-4	8.7
13.	Sikar (KhatuShyam)-S-1	7.90
14.	Sikar (Rengus)-S-2	8.01
15.	Sikar (Bai)-S-3	7.63
16	Sikar (Banthala)-S-4	11.01

14.6 Hexane Extractable

During present investigations, certain differences in hexane extractable in *C. gigantea* and *C. procera* and also among different samples collected from different places were recorded (Table 14.2). These differences were also reflected in their AFLP profiles.

14.7 Genomic Characterization

The genomic characterization of *Calotropis* spp. from different parts of Rajasthan was carried out using AFLF (amplified fragment length polymorphism) (Figs. 14.23 and 14.24). *Calotropis gigantea* has generally white flowers, while *Calotropis procera* or its subdivisions have purplish white flowers. However, no subdivisions or subspecies of *Calotropis procera* have been recognized in literature. During present investigations, certain differences in AFLP indicated differences in *C. gigantea* and *C. procera* and also among different samples collected from different places. This difference was also reflected in their contents of hexane extractables.

Molecular approach has proved increasingly valuable in the identification of plant varieties (Ruan and Diaqiong 2005). Different types of markers have been used to assess phylogenetic relationships and diversity in different plant species,

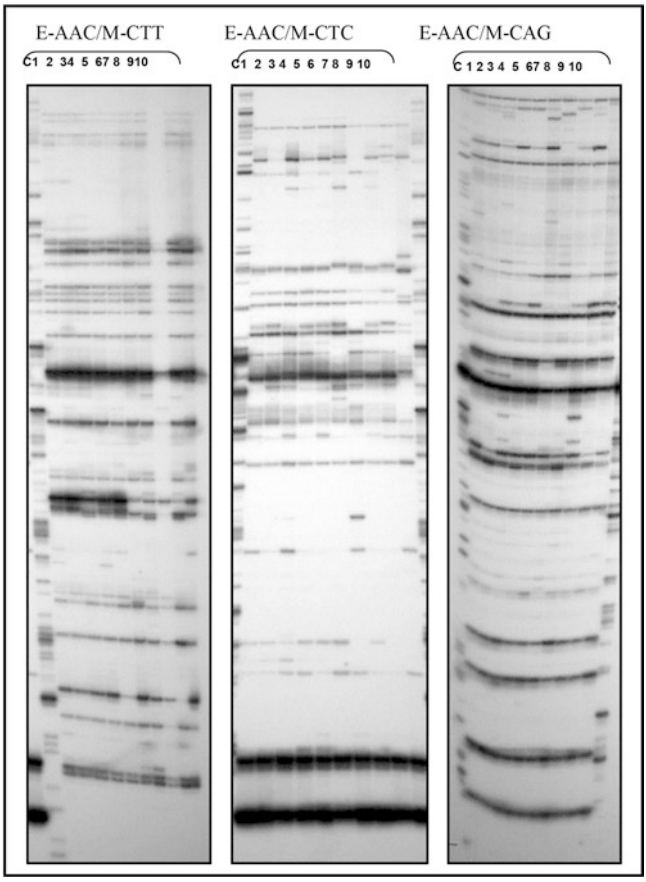

Fig. 14.23 AFLP profiles of *Calotropis* genotypes with three primer pairs. Lane C is control lane. Lanes 1 to 9 are *Calotropis procera* genotypes and lane 10 is *Calotropis gigantea*

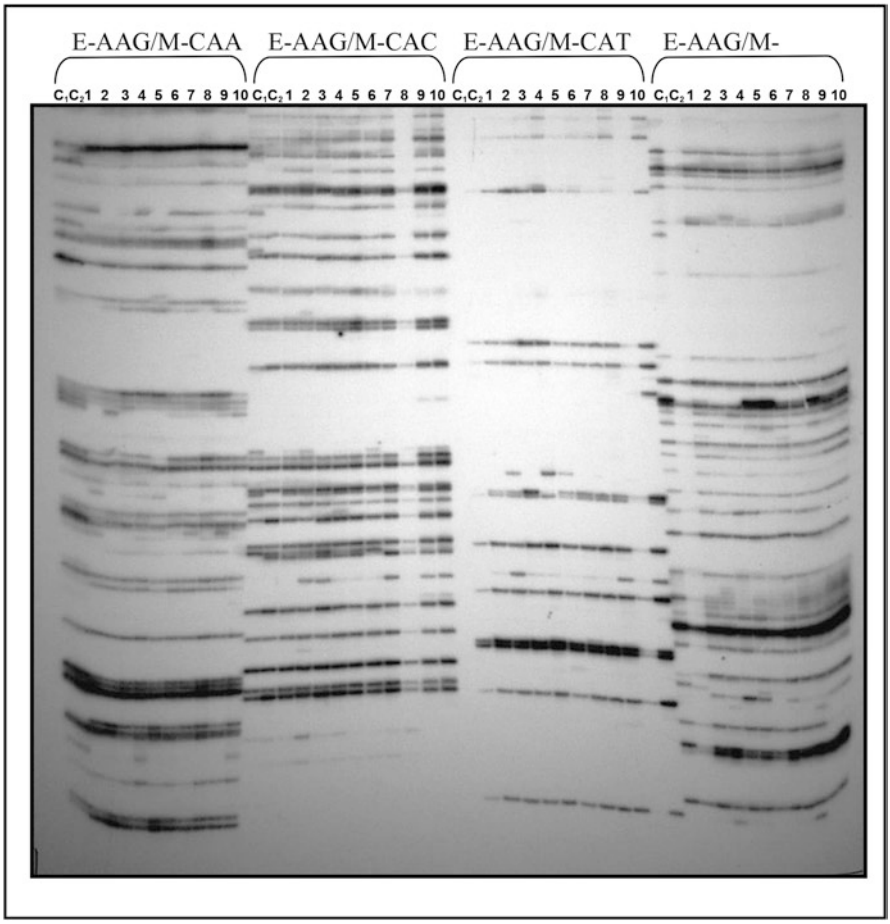

Fig. 14.24 AFLP profiles of *Calotropis* genotypes with four primer pairs. Lanes C1 and C2 are control lanes. Lanes 1 to 9 are *Calotropis procera* genotypes and lane 10 is *Calotropis gigantea*

viz., initially isozyme, later molecular markers such as randomly amplified polymorphic DNA (RAPD), and recently chloroplast DNA and internal transcribed spacer (ITS) sequences. Both RAPD and AFLP markers are useful for studying relationships among DNA sequences, independently of their location in the chromosomes or of particularities in their nucleotide sequence (Cabrita et al. 2001; Ruan and Diaqiong 2005).

However, the higher multiplex ratio of AFLP markers makes them more suitable for distinguishing between closely related genotypes, such as different clones within a given cultivar which often, having diverged by small mutational events, present minimal genetic differences (Cabrita et al. 2001).

During present analysis, genetic relationships in five populations were studied. The preliminary results were confirmed by repeating the experiments. The present

report suggests usefulness of AFLP markers for analyzing DNA fingerprint patterns and genetic relationships among five populations collected from different parts of Rajasthan, and *C. gigantea* was used as outliner. The data provides scientific basis for identification of improved germplasm, and further characterization could lead to identification of certain genes or expressions of gene combinations which could be the source of drought and salinity tolerance. These could be utilized for further genetic manipulations in plant species or producing specific polymers or plant proteins.

An extensive survey was conducted to select the candidate plant (CP) of *Calotropis procera* from different districts of Rajasthan and *Calotropis gigantea* obtained locally. The selection was made on phenotypic assessment of characters of economic interest, i.e., plant height, stem girth, crown spread, and total biomass.

14.7.1 AFLP Analysis of *Calotropis procera* to Assess Genetic Diversity

The genus *Calotropis* includes two species and no subspecies have been reported in the world. These species are all diploid (2n = 22). *Calotropis* spp. are worldwide in distribution and are reported from different tropical regions like in Caribbean, Africa, Middle East, and Southeast Asia. They are very hardy plants and are able to grow under extremes of environmental conditions. Both the species *Calotropis procera* and *C. gigantea* have great ethnobotanical, ecological, medicinal, and ornamental value. Commercially, *Calotropis* species have been suggested to have valuable hydrocarbons which could be converted into petroleum-like substances. *Calotropis* is a cross-pollinating plant and propagates through seeds. The size of plants, stem girth, leaf size, growth pattern, flower colors, and flower size are important morphological parameters for determining plant species. During previous investigations, considerable variations were recorded in *Calotropis* spp. in morphological and physiological characteristics.

Some high-yielding populations were recorded from different regions of Rajasthan, and investigations were carried out on their morphological and genetic characterizations. Some of these high-yielding plant populations were multiplied at the Energy Plantation Demonstration Project Centre, University of Rajasthan, Jaipur. However, investigators often look only at morphological characters, which do not always yield clear answers concerning identification of any plant varieties. Molecular approach has proved increasingly valuable in the identification of plant varieties (Ruan and Diaqiong 2005).

14.8 Materials and Methods

An extensive survey was conducted to select the candidate plant (CP) of *Calotropis procera* from different districts of Rajasthan and *Calotropis gigantea* obtained locally. The selection was made on phenotypic assessment of characters of

Table 14.3 Plant materials used in the first experiment

S No	Samples	Seed parent
1.	AK1-1	AK1-Jaisalmer-CP1
2.	AK1-2	
3.	AK2-1	AK2-Barmer-CP2
4.	AK2-2	
5.	AK3-1	AK3-Banthala-CP3
6.	AK3-2	
7.	AK4-1	AK4-Jaipur-CP4
8.	AK4-2	
9.	AK-Jaisalmer	AK- Jaisalmer
10.	*Calotropis gigantea* (Jaipur)	*Calotropis gigantea* (Jaipur)

economic interest i.e., plant height, stem girth, crown spread, and total biomass. Seeds from these elite plants were collected and grown at the EPDP Centre under uniform climatic and edaphic conditions. Total genomic DNA was isolated from fresh leaf tissue of 2-year- and 6-month-old sampling plants (Table 14.3).

Two samples each of AK1-Jaisalmer-CP1, AK2-Barmer-CP2, AK3-Banthala-CP3, and AK4-Jaipur-CP4 were obtained from the plants raised from the seeds collected from these regions at the EPDP Centre. AK-Jaisalmer was collected from Jaisalmer directly from the parental elite plant. *Calotropis gigantea* was collected from the EPDP Centre and used as outliner.

14.8.1 DNA Extraction

CTAB method of Doyle and Doyle (1990) was used to isolate total genomic DNA which is a standard protocol for obtaining highly pure DNA:

1. 0.1 gm of lyophilized plant material was ground to fine powder in mortar and pestle using quartz sand.
2. The powder was transferred immediately into 5 ml of pre-warmed (65 °C) isolation buffer, incubated for 30 min at 65 °C in a water bath, and mixed gently every 10 min.
3. Equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed. The slurry was centrifuged at 10,000 rpm at 24 °C for 10 min. Aqueous phase was transferred to fresh tubes.
4. 20 µl of RNaseA (10 mg/ml) was added and incubated for 30 min at 37 °C.
5. Again equal volume of chloroform-isoamyl alcohol was added, mixed gently, and centrifuged at 10,000 rpm at 24 °C for 20 min. Upper aqueous phase was transferred to glass centrifuge tubes using large bore pipette.
6. 0.6 volume of ice cold isopropanol was added and mixed gently but thoroughly by inverting the tubes several times. At this stage, the DNA-CTAB complex was precipitated out and then centrifuged at 6000 rpm at 4°C for 10 min to pellet out the DNA.

7. The pellet was washed with washing solution (70% ethanol containing 10 mM ammonium acetate) by gently agitating and collected by centrifugation (10 min, 5000 rpm, 4 °C). The pellet was dried, and 500 µl of M. Q. water was added to the DNA to dissolve overnight.

14.8.2 Purification of DNA

1. Phenol purification was used to purify all the crude DNA samples.
2. Equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to 500 µl of crude DNA prep.
3. The slurry was mixed gently with inversion and centrifuged for 15 min in micro-centrifuge. The aqueous upper layer was transferred to new Eppendorf tube using a blunt/cut tip.
4. Equal volume of chloroform-isoamyl alcohol was added to the aqueous layer. It was mixed gently and centrifuged for 15 min.
5. Aqueous upper layer was taken out, and one tenth volume of 3M sodium acetate (pH 4.8) was added. It was mixed by inversion and incubated at 4 °C for 30 min. A 2.5 volume of absolute alcohol was added, mixed by inversion, and incubated for half an hour at -20 °C. DNA was pelleted out by centrifugation.
6. The DNA pellets were washed with 70% alcohol for a few minutes. The alcohol was drained out on a paper towel, and DNA was kept for drying at 37 °C.
7. To the DNA, 200 µl of M.Q. water was added after proper drying and left overnight at room temperature to dissolve.
8. All purified DNA were quantified on agarose gel by comparing with uncut lambda DNA of known concentration.

14.8.3 AFLP Assay

The AFLP analysis was based on the protocols and kits of Life Technologies, CAT Nos. 10544-013, 10483-014 (GIBCO BRL), which follow the principle as developed by Zabeau and Vos (1993). In brief, the protocol had the following steps:

1. *Restriction of genomic DNA:* Genomic DNA (300 ng) was restricted simultaneously with *Eco* RI and *Mse* I (1.25 U/µl each) in a total reaction volume of 25 µl.
2. *Adapter ligation*: The restricted fragments were ligated to *Eco* RI and *Mse* I adapters.
3. *Preamplification*: The ligated products were subsequently amplified using primers complementary to the adapter sequences and the restriction sites. The following cycling parameters were used for preamplification:

$$90^{o}\,\text{C for } 30\,\text{sec}$$
$$56^{o}\,\text{C for } 60\,\text{sec}) \text{ for } 20\,\text{cycles}$$
$$72^{o}\,\text{C for } 60\,\text{sec}$$

An aliquot of the preamplified product was checked on the gel and smears were observed.

4. *Primer labeling:* Only the *Eco* RI primer was end-labeled using γ- ^{32}P ATP and T_4 polynucleotide kinase.
5. *Selective amplification:* A diluted aliquot of the preamplified mix was used as a template for selective amplification. The primers used are *Eco* RI + 3 and *Mse* I + 3 primers. The following cycling parameters were used:

$$94^{o}\,C \text{ for } 30\,sec$$
$$65^{o}\,C \text{ for } 30\,sec) 1\,cycle$$
$$72^{o}\,C \text{ for } 60\,sec$$

This cycle was followed by 12 cycles in which the annealing temperature was lowered by 0.7 °C per cycle. Following this, 23 more cycles were performed with the following profile:

$$94^{o}\,C \text{ for } 30\,sec$$
$$56^{o}\,C \text{ for } 30\,sec) \text{ for } 23\,cycles$$
$$72^{o}\,C \text{ for } 60\,sec$$

After completion of PCR, equal volume of 98% formamide dye was added, and the samples were then loaded onto 6% polyacrylamide gel. After electrophoresis, the gel was transferred onto Whatman 3, dried, and exposed to an X-ray film for 16 h. The film was developed to obtain the autoradiogram.

14.8.4 Primer Sequences

The sequences of the primers provided by GIBCO BRL are given in Table 14.4. The preamplification primers have one selective nucleotide, whereas the selective amplification primers have three selective nucleotides. There are eight *Eco* RI primers and eight *Mse* I primers for selective amplification. A total of 64 primer combinations are possible using these 16 primers (Table 14.4).

14.8.5 Data Analysis

The amplified fragments (bands) generated using 11 primer combinations with the ten samples were scored manually for their presence (denoted as 1′) and absence (denoted as 0′) (Figs. 14.23 and 14.24). The genetic similarity (GS) values between pairs of samples were estimated according to Jaccard's similarity coefficient ($Gs_{ij} = a/a + b + c$), where Gs_{ij} is the measure of genetic similarity between individuals i and j, a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. The matrix was used to construct a phenetic dendrogram using the UPGMA (unweighted pair-group method of arithmetic averages; Sneath and Sokal

Table 14.4 Sequence of the primers employed for the AFLP analysis

	Code	Sequence
Preamp primers		
Eco RI+1	E-A	5′-GAC TGC GTA CCA ATT CA-3′
Mse I+1	M-C	5′-GAT GAG TCC TGA GTA AC-3′
Selective primers		
Eco RI+3 –AAC	E-AAC	5′-GAC TGC GTA CCA ATT C **AAC**-3′
Eco RI+3 –AAG	E-AAG	5′-GAC TGC GTA CCA ATT C **AAG**-3′
Eco RI+3 –ACC	E-ACC	5′-GAC TGC GTA CCA ATT C **ACC-**3′
Eco RI+3 –ACG	E-ACG	5′-GAC TGC GTA CCA ATT C **ACG**-3′
Eco RI+3 –AAG	E-AAG	5′-GAC TGC GTA CCA ATT C **AAG**-3′
Mse I+3 –CAA	M-CAA	5′-GAT GAG TCC TGA GTA A **CAA**-3′
Mse I+3 –CAC	M-CAC	5′-GAT GAG TCC TGA GTA A **CAC**-3′
Mse I+3 –CAG	M-CAG	5′-GAT GAG TCC TGA GTA A **CAG**-3′
Mse I+3 –CAT	M-CAT	5′-GAT GAG TCC TGA GTA A **CAT**-3′
Mse I+3 –CTA	M-CTA	5′-GAT GAG TCC TGA GTA A **CTA**-3′
Mse I+3 –CTC	M-CTC	5′-GAT GAG TCC TGA GTA A **CTC**-3′
Mse I+3 –CTG	M-CTG	5′-GAT GAG TCC TGA GTA A **CTG**-3′
Mse I+3 –CTT	M-CTT	5′-GAT GAG TCC TGA GTA A **CTT**-3′

1973) in order to cluster the samples. The statistical analysis was performed using NTSYS-pc software (version 2.02, Rohlf 1998).

14.8.6 Results and Analysis

AFLP analysis was performed to obtain fingerprint profiles of nine samples of *Calotropis procera* and one accession of *Calotropis gigantea*. Eleven *Eco* RI/*Mse* I primer combinations were used (Table 14.5) each containing three selective nucleotides. The 11 AFLP primer combinations identified a total of 288 bands, of which 103 were polymorphic (36% polymorphism). The percentage of polymorphic bands with individual combination varied from 21% (E-AAG/M-CTT) to 68% (E-AAC X M-CTC). The size of the fragments ranged from 50 to 350 bp. AFLP profiles generated with seven primer combinations are shown in Figs 14.23 and 14.24. Twenty-two rare bands (which were either present or absent in one genotype) were also obtained. Details of the bands obtained along with the primer combinations used are shown in Table 14.5.

Similarity matrix based on Jaccard's coefficient was computed (Table 14.6), and cluster analysis was performed using the UPGMA algorithm in order to obtain a phenetic dendrogram. The lowest genetic similarity obtained was 76% between samples AK1-1 and *C. gigantea*, while the highest genetic similarity was 93% between AK2-2 and AK3-1 (Fig. 14.25). The genetic diversity thus obtained is very less considering the fact that an entirely different species, *C. gigantea*, was included in the analysis. Moreover, the samples were collected from widely spaced locations. However, most of the genetic diversity obtained was *within C. procera* species.

Table 14.5 Monomorphic, polymorphic, and total number of bands detected by 11 AFLP primer combinations among the *Calotropis* samples

Sl no.	Primer combination	Total nos. of bands	Monomorphic bands	Polymorphic bands	% polymorphism	Rare present/ absent bands
1	E-AAG X M-CAA	36	26	10	27.78	1
2	E-AAG X M-CAC	34	26	8	23.53	1
3	E-AAG X M-CAT	15	11	4	26.67	1
4	E-AAG X M-CTT	34	27	7	20.59	1
5	E-ACG X M-CAA	14	6	8	57.14	4
6	E-ACG X M-CAA	23	16	7	30.43	2
7	E-ACG X M-CAT	15	9	6	40.00	3
8	E-ACG X M-CTG	13	10	3	23.08	3
9	E-AAC X M-CTG	32	20	12	37.50	1
10	E-AAC X M-CTC	34	11	23	67.65	3
11	E-AAC X M-CTT	38	23	15	39.47	2
	Total	288		103		22

Table 14.6 Similarity index between the pairs of genotypes of *Calotropis procera* and *Calotropis gigantea* based on Jaccard's coefficient

	AK1-1	AK1-2	AK2-1	AK2-2	AK3-1	AK3-2	AK4-1	AK4-2	AK-J.	*C.gig.*
AK1-1	1.0000									
AK1-2	0.8810	1.0000								
AK2-1	0.8627	0.8784	1.0000							
AK2-2	0.8964	0.9048	0.8789	1.0000						
AK3-1	0.8770	0.8854	0.8893	0.9315	1.0000					
AK3-2	0.8438	0.8521	0.8206	0.8527	0.8199	1.0000				
AK4-1	0.8465	0.8405	0.8810	0.8411	0.8510	0.8543	1.0000			
AK4-2	0.8810	0.8819	0.8711	0.8898	0.8780	0.8378	0.8622	1.0000		
AK-J	0.8108	0.8333	0.8092	0.8269	0.8295	0.8765	0.8720	0.8405	1.0000	
C.gig.	0.7672	0.7757	0.7863	0.8038	0.7786	0.7816	0.7977	0.7893	0.8189	1.0000

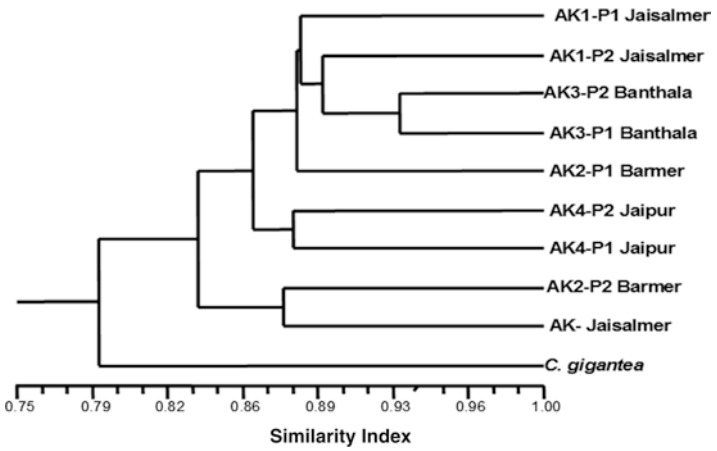

Fig. 14.25 Phenetic dendrogram of different *Calotropis* spp.

14.9 Conclusion

The use of biomass for energy and industry allows a significant quantity of hydrocarbons to be consumed without increasing the CO_2 content of the atmosphere. *Calotropis* is distributed in western India and central India mainly in semiarid and arid regions. It contains latex which is 30% hydrocarbons mainly triterpenoids. These hydrocarbons can be converted into biofuels. Its cultivation in these semiarid and arid regions will create new employment opportunities within the community and particularly in rural areas and will promote the use of biomass for energy, industry, and environment. Improved agronomic practices and well-managed biomass plantations of hydrocarbon-yielding plants in adverse climatic conditions will provide a basis for environmental improvement by helping to stabilize certain soils, avoiding desertification which is already occurring rapidly in tropical countries.

During present investigation, studies were conducted on characterization of bioenergy resources in the semiarid region of Rajasthan. The present investigations carried out with an object of biomass production and utilization in less fertile areas will provide satisfactory answers to the double challenge of energy crisis and forced deforestation in the country and semiarid and arid regions of Rajasthan.

The overall diversity observed among the samples of the present study was very less. This may be due to very narrow genetic base of the species, which may be related to its biology (prevalent self-pollination). However, as the number of samples used in this study was very small, further studies with more samples may be carried out to get more reliable results.

The plants from Jaisalmer region morphologically resembled in several characteristics to the *Calotropis gigantea* like plant height, stem girth, branching pattern, leaf diameter, leaf length, flowering, and fruiting. The seeds from these plants were of large size, and 100 seed weight was much greater. However, they showed maximum genetic diversity indicating that these are two distinct species.

The seeds were sown in the Energy Plantation Demonstration Project Centre, University of Rajasthan, Jaipur. The seeds germinated and produced plants which were very tall and robust. Thus there could still be some cultivars or even subspecies within *Calotropis procera* with higher yield potential but further characterization is required to establish this.

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Potential of Lignocellulosic Materials for Production of Ethanol

15

Ashwani Kumar and Nidhi Gupta

Abstract

Increasing greenhouse gases, mainly due to anthropogenic causes, are the major cause of climate change. The need for renewable fuels is becoming paramount largely because of environmental reasons. Presently ethanol and biodiesel are predominantly produced from corn kernels, sugarcane, or soybean oil which might create food vs fuel competition and destabilize land use pattern for agriculture. Recently cellulosic biofuels and algal biodiesels are prominent biological approaches to sequester and convert CO_2. In order to avoid this biofuel feedstock, lignocelluloses—the most abundant biological material on earth—are being explored. Lignocelluloses are omnipresent—wheat straw, corn husks, prairie grass, discarded rice hulls, or trees. The race is on to optimize the technology that can produce biofuels from lignocellulose sources more efficiently—and biotech companies are in the run. There is a campaign which advocates that 25% of US energy come from arable land by 2025. Present review attempts in presenting state-of-the-art report on biofuel production from lignocellulosic materials.

Keywords

Lignocellulose • Ethanol • Cellulose • Green house gases

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15.1 Introduction

Fossil fuels account for about 88% of the global primary energy consumption (Brennan and Owende 2010). Depletion of fossil fuels and increase in environmental concerns lead to the search for alternative fuels. A 2007 UN report estimated that these biofuels would be commercialized by 2015 and become competitive with petroleum-based fuels in the next 10–15 years. The International Energy Agency (IEA), based in Paris, laid out a "roadmap" to ramp-up the use of biofuels from around 2% of global transport fuel today to 27% by the year 2050. Biofuels are fuels made from recently living organisms.

Considerable work has been done on sustainable biofuels during recent years and development of sustainable energy systems from renewable biomass feedstocks is now a global effort (Hill et al. 2006; Kumar 2001, 2007, 2008, 2011, 2014; Kerr 2007; Nigam and Singh 2011; John 2013). Fuel production from renewable sources is becoming crucial due to the increased demand on crude oil, depletion of fossil fuel reservoirs, and the need to reduce greenhouse gas emissions (Travaini et al. 2016). Biomass is the only suitable and renewable primary energy resource than can provide alternative transportation fuels such as bioethanol or biodiesel in the short term for the development of biorefineries concerning environmental sustainability, energy, and economy (Alvira et al. 2010; Aditiya et al. 2016). Potential of a number of feedstocks in two categories—lignocelluloses and lipids—has been examined. Lignocelluloses include forest-based plants; agricultural waste and food wastes like corn stover, wheat straw, rice straw, and sugarcane bagasse; agricultural by-products; and purpose-grown energy crops, such as switch grass. Utilization of nonfood lignocelluloses therefore presents a necessary direction for large-scale biofuel production. Lipids can be produced from algae and other microbes, as well as extracted from nonedible oilseed-bearing plants, such as *Camelina* and *Jatropha* (Maitan-Alfenas et al. 2015). As for biomass lipids, biodiesel has yet to significantly substitute for petroleum-based diesel fuel due to the limited availability of oil plant feedstocks (Atsumi et al. 2009).

Cultivating biofuel crops, trees, and grasses for biofuels may compete with agriculture especially in developing countries, there can be unfavorable ramifications of the "food versus fuel" use of plant products (Negash 2012; Shaik and Kumar 2014; Boakye-Boaten et al. 2016). Lignocellulosic biomass offers great environmental benefits without threatening food supplies and biodiversity (Demain et al. 2005: Zhang et al. 2012). Among potential biorefinery products, bioethanol from nonfood biomass could be used as a renewable fuel in the automotive sector (Tan and Lee 2016). There is currently a great deal of research being carried out to convert lignocellulosic biomass to fuels (Chen et al. 2016) and chemicals (Shen et al. 2015).

The first-generation (1G) fuel ethanol production refers to the hexose sugars released from, for instance, corn starch (Fig. 15.1) or sugarcane-derived sucrose and their direct fermentation to ethanol by yeast. Liquid biofuels, bioethanol in particular, are currently produced from the freely accessible sucrose in sugarcane and from starch in maize grain in Brazil and the USA, respectively. Approximately 60% of the world's ethanol is produced in the USA (Renewable Fuels Association

Fig. 15.1 Corn was the major source of starch biofuel in the USA. E85 sold at the gas station in the USA

Fig. 15.2 Soy biodiesel bus being run in Illinois

2015) and 90% of US biorefineries use corn as a feedstock (Ethanol Producer Magazine 2015). Biodiesel is obtained from soybean oil in the USA. E85 is 85 percent Ethanol mix with gasoline obtained from "Homegrown corn" and is sold at selected gas stations in USA (Fig. 15.1). Soy biodiesel produced from Soya oil is also used in public transport in Illinois, USA (Fig. 15.2).

However, recent trends suggest moving away from first-generation biofuels derived from food crops such as corn, sugarcane, and oilseed, and toward the next generation, made from more plentiful lignocellulosic feedstocks such as corn stover, grasses, and wood chips (Waltz 2010). Second-generation biofuels are made from nonedible plant materials. Third-generation biofuels are made from algae and other microbes. Second- and third-generation biofuels, which we refer to as "advanced biofuels." could play an important role in diversifying the world's energy supplies and curbing greenhouse gas emissions.

Sugarcane molasses or starch-based materials, corn seeds provide first-generation feedstocks for production of ethanol. Currently, Brazil and the USA are the two countries that produce large quantities of fuel ethanol from sugarcane and maize, respectively, and they together account for about 70% of the world's bioethanol production (Balat et al. 2008). According to Carr and Hettenhaus (2009), in the USA, ethanol production, primarily from corn grain, has more than tripled since 2000. Annual US production of ethanol is expected to exceed 7 billion gallons in 2008, displacing nearly 5% of the projected 145 billion gallons of US gasoline demand. Sales of biobased plastics are also expanding. Ninety billion liters of fuel ethanol is currently produced worldwide (Renewable Fuels Association; URL: www.ethanolrfa.org) using almost exclusively starch or sucrose-containing feedstocks (see review Gombert and van Maris 2015).

15.1.1 Lignocellulosic Biomass

Lignocellulose is the most abundant renewable biomass; its annual production has been estimated in 1×10^{10} MT worldwide (Sánchez and Cardona 2008). Annual production of lignocellulosic biomass is between 10 and 50 billion tons (Sánchez and Cardona 2008). A large number of pretreatment approaches have been investigated on a wide variety of feedstock types, and there are several recent review articles which provide a general overview of the field (Hendriks and Zeeman 2009; Roy and Kumar 2013; Cabrera et al. 2015, Saini et al. 2015). Lignocellulosic biomass is composed of cellulose (38–50%), hemicelluloses (23–32%), and lignin (15–25%) and also water, soil, salts, extractives, and other materials (McKendry 2002) (Fig. 15.3). The polysaccharides in lignocellulosic biomass are not readily enzyme accessible mainly because of the presence of lignin. To improve the accessibility of the polysaccharides for enzymatic digestion, the biomass is pretreated, typically

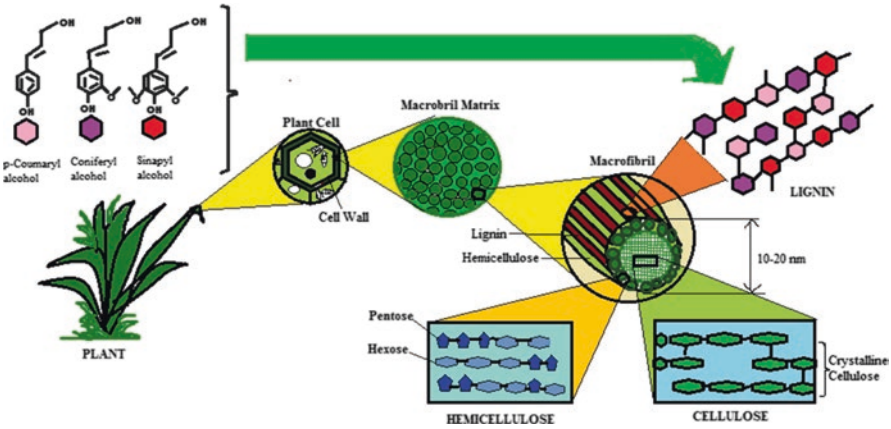

Fig. 15.3 Composition of lignocellulose (Source: Roy and Kumar 2013)

with acid or alkali, to disrupt the bonds between lignin and hemicelluloses or to break down and/or remove the lignin itself (Vanholme et al. 2013).

15.1.2 Cellulose

The cellulose and noncellulosic polysaccharides of plant cell walls represent the largest source of renewable carbohydrate on Earth (Pauly and Keegstra 2008). So far, cellulose is the only polysaccharide that has been used for commercial cellulosic ethanol production, probably because it is the only one for which there are commercially available deconstructing enzyme mixtures. However, the key difficulty of industrial production of cellulosic bioethanol is in converting cellulose into fermentable sugars (Himmel et al. 2007). In a traditional process, cellulosic biomass is synergistically hydrolyzed by commercial cellulases, but the large consumption of cellulases and the independent steps of saccharification and fermentation make it costly and time-consuming (Lynd et al. 2002). Lynd et al. (2005) proposed a method known as consolidated bioprocessing (CBP) that combines enzyme production, cellulose hydrolysis, and fermentation into a single process. It has been reported to significantly reduce the cost of cellulosic ethanol production.

Conversion of cellulosic materials into ethanol consists of pretreatment, enzymatic hydrolysis, and fermentation steps (Goswami et al. 2015; He et al. 2015c). The biomass is dried and ground to fine-sized particles for better hydrolysis. Following step is delignification, which is breaking lignin layer of the ground biomass to expose cellulose, which is done either thermochemically, using high heat, or steam explosion, combined with alkali or dilute acid, or biologically, using fungi such as *Pleurotus ostreatus.* Menon and Rao (2012) reported that off late biomass pretreatment process have focused on novel methodologies more environmentally friendly using a microbiological-based bioconversion system in contrast to the conventional physicochemical processes. Lignocellulose-degrading fungus, *Phanerochaete chrysosporium*, is most extensively studied for its ability to degrade biomass feedstock (Bak 2014). The released cellulose is hydrolyzed to sugars (saccharification) which are fermented by certain microorganisms, such as *Saccharomyces cerevisiae* and *Zymomonas mobilis*, to bioethanol (Faraco 2013).

Saccharomyces cerevisiae is an ideal engineered candidate for CBP to achieve the simultaneous cellulose saccharification and ethanol fermentation, because it has high ethanol productivity, strong ethanol tolerance, and clear hereditary information (Nevoigt 2008; Alper et al. 2006). In fact, as early as in the 1990s, researchers have begun to use cell secretion or cell surface display to endow the *S. cerevisiae* with noncomplex cellulase systems (van Zylet al 2007; Elkins et al. 2010). In such systems, cellulases were either cell secreted into culture medium as free forms or independently displayed on yeast cell surface. The engineered *S. cerevisiae* were able to use amorphous cellulose directly, but ethanol yields were quite low. Compared with the noncomplex cellulase system, enzymes in complex cellulase system (cellulosome) are assembled by nonanalytic scaffolding.

The most promising strategy for converting cellulose to ethanol in yeast is the concerted heterologous expression of all types of cellulolytic enzymes and to maximize their synergies. Genes for endo-/exo-glucanase and β-glucosidase were chromosomally integrated, generating a strain, *S. cerevisiae* L2612δGC, able to grow and produce ethanol in cellulose-containing media.

15.1.3 Hemicellulose

Cellulose microfibrils are coated with other polysaccharides such as hemicellulose or xyloglucans. Depending on the plant species, 20–40% of the plant cell wall polysaccharides are hemicellulose. Like cellulose, hemicellulose could be converted into fermentable sugars by enzymatic hydrolysis for the production of cellulosic ethanol. To date, numerous studies regarding the metabolic engineering of *S. cerevisiae* for xylose utilization have been reported, and many reviews have already addressed the current advancement in metabolic engineering of xylose-fermenting strains and factors which affect xylose metabolism in yeasts.

15.1.4 Pectin

About 35% of dicotyledonous plant dry matter is made up of pectin, a mixed group of various branched, hydrated polysaccharides that are abundant in galacturonic acid. Pectin is not considered important for the production of cellulosic ethanol.

15.1.5 Lignin

Lignin is a major constituent of secondary cell walls and has an important role in protecting the plants against invasion by pathogens and insects. It accounts for about 10–25% of total plant dry matter. Lignin is composed of a complex of phenylpropanoids (aromatic compounds) linked in a network to cellulose and xylose with ester, phenyl, and covalent bonds. There is interest in using genetic engineering to modify the way plants make lignin to make it easier to remove lignin from wood pulp and to increase the digestibility of forage species (Van Acker et al. 2014).

Sugars are natural intermediates in the biological and chemical conversion of lignocellulosic biomass (Hahn-Hägerdal et al. 2006; Lange 2007; Himmel et al. 2007; Stephanopoulos 2007; Binder and Raines 2009; Carroll and Somerville 2009), but access to sugars is hindered by the recalcitrance of plant cell walls. Deriving sugars from this heterogeneous feedstock requires both physical and chemical disruptions. Enzymatic methods of saccharification are the most common, and use physical and chemical pretreatment processes followed by hydrolysis with cellulases to produce sugars. The proper combination of pretreatment and enzymes for a given feedstock enables high yields of sugars from both hemicellulose and cellulose components (Wyman et al. 2005; Lau and Dale 2009. Nonetheless, the

Fig. 15.4 Tissue culture raised *Miscanthus giganteus* transplanted to field conditions. (Professor Jack Widholm is also seen in picture)

costs of both pretreatment and enzymes [one-third of the cost of ethanol production from cellulose (Lau and Dale 2009)] and low rates of hydrolysis are potential drawbacks to enzymatic hydrolysis.

15.1.6 Sources of Lignocellulosic Materials

Sweet sorghum (*Sorghum bicolor* L. Moench), a potential crop for biomass, has high biomass yield and richness of carbohydrates. Sweet sorghum stalk is similar to sugarcane, releasing sweet juice with high levels of sugars (12–20%) which provide good substrates for fermentation to ethanol and leave behind lignocellulosic biomass, the bagasse (Serna-Saldıvar et al. 2012). *Miscanthus giganteus* is also potential source of lignocellulosic biomass. Professor Jack Widholm (UIUC, Illinois) multiplied large number of triploid plants of *Miscanthus giganteus* through tissue culture and raised them under field conditions (Fig. 15.4).

15.1.7 Lignin Conversion Technologies

Lignin can then be treated by conventional or new technologies. Pyrolysis or gasification yield syngas, which, by current use in petroleum technology, can be converted to fuels and chemicals. A second possibility is cracking to platform chemicals by elimination of most functional groups present in lignin. In this way, it is possible

to obtain platform chemicals to be further converted by petroleum technologies into fuels and block and fine chemicals (Aden 2005; Koppram et al. 2014).

15.1.8 Pretreatment Methods for Lignin

Biomass origin and pretreatment methods determine the composition of feed streams, and this has to be adapted to local environments. Pretreatment is considered to be a necessary process to break the lignin seal and disrupt the crystalline structure of cellulose (Choi et al. 2013; He et al. 2014). Recently Raghavi et al. (2016) reviewed pretreatment methods and reported sequential pretreatment of sugarcane trash using crude glycerol-assisted ferric chloride and sodium hydroxide. This treatment was highly effective in removing hemicelluloses and lignin, and the hydrolysate serves as a good source for bioethanol production by *S. cerevisiae.* Sindhu et al. (2016) reported a sono-assisted acid pretreatment strategy for the effective removal of lignin and hemicelluloses and to improve the sugar yield from chili postharvest residue. The major fermentation inhibitors like furfural, 5-hydroxymethyfurfural, and organic acids, like citric acid, succinic acid, and propionic acid, were absent. Absence of inhibitors will eliminate the detoxification step. This strategy showed a better hemicelluloses and lignin removal.

Pretreatment methods may differ for different plant materials. The Alfa is a complex lignocellulosic substrate (Cao et al. 2012). It is mainly composed of cellulose (49.09% ± 0.015), hemicellulose (18.20% ± 0.3), and lignins (16.25% ± 0.475). Cellulose and hemicellulose are attractive components for the production of biofuels or synthons, because these polysaccharides can be biologically hydrolyzed to simple sugars, which, in turn, can be converted into ethanol or other high-value chemicals (Ragauskas et al. 2006; Lynd et al. 2008). On the other hand, lignin is mostly considered to be a hindrance in bioethanol production processes (Rubin 2008; Davidi et al. 2016).

Zaafouri et al. (2016) optimized the pretreatment of Alfa (*Stipa tenacissima*) fibers for the production of second-generation bioethanol.

Fujita et al. (2016) studied the growth and starch accumulation ability of two types of duckweeds (*Wolffia globosa*), a freshwater plant that accumulates starch as a renewable biomass for the production of third-generation biofuels. Mussoline et al. (2017) reported that an industrial sweet potato (*Ipomoea batatas* L.) cultivar, CX-1, offers several advantages as an alternative crop for bioethanol production, including high agronomic productivity and high starch content as well as viable coproducts for additional bioenergy recovery.

15.1.8.1 Enzymatic Treatment of Lignocellulose Material

The enzymatic pretreatment aims at the delignification and the hydrolysis of the hemicellulose. The hydrolysis of the hemicelluloses, in spite of the specificity of xylanases, is restricted due to hurdles in the form of high cost of enzyme and adequate scale-up to industrial level. There are two major types of lignolytic enzymes widely used: *phenol oxidase (laccase)* and *peroxidases (lignin peroxidase, LiP, and*

manganese peroxidase, MnP). Laccase (benzenediol: oxygen oxidoreductase 1.10.3.2) shows a high thermal resistance (stable at 60 °C), low substrate specificity, and high oxidation rates that make this enzyme an ideal candidate for the development of efficient processes for lignin modification. Laccase is able to oxidize phenolic systems by an outer sphere electron transfer process that generates a radical cation, which by fast deprotonation generates a reactive phenoxy radical. The phenoxy radical intermediates formed during this process can further disproportionate and consequently initiate lignin degradation.

15.1.9 Metabolic Engineering for Biofuel

In contrast to simple genetic engineering, metabolic engineering considers metabolic and cellular system as an entirety and accordingly allows manipulation of the system considering efficiency of overall bioprocess (Burton and Fincher 2014). The natural pathways for ethanol production from sugars in *S. cerevisiae* and *Z. mobilis* have led to yields exceeding 95% of theoretical maximum, which is 0.51 g of ethanol per g of glucose. However, natural ethanologenic hosts *S. cerevisiae* and *Z. mobilis* lack the ability to ferment pentoses, which are significant hydrolysis products of lignocellulose biomass. Furans, like 5-hydroxymethylfurfural (HMF) and furfural, are known to inhibit yeast growth and viability and to reduce ethanol productivity. To tackle this problem, one possibility is to introduce pentose-metabolizing pathways into *S. cerevisiae* and *Z. mobilis*. However, one can express the ethanol genic pathways into *E. coli*, whose broad range of carbohydrate metabolizing capacity makes it a top candidate for biocatalyst engineering. *S. cerevisiae* (baker's yeast), although slower growing than *E. coli*, can be transformed readily; efficient homologous recombination enables stable integration of genes into the chromosome, and a host of genetic tools are available. Numerous metabolic engineering approaches have been developed for *S. cerevisiae* (Siddiqui et al. 2012; Krivoruchko and Nielsen 2014). Yeast as a heterologous host has been particularly important for plant-derived pathways such as complex terpenes (Takahashi et al. 2007); because plant cytochrome P450s can be challenging to express in *E. coli*, yeast remains the more common choice of host for plant-derived pathways. Additionally, proteins can be targeted to different cellular compartments in a yeast host, which can be an important aspect of a metabolic engineering strategy.

For alleviation of the phenolic inhibition on ethanol fermenting strains, washing or over-liming methods do not work effectively because of the low water solubility and the increased hydrophobicity of phenolics after pretreatment (Gu et al. 2014). According to Gu et al. (2014, 2015), corn cob residue as the lignocellulosic biomass accumulated phenolic compounds generated from xylitol production industry. For utilization of this biomass, *Zymomonas mobilis* ZM4 was tested as the ethanol fermenting strain and presented a better performance of cell growth and ethanol fermentability in the simultaneous saccharification and fermentation (SSF) than the typical robust strain *Saccharomyces cerevisiae.*

15.1.10 Wood Biomass

According to Van Acker et al. (2014), lignin is one of the main factors determining recalcitrance to enzymatic processing of lignocellulosic biomass. Lienqueo et al. (2016) reported second generation bioethanol from *Eucalyptus globulus* Labill and *Nothofagus pumilio.* Poplars (*Populus tremula* × *Populus alba*) downregulated for cinnamoyl-CoA reductase (CCR), the enzyme catalyzing the first step in the monolignol-specific branch of the lignin biosynthetic pathway, grown in field under short rotation coppice culture showed up to 161% increased ethanol yield. Thus, Van Acker et al. (2014) concluded that CCR downregulation may become a successful strategy to improve wood processing to fermentable sugars if it can be targeted and stabilized to fibers only.

15.1.11 Sugarcane and Energy Cane for Second-Generation Biofuels

The world yield average is 80 tons/ha, but the calculated theoretical yield potential of sugarcane has been noted to be over 380 tons/ha (Waclawovsky et al. 2010; Nakashima and Ishikawa 2016), so there are still gains to be expected. Improvement of the crop performance in genetically modified sugarcane has been achieved in terms of increased yield and increased sugar contents besides pest and disease resistance with improvement of the crop performance (Arencibia et al. 1998; Dal-Bianco et al. 2012). Arruda (2012) reviewed strategies to increase sugar content in mature sugarcane stalk have also been developed by overexpressing genes that encode enzymes that convert sucrose into other disaccharides or sugar polymers. Plants with these modifications show an increase in overall sugar yield.

With the recent interest in feedstock for second-generation cellulosic ethanol, the energy cane concept reemerged as an important topic of some breeding programs. Instead of accumulating high levels of soluble sugar in the stalks, energy cane is bred to produce plants with high fiber content and high biomass. These traits can be obtained, for example, by backcrossing elite sugarcane varieties to *Saccharum spontaneum* (Matsuoka et al. 2009).

15.1.12 Direct Production of Ethanol from Biomass

Chung et al. (2014) reported the direct conversion of switchgrass, a nonfood, renewable feedstock, to ethanol using thermophilic, anaerobic, cellulolytic bacterium *Caldicellulosiruptor bescii*. This bacterium was engineered to utilize unpretreated biomass and produce ethanol. This process was accomplished by deletion of lactate dehydrogenase and heterologous expression of a *Clostridium thermocellum* bifunctional acetaldehyde/alcohol dehydrogenase. Although wild-type *C. bescii* lacks the ability to make ethanol, 70% of the fermentation products in the engineered strain were ethanol [12.8 mM ethanol directly from 2% (wt/vol) switchgrass, a real-world

substrate] with decreased production of acetate by 38% compared with wild type. Direct conversion of biomass to ethanol represents a new paradigm for consolidated bioprocessing, offering the potential for carbon neutral, cost-effective, sustainable fuel production. Sato and Umagai (2013) developed microbial platform for the production of plant isoquinoline alkaloids involving the unification of the microbial and plant metabolic pathways into a single system. They could have potential applications for biofuel production.

15.1.13 Oxidation of Lignin by Biocatalysis Processes

In nature, lignin is selectively oxidized by white-rot fungi. These species excrete a pool of lignin lytic enzymes that activate both dioxygen and hydrogen peroxide at the degradation of lignins. Among them, laccases and peroxidases, more specifically Mn peroxidase and lignin peroxidases, are the most active. Strategies for enzyme immobilization and use in mixtures to reproduce natural cascades have also been reported.

Anaerobic fungi harbor a rich reservoir of undiscovered cellulolytic enzymes and enzyme complexes that can potentially transform the conversion of lignocellulose into bioenergy products. According to Haitjema et al. (2014), anaerobic gut fungi are commonly found in the digestive tract of ruminants and monogastric herbivores, and they are the primary colonizers of ingested plant biomass. These gut fungi significantly contribute to the decomposition of plant biomass into fermentable sugars. Stable genetic transformation is critical for understanding and engineering microbial systems, and it would allow for endogenous pathways to be enhanced, eliminated, or tuned according to need. Nucleic acids, as the precursors of cellular protein components, can provide profound insight into the physiology and metabolism of gut fungi (Solomon et al. 2014). In addition, foreign genes and gene ensembles could be introduced into anaerobic fungi that could allow for synthesis of nonnative compounds. These techniques are key to developing anaerobic fungi as novel lignocellulose-degrading platform microbes, especially since native gut fungal cellulolytic genes do not always retain activity when expressed in other model microbes (Haitjema et al. 2014).

15.1.14 Fermentation Process

Fermentation of the hydrolyzed biomass can be carried out in a variety of different process configurations. Separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and cofermentation (SSCF), and consolidated bioprocessing (CBP) are the fermentation configurations available for bioethanol production from lignocellulosic biomass. In SSF, reducing sugars produced in cellulose hydrolysis or saccharification are simultaneously fermented to ethanol, which greatly reduces the product inhibition to the hydrolysis. CBP approach has been contemplated to hold promise for commercial

cellulosic bioethanol production because of its characteristic features that avoid the need for costly cellulase enzymes by using engineered microorganisms that produce cellulases and ethanol in a single step.

Conversion of monosaccharides to bioethanol is accomplished metabolically via glycolysis and fermentation (Singh et al. 2016). Sequential enzymatic hydrolysis and fermentation is defined as separate hydrolysis and fermentation (SHF). On the other hand, if the two process steps are performed simultaneously, the process is referred to as SSF. This offers the possibility of cell recycling (Roca and Olsson 2003; Cotana et al. 2015). However, SSF allows the sugars released via hydrolysis to be rapidly consumed by the microorganism, thereby minimizing feedback inhibition of cellulolytic enzymes (Cotana et al. 2015). Efforts to reduce capital and certain operating costs in the saccharification of pretreated biomass and fermentation of the resultant sugars have focused on conducting both steps in a single vessel. Simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and co-fermentation (SSCF, where xylose is co-fermented with glucose) have been developed. Reducing cellobiose inhibition of endoglucanase and cellobiohydrolases in SSCF processes can be accomplished by the expression of a cellobiose transporter and β-glucosidase in *Saccharomyces cerevisiae* strains able to ferment xylose (Li et al. 2010; Galazka et al. 2010).

15.1.15 Synthetic Biology Approach

The challenge is to find the right biomaterial cheap enough to turn a profit when manufactured on a large scale. At the end of the day though, producing hydrocarbons, by whatever method, puts carbon into the atmosphere and exacerbates the greenhouse effect.

Although it is still in its infancy, metabolic engineering and synthetic biology offer great potential to overcome the challenges associated with lignocellulose bioconversion. Synthetic biologists have far greater ambitions. They aim to design entirely new life forms with preselected functions, like the microbes which will digest trees and grasses and ferment them into biofuels or the algae which will harvest solar energy to produce oil. The idea of advanced biofuels is based on using bioengineered microorganisms to digest organic materials to produce hydrocarbons. One example of this is methanotroph, a bacterium which eats biomaterial and coverts it into carbon gas which is fermented to produce diesel fuel.

Synthetic biology approach for co-fermentation of hexose and pentose sugars has been successfully demonstrated by incorporating two mutants of *E.coli* in a single system—one capable of utilizing only glucose while the other capable of utilizing only xylose as a carbon source. Another promising alternative for simultaneous utilization of pentoses and hexoses is the conversion of xylose into xylulose by xylose isomerase or oxidoreductase. The fermentation of xylulose begins by its intracellular phosphorylation to xylulose-5-phosphate, which enters the pentose phosphate pathway and eventually forms ethanol. Yeasts like *S. cerevisiae* and *Schizosaccharomyces pombe* are capable of fermenting xylulose to ethanol, along

with glucose. The science behind these new biofuels is synthetic biology. Synthetic biology has rapidly grown out of genetic engineering into a new science with new risks. Genetic engineers merely modify existing organisms by splicing a few genes from one organism into another (see also Chap. 24).

15.2 Discussion

At present a driver for biofuel production is also the opportunity to reduce GHG emissions. Gombert and Van Maris (2015) suggested three types of strategies used to achieve this goal: engineering free-energy conservation, engineering redox metabolism, and decreasing sugar losses in the process. They suggested that whereas the two former strategies lead to decreased biomass and/or glycerol formation, the latter requires increased process and/or yeast robustness. Efficient breakdown of lignocellulose polymers into simple molecules is a key technological bottleneck limiting the production of plant-derived biofuels and chemicals. To achieve an economical and environmentally friendly system of bioethanol production from straw, a number of breakthroughs are needed, not only in individual process steps but also in the balance and combination of these processes (Chen and Qiu 2010; Gallego et al. 2015). The rollout of cellulosic biofuels, which have superior greenhouse gas emission profiles to corn ethanol, has been hampered by lack of finance, technological immaturity, and market risk. A growing number of cancelled or delayed projects has weighed heavily on a sector that pioneered sophisticated metabolic engineering approaches but that has so far failed to deliver on billions of dollars of investment (Sheridan 2013). However, its political opposition to biofuels is matched by several recent exits from large-scale biofuels projects. According to Albers et al. (2016), a look at the global landscape of biofuel patenting shows that, after surging between 2004 and 2008, the invention of biofuel technologies slowed considerably and in many countries went into decline. Global trends point to an uncertain future, in particular, for advanced biofuels. Strategies to increase sugar content in mature sugarcane stalk have also been developed by overexpressing genes that encode enzymes that convert sucrose into other disaccharides or sugar polymers to obtain plants with increase in overall sugar yield (Arruda 2012). However, although research has provided some insight into the mechanisms of transgene silencing in sugarcane, there is still much to be understood about the production of long-term stable, genetically modified sugarcane. Engineering of secondary metabolism, of a plant might confer increased disease resistance or alternatively increase production of biofuels. In these cases, the host for which the application is being designed may not be sequenced or have appropriate genetic tools available, and embedded regulatory networks can tightly and redundantly control the levels of product production (see review Connor 2015). In addition, regulatory issues associated with the crop propagation model will also be a challenge to the commercial approval of genetically modified sugarcane (Arruda 2012; Dal-Bianco et al. 2012).

Although lignin is the most important factor limiting the conversion of plant biomass to fermentable sugars, the wood from transgenic poplar, downregulated in

cinnamoyl-CoA reductase (CCR), an enzyme in the lignin biosynthetic pathway, is easier to process into ethanol. However, strong downregulation also affected biomass yield. In conclusion, CCR downregulation may become a successful strategy to improve biomass processing if the yield penalty can be overcome (Chen and Dixon 2007; Van Acker et al. 2013; Van Acker et al. 2014).

Commercial transgenic plants may yet take years to come to commercialization and will probably be targeted at insect and drought resistance (Dal-Bianco et al. 2012). A reference sequence will be important to define gene promoter sequences that may allow gene networks to be defined as well as speed up gene discovery projects and the development of tools for transgenic plant generation. SNP discovery and QTL determination will also profit from a reference genome. With the aid of modern tools of genetics for polyploids and the introduction of new genotypes, breeding is expected to progress much further toward achieving higher levels of productivity (Dal-Bianco et al. 2012).

Although the first 2G fuel ethanol factories have started, 1G ethanol production will probably remain either as a stand-alone technology or as part of biorefineries. Food production waste can also be used as carbon and nutrient sources for the bioproduction of fuels and chemicals (Girotto et al. 2015). With a high contribution (up to 70%) of the feedstock to the final production cost, high production volumes, and small profit margins, the overall conversion yield of the raw material into ethanol is crucial for the process economy (Gombert and Van Maris 2015). The economic production of cellulolytic enzymes and reducing the enzyme-to-biomass ratio required for hydrolysis remain key to commercializing biomass-derived fuels. *Trichoderma reesei* remains the most effective commercial host for cellulase production (Merino and Cherry 2007), although other fungal hosts may be attractive.

In vitro synthetic biosystems are emerging as an alternative solution for accomplishing a desired biotransformation without concerns of cell proliferation, complicated cellular regulation, and side product formation. The US bioethanol industry depends on glucose conversion by yeast wherein pyruvate (C3) is decarboxylated to acetaldehyde and then reduced to ethanol (C2) by a monofunctional alcohol dehydrogenase. Bioethanol production is achieved by only two metabolic pathways and only at moderate temperatures. Herein a fundamentally different synthetic pathway for bioalcohol production at 70 °C was constructed by insertion of the gene for bacterial alcohol dehydrogenase (*AdhA*) into the archaeon *Pyrococcus furiosus.* The engineered strain converted glucose to ethanol via acetate and acetaldehyde, catalyzed by the host-encoded aldehyde ferredoxin oxidoreductase (*AOR*) and heterologously expressed *AdhA*, in an energy-conserving, redox-balanced pathway (Basen et al. 2014). Great potential markets of biocommodities will help in overcoming obstacles pertaining to cost and stability of enzymes and coenzyme biosystem optimization and scale-up, with next several decades (Zhang 2014). Zhang et al. (2016) reviewed latest research progress on biofuel production using food processing wastes.

Competition of genetically engineered yeast strains with wild yeast strains in non-aseptic processes provides an important challenge. The development of recombinant DNA technology protocols suitable for industrial yeast strains, which are not

necessarily as amenable to genetic modifications as laboratory strains (Le Borgne 2012; Reis et al. 2012), is of high importance. Interestingly, recent developments, such as the CRISPR/Cas9 system and simultaneous integration of multiple genes (Kuijpers et al. 2013; DiCarlo et al. 2013), might result in increased accessibility of industrial yeasts for metabolic engineering (see also Gombert and van Maris 2015).

15.3 Conclusion

Biorefinery of microbial biomass to value-added products will also improve the process economics for biodiesel production. Many of the potential risks and harms of next-generation biofuels are environmental. For example, a report by the UN Convention on Biological Diversity (CBD) Secretariat noted that several candidate species for future biofuel production show the traits of invasive species. Building "terminator genes" into synthetic organisms or making them dependent on artificial substances may decrease the likelihood of uncontrolled proliferation. But uncontrolled proliferation may occur despite best efforts at containment. Synthetic microorganisms released into the environment, accidentally or intentionally, could share genes with other microorganisms through horizontal gene transfer or evolve beyond their functionality.

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Agro-industrial Lignocellulosic Waste: An Alternative to Unravel the Future Bioenergy

16

Nidhi V. Maheshwari

Abstract

Dwindling reserves of fossil fuel and petroleum derivatives, rising oil prices, concern about environmental impact, and supply insecurity demand environmentally sustainable energy sources. Production of fuels and chemicals through microbial fermentation of plant material that uses renewable feedstock is a desirable alternative to petrochemicals. Lignocellulose represents the most widespread and abundant source of carbon in nature and is the only source that could provide a sufficient amount of feedstock to satisfy the world's energy and chemical needs in a renewable manner. Typically, most of the agricultural lignocellulosic biomass is comprised of about 10–25% lignin, 20–30% hemicellulose, and 40–50% cellulose. The processing and utilization of this substrate are complex, differing in many aspects from crop-based ethanol production. Sustainable and economically viable manufacturing of bioethanol from lignocellulose raw material is dependent on the availability of a robust ethanol-producing microorganism, able to ferment all sugars present in the feedstock. Thus, an obvious target in the field of metabolic engineering has been the tailoring of such a microorganism, combining advantageous traits from different microorganisms with classical procedures such as random mutagenesis. Nowadays research is being directed to develop metabolically and genetically engineered *Saccharomyces* strains and other ethanol-fermenting microbes that has the potential to utilize wide range of substrates including pentose and hexose sugars, ability for direct and efficient ethanol production from cellulosic materials, and ability to tolerate ethanol stress. Although it is still in its infancy, metabolic engineering and synthetic biology offer great potential to overcome the challenges associated with lignocellulose bioconversion.

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Bioethanol • Lignocellulose • Fermentation • *Saccharomyces cerevisiae*

16.1 Introduction

Rapid depletion of bioenergetics resources and the environmental compliance concerning the greenhouse gases has attracted the awareness in nonconventional fuel from natural resources. Production of fuels and chemicals through microbial fermentation of plant material that uses renewable feedstock is a desirable alternative to petrochemicals. Mature technologies for ethanol production are crop based, utilizing substrates such as sugarcane juice and corn starch. But there are huge concerns regarding the increasing diversion of starch- or sucrose-rich crop materials and land from food to biofuel production. This has shifted attention to the use of lignocellulose-derived bioethanol as a biofuel (Morales et al. 2015). For the past few years, the biomass-based ethanol has caught the attention of global industry. Agro-industrial biomass comprised on lignocellulosic waste is an inexpensive, renewable, and abundant and provides a unique natural resource for large-scale and cost-effective bioenergy collection. Recently lignocellulosic biomasses have gained increasing research interests and special importance because of their renewable nature (Chen 2014). To expand the range of natural bioresources, the rapidly developing tools of genetic engineering can lower the conversion costs and also enhance target yield of the product of interest.

Bioethanol can be used as fuel with significant characteristics like high octane number, low cetane number, and high heat of vaporization. Its main drawbacks are the corrosiveness, low flame luminosity, lower vapor pressure, miscibility with water, and toxicity to ecosystems. One crucial problem with bioethanol fuel is the availability of raw materials. The supply of feedstocks for bioethanol production can vary season to season and depends on geographic locations. Lignocellulosic biomass, such as forest-based woody materials, agricultural residues, and municipal waste, is prominent feedstock for bioethanol because of its high availability and low cost (Dominguez et al. 2015). In addition, the supply and the attentive use of microbes render the bioethanol production process highly peculiar.

Lignocellulosic (cellulosic) biomass-derived ethanol is often termed as “second generation” or “2G” as the “first generation” or “1G” ethanol is derived from sugarcane, corn, wheat, and other starchy feedstocks (Jordan et al. 2012), the most promising near-/long-term fuel candidate. In addition, cellulosic biomass-derived ethanol may serve as a precursor to other fuels and chemicals that are currently derived from unsustainable sources and/or are proposed to be derived from cellulosic biomass. Bioethanol production will be probably the most successful biofuel because it has plenty of usable forms (heat, power, electricity, or vehicle fuel). The benefits estimated from mandated use of cellulosic biofuels include nation’s energy security through domestic production of transportation fuel and environmental improvement through greenhouse gas mitigation and other particulate emissions associated with

fossil fuel combustion. Additional benefits include creating new markets for agricultural products, keeping productive farmland in use, and improving trade balances.

16.2 Physicochemical Characteristics of Lignocellulosic Biomass

Biomass refers to renewable organic materials, including agricultural products and agricultural wastes, wood and its wastes, animal wastes, urban wastes, aquatic plants, and so on. Lignocellulosic complex is regarded as the most abundant biopolymer in the earth constantly generated through photosynthesis and as one of the potential raw materials for ethanol production (da Silva 2016). About 50% of the world biomass is considered as the lignocellulosic biomass, and its total annual production is estimated to be approximately 10–50 billion ton (Mood et al. 2013). Generally, lignocellulosic biomass for bioethanol (as fuel) production can be differentiated into the following six groups: crop residues (sugarcane bagasse, sweet sorghum bagasse, pulp, wheat straw, rice straw, rice hulls, and barley straw), softwood (pine and spruce), hardwood (aspen and poplar), cellulose wastes material such as newsprint and waste office paper, herbaceous biomass material (timothy grass, alfalfa hay, switch grass, coastal Bermuda grass), and municipal solid wastes (Singh et al. 2012).

Lignocellulose, the principal component of the plant cell walls, is mainly composed of cellulose (40–60% of the total dry weight), hemicellulose (20–40%), and lignin (10–25%) (Dionisi et al. 2015), together with small amounts of other components, like acetyl groups, minerals, and phenolic substituents. Depending on the type of lignocellulosic biomass, these polymers are organized in complex non uniform three-dimensional structures to different degrees and varying relative composition. Lignocellulose has evolved to resist degradation, and this robustness or recalcitrance of lignocellulose stems from the crystallinity of cellulose, hydrophobicity of lignin, and encapsulation of cellulose by the lignin-hemicellulose matrix (Agbor et al. 2011). Its composition depends not only on the type of plant but also on the selected part of the plant (Brown 1999) and on growth conditions (Wiselogel and Johnsson 1996). This material differs from products with high sugar and starch content (McMillan 1997).

The major component of lignocellulosic biomass is cellulose, being the most abundant and easily available carbohydrate polymer all around the earth which is a major polysaccharide constituent of plant cell wall, composed of repeating (1,4)-D-glucopyranose units, which are attached by β-1,4 linkages with an average molecular weight of around 100,000 (Himmel et al. 2007). Naturally cellulose molecules exist as bundles which aggregated together in the form of microfibrils order, i.e., crystalline and amorphous regions (Taherzadeh and Karimi 2008).

The second most abundant polymer after cellulose is hemicellulose which is heterogeneously branched in nature. The backbone of the hemicellulose polymer is built up by sugar monomers like xylans, mannans, and glucans, with xylans and

mannans being the most common (Ladisch and Lee 2005); in this case xylanases are the enzymes involved in its degradation. Hemicelluloses differ in composition too; hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses contain mostly glucomannans. The heteropolymers of hemicellulose are composed of different 5- and 6-carbon monosaccharide units: pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and acetylated sugars. Hemicelluloses are embedded in the plant cell walls to form a complex network of bonds that provide structural strength by linking cellulose fibers into microfibrils and cross-linking with lignin (Schellar and Ulvskov 2010). Cellulose and hemicellulose bind tightly with noncovalent attractions to the surface of each cellulose microfibril. Hemicellulose degrades quickly due to its amorphous nature (Hamelinck et al. 2005). Among other important aspects of the structure and composition of hemicellulose are the lack of crystalline structure, mainly due to the highly branched structure, and the presence of acetyl groups connected to the polymer chain.

Lignin is generally the most complex and smallest fraction of the biomass. Lignin is a three-dimensional polymer of phenylpropanoid units. The oxidative coupling of three different phenylpropane building blocks, monolignols: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, forms the structure of lignin. The corresponding phenylpropanoid monomeric units in the lignin polymer are identified as p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively. Lignin acts like a glue by filling the gap between and around the cellulose and hemicellulose complexion with the polymers. It is present in almost all kind of cellulosic plant biomass and acts as a protective sheet against cellulosic and hemicellulosic components of the biomass materials. It adds compressive strength to the plant tissue and the individual fibers, stiffness to the cell wall, and resistance against insects and pathogens (Rubin 2008).

16.3 Cellulosic Biomass to Ethanol

In 2014 the global production of bioethanol reached 24.5 billion gallon, up from 23.4 billion gal in 2013 which shows the international bioethanol market is at a very dynamic stage. More than half (60%) of global bioethanol production is based on sugarcane conversion, and the rest (40%) comes from other crops (Dufey 2006). The United States (corn) and Brazil (sugarcane) are the global producers as they produce 70% of the global bioethanol production.

Thus most ethanol produced to date as biofuel is generated from edible crops. However, this "first generation" approach led to the "food versus fuel" conflict and dilemma leading to search for alternative biomass sources for the "next generation biofuels" mostly based on cellulose (Valentine et al. 2012). Plant cell walls are the most abundant renewable resource on our planet with $150\text{-}170\times10^9$ tons produced annually (Pauly and Keegstra 2008). The major components of plant cell walls are cellulose, hemicellulose, and lignin that comprise around 90% of its dry biomass (Gibson 2012). Lignocellulose offers several benefits over sugar and starch as a substrate for bioethanol production (Morales et al. 2015). Lignocellulosic biomass,

which covers a large category of agricultural biomass, has minimized the potential conflict between land use for food production and production of energy feedstock. Thus, the production of ethanol from the cell walls of non-crop plants or nonedible parts of plants is considered a sustainable solution for biofuel production. This is despite the current difficulties related to the costs, high energy inputs, and harsh conditions required to process the complex cell wall polymers into fermentable sugars. The raw material is cheaper compared to conventional agricultural feedstock and can be produced along with lower involved fertilizers, pesticides, and energy. Biofuels from lignocellulosic biomass have low emissions of greenhouse gas and reduce environmental impacts, including climate change. Biofuels might also provide employment in rural areas. A large number of studies for developing large-scale ethanol production from lignocellulosic biomass have been carried out worldwide. The complex composition of lignocellulosic materials is a key factor affecting the efficiency of bioethanol production during the conversion processes (Dixon 2013), and this is directly related to the composition of lignocellulosic material. Cellulose and hemicellulose are the two main polymers of the biomass that break down into fermentable sugars, which are further converted into ethanol, but the breakdown of lignocellulosic biomass is a complicated and energy-consuming process (Kaur et al. 2013).

Low-carbon biofuels from commercial-scale cellulosic ethanol have become a reality in recent times. Numerous cellulosic ethanol refineries have now come online worldwide with several more in the pipeline (European Biofuels Technology Platform 2015). To date, the largest cellulosic ethanol industrial-scale refinery is the Beta Renewables/Novozymes-funded plant situated at Crescentino in Northwestern Italy which commenced operations in October 2013. The facility is entirely self-sufficient, using the lignin and biogas by-products to power the plant which generates 75 million liters annually of cellulosic ethanol, enough fuel for more than 50,000 cars. In the United States, there are over 200 corn-based ethanol plants in operation (Gnansounou 2010). Many of these bioethanol plants are evolving to become cellulosic ethanol production facilities utilizing cheaper agricultural residues and nonfood substrates. Encouraging yields ranging from 68 to 83 gallons per tonne of biomass have recently been reported by several bioenergy groups such as Abengoa Bioenergy, Iogen Energy, and Poet, LLC from their respective pilot cellulosic ethanol plants (Guo et al. 2015). With the inevitable upsurge in oil price back to pre-2014 levels an unavoidable reality allied with advancements in the relevant technology, industrial-scale lignocellulosic bioethanol will continue to spread worldwide in the near future.

16.4 Processes for Bioethanol Production

One of the most important goals of lignocellulosic biomass refining is to fractionate lignocellulose into its three major components: cellulose, hemicelluloses, and lignin. Single-step treatment methods, like pyrolysis, are not efficient. Although they render lower costs, deconstruction of the lignocellulosic biomass takes place since

these methods generally rely on high temperatures. It is highly inconvenient and difficult to separate the targeted chemicals and fuels via single-step methods because the produced bio-oil consists of a mixture of hundreds of compounds. For downstream and efficient separations, additional costs and various pretreatment methods are required. Application of the pretreatment methods changes the natural-binding characteristics of lignocellulosic materials by modifying the supramolecular structure of cellulose-hemicellulose-lignin matrix. Therefore, this robust and complex structure in order to be converted into bioalcohols requires a multistep process, including pretreatment, enzymatic hydrolysis, and fermentation that increases the cost of biofuels production significantly (Kumagai et al. 2014).

Pretreatment is a processing step to make lignocellulosic biomass more amenable to biological conversion at high yields that otherwise suffers from low yields and high processing costs (Wyman et al. 2013). Pretreatment methods are divided into different categories such as mechanical, chemical, physicochemical, and biological methods or various combinations of these (Barakat et al. 2013). Various pretreatment options were reported to fractionate, solubilize, hydrolyze, and separate cellulose, hemicellulose, and lignin components. Some of them include milling, irradiation, microwave, steam explosion, ammonia fiber explosion (AFEX), supercritical CO_2 and its explosion, SO_2, alkaline hydrolysis, liquid hot-water pretreatment, organosolv processes, wet oxidation, ozonolysis, dilute- and concentrated-acid hydrolyses, and biological pretreatments (Saha 2005). A few new promising pretreatments that have recently been developed include cosolvent-enhanced lignocellulosic fractionation (CELF) (Nguyen et al. 2015a, b), cosolvent-based lignocellulosic fractionation (COSLIF) (Zhang et al. 2007), extractive ammonia (EA) pretreatment (Chundawat et al. 2013), γ-valerolactone (GVL) pretreatment (Shuai et al. 2016; Wu et al. 2016), pretreatment applying ionic liquid(s) (Konda et al. 2014), sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) (Zhu et al. 2009), and switchable butadiene sulfone pretreatment (de Frias and Feng 2013). The common goal of these methods is to reduce the biomass in size and open its physical structure. Each of these methods has been reported to have distinct advantages and disadvantages.

The performance of the hydrolysis is highly associated to the pretreatment process (Girio et al. 2010). During this reaction, cellulose and hemicellulose are hydrolyzed into simple and soluble compound available for further conversion (fermentation) to ethanol (Chandel et al. 2007). There are two different methods of hydrolysis processes that involve either acidic or enzymatic reactions. Acidic reactions require high quantity of acid which makes its usage less attractive (Hamelinck et al. 2005). Also, when acids are used in the hydrolysis, the phenomenon of chemical dehydration occurs on monosaccharide resulting in the appearance of other compounds like aldehydes. These limitations led to the researchers' interest to focus on enzymatic hydrolysis. Compelling pretreatment is fundamental to an efficient enzymatic hydrolysis. Eggeman and Elander (2005) have demonstrated that *Trichoderma reesei* is a very efficient fungus to produce industrial grade cellulolytic enzymes. The use of metallic compounds like Ca^{2+} and Mg^{2+} could intensify the enzymatic hydrolysis (Liu et al. 2010).

High cost of cellulase and other accessory enzymes required for biological conversion of pretreated lignocellulosic biomass into sugars is another major impediment in the commercialization of lignocellulosic biomass to fuels and chemicals (Culbertson et al. 2013). In addition to enzymes, low accessibility to (hemi) cellulose, their strong inhibition by components generated during pretreatment (e.g., phenols) (Kim et al. 2011), and enzymatic saccharification (Kumar and Wyman 2014) are one of the main reasons for high loading of enzymes required for commercially viable sugar yields. In addition, enzymes' unproductive binding to lignin (Li et al. 2013) and pseudo-lignin (Kumar and Wyman 2013) also lowers the amount of enzymes available and affects their effectiveness. Although cellulase end-product inhibition by glucose can be alleviated in a process configuration called simultaneous saccharification and fermentation (SSF), and inhibition by cellobiose and hemicellulose oligomers can be alleviated by supplementing cellulase with accessory enzymes, low reaction rates at fermentation temperatures (32–37 °C) (Elia et al. 2008) and inhibition by ethanol still pose a challenge to high yields and titers at low enzyme loadings (Podkaminer et al. 2011). The discovery of novel non-hydrolytic enzymes like polysaccharide monooxygenases (LPMOs) appears to be highly promising in reducing cellulase and ultimately overall processing costs (Agger et al. 2014). LMPOs have certain limitations like they require electron donor (Muller et al. 2015) and make cellulase cocktails less stable (Scott et al. 2016); in addition to loss of some of the carbohydrates and requirement of different process configurations, the aldonic acids resulting from polysaccharides oxidation by LPMOs can be inhibitory to enzymes as well as microbes (Cannella et al. 2012). Thus, it is still to be seen whether these new non-hydrolytic enzymes would be advantageous in the long run.

Fermentation is the following step and requires the presence of microorganisms to degrade sugars into alcohols and other end products. Typically *S. cerevisiae* converts the sugars into ethanol under anaerobic conditions at a temperature of 30 °C. In this pathway other by-products are also generated in the form of CO_2 and N-based compounds. *S. cerevisiae* is known as the most studied microorganism for the fermentation of lignocellulosic hydrolyzates that ferment the glucose contained in hydrolyzate while unable to ferment pentose sugar such as xylose. *S. cerevisiae* is a prevalent microorganism and provides a high yield of ethanol (12.0–17.0% w/v; 90% of the theoretical yield) from sugars (Kumar et al. 2009).

Fermentation of biomass hydrolyzates involves processes done in separate units (hydrolysis and fermentation). This system is known as separate hydrolysis and fermentation (SHF). However, on the other hand, simultaneous saccharification and fermentation (SSF) is a process which is performed in a single unit.

16.4.1 Separate Hydrolysis and Fermentation (SHF)

The SHF is the traditional method for bioethanol production in which hydrolysis and fermentation are performed in separate units. In this process, a fraction contains the cellulose in an accessible form after pretreatment is subjected to hydrolysis.

After completion of hydrolysis, the obtained cellulose hydrolyzate is converted into ethanol by fermentation. The interesting feature of SHF is that every step could be conducted at its optimal conditions so that the probability of product recovery is more (Sharma et al. 2011). The most important parameters to be taken into consideration for saccharification step are availability of cellulose (for glucose conversion), reaction time, temperature, pH, optimal enzyme unit, and substrate loading (Oberoi et al. 2012). Several studies have reported the weakness of *S. cerevisiae* to ferment only hexose sugars and the interest for versatile-acting microorganisms increased. To date, extensive research has been conducted to develop microorganisms which enable to (i) ferment pentose and hexose sugars synchronously available from the hemicellulose fraction and (ii) endure under inhibitory conditions.

16.4.2 Simultaneous Saccharification and Fermentation (SSF)

The SSF process is more attractive than the SHF due to high ethanol yield and less energy consumption. In this process, cellulases (for hydrolysis) along with microorganisms (for fermentation) are added in the same process unit allowing glucose formation and immediate consumption of glucose by microbial cells, resulting into ethanol production. Therefore, the inhibitory effect of sugars on cellulases is neutralized. However, the use of more diluted media makes the final product of low concentration. Moreover, this process proceeds at nonoptimal parameters of hydrolysis, and, at the same time, higher enzyme dosages are required, which enhance substrate conversion rate as well as the cost of the process. Alkasrawi et al. (2003) reported that the addition of nonionic surfactants, such as Tween-20 and Tween-80, to the steam-exploded wood in a batch of SSF by using *S. cerevisiae* gave 8% increment in ethanol yield, 50% reduction in cellulases dosage (from 44 FPU/g to 22 FPU/g of cellulose), and decrease in the time period required for reaching maximum ethanol concentration. It is felt that the surfactant prevents the unuseful adsorption of cellulases to lignin.

16.5 Consolidated Bioprocessing (CB)

Three main steps in lignocellulosic biomass conversion – enzymes production, biological hydrolysis of biomass to sugars and oligomers, and fermentative metabolites (e.g., ethanol) production – can be combined into a single bioprocessing system "direct microbial conversion (DMC)" (Demain et al. 2005) or lately known as "consolidated bioprocessing (CBP)" (Li et al. 2014).

A novel development, the consolidated bioprocessing (CBP) proceeds by producing all required enzymes and ethanol using a single type of microorganisms in a single reactor. CBP is considered as the ultimate evolution of biomass-to-bioethanol conversion technology, since it implies neither capital nor operating costs (Lynd et al. 2008) for dedicated enzyme production together with a reduced consumption of substrate for enzyme production.

Increasing evidence suggests that CBP may be feasible (Okamoto et al. 2014). Ever since the concept of CBP was proposed in 1996, CBP research has focused on the development of new and even more effective CBP microorganisms, which has been a key challenge (Lynd et al. 2005). Bacteria and yeast have been the primary candidates for CBP research, and some progress has been made in this regard (den Haan et al. 2015). There are several cellulolytic/non-cellulolytic and thermophilic/mesophilic candidate microorganisms for CBP including bacteria, e.g., *Clostridium thermocellum* (Shao et al. 2011), *Thermoanaerobacterium saccharolyticum* (Shaw et al. 2008), *Clostridium phytofermentans* (Jin et al. 2012), and *Caldicellulosiruptor bescii* (Chung et al. 2014), and yeasts, e.g., *S. cerevisiae* and thermotolerant *K. marxianus* (Yamada et al. 2013). Thermophiles have an added advantage of higher hydrolysis rates and less probability of contaminations at fermentation temperatures of >60 °C than mesophiles that usually operate at temperatures <50 °C (Olson et al. 2012). *Caldicellulosiruptor bescii* has recently been engineered to produce ethanol at high metabolic yield; however, the productive yields are too low for commercial application yet (Chung et al. 2014). In addition to thermophilic and other bacteria, research is also underway in modifying yeasts to convert them into CBP organisms (Yamada et al. 2013). However, most of these genetically engineered strains still need some supplementation of exogenous enzymes for high ethanol yields.

Fungi have not been widely proposed as CBP microorganisms, but there are a few recent reports of researchers developing strains of the fungi *Fusarium oxysporum* and *Trichoderma reesei* with enhanced CBP potential (Huang et al. 2014).

16.6 Genetic Engineering for the Fermentation of Lignocellulose into Ethanol

The utilization of lignocellulose as a raw material for a fermentation process imposes many demands on the potential microorganism, which therefore must display many of the features, viz., broad substrate utilization range, high ethanol yields and productivity, minimal by-product formation, high ethanol tolerance, etc.

The ability to utilize all sugars present in lignocellulose substrate is a prerequisite for the efficient production of ethanol from the raw material. Given the high ethanol on glucose (and sucrose) as well as the high ethanol tolerance of *S. cerevisiae* and *Z. mobilis*, an obvious approach was to expand their substrate utilization range, so that all monosaccharides in lignocellulosic materials are utilized. The preferred microorganism in crop-based processes, *Saccharomyces cerevisiae*, is unable to ferment pentoses and is therefore of limited use for lignocellulose substrates with a high content of pentoses, unless the necessary pathways are inserted and expressed. The same restriction applies to the ethanologen bacterium *Zymomonas mobilis*. Thus, the efficient utilization of xylose in hemicellulose in addition to glucose in cellulose by a recombinant xylose-fermenting *S. cerevisiae* strain would offer an opportunity to reduce the production cost of bioethanol significantly (Oh et al. 2013). To date, numerous studies regarding the metabolic engineering of *S. cerevisiae* for xylose utilization have been reported, and many reviews have already

addressed the current advancement in metabolic engineering of xylose-fermenting strains and factors which affect xylose metabolism in yeasts (Xin et al. 2014).

With ethanol being a low value-added product, the overall yield in the conversion of sugars to ethanol is crucial. Utilizing crop sugars as substrates for ethanol production, yields of 90–95% of the theoretical can be obtained using *S. cerevisiae* or *Z. mobilis*, and yields in this range are also required for an economically feasible process based on lignocellulose as raw material. However, of equal importance to the yield is a high productivity, since the depreciation of capital investments also contributes significantly to the cost of ethanol production (Zaldivar et al. 2001). *E. coli* and several enteric bacteria naturally possess a broad substrate utilization range, converting hexoses (glucose, mannose, galactose, fructose), pentoses (xylose and arabinose), and uronic acids (galacturonic acid, glucuronic acid) to the central metabolite, pyruvate. This compound is further converted to a near-equal mix of ethanol, lactate, acetate, and formate (H_2O plus CO_2). Normally, fermentations are carried out at pH 7.0 and at temperatures between 30 and 35 °C. The main strategy to increase ethanol production in *E. coli* and make it suitable for lignocellulose processes was to redirect the carbon flux toward ethanol production, which was achieved in three main steps. The insertion of *pdc* and *adhB* genes from *Z. mobili*s, encoding for highly active ethanologenic enzymes; enabled *E. coli* to produce ethanol and CO_2 from hexoses and pentoses at high efficiency; Control of a single promoter creating the PET (production of ethanol) operon (Ingram et al. 1987). The PET operon was subsequently introduced into several bacterial hosts. The direction of carbon flux toward ethanol formation was favored by the expression of high levels of heterologous *pdc* and *adhB* as well as by the fact that the original PDC from *Z. mobilis* has an affinity toward pyruvate higher than other homologous enzymes competing for pyruvate in *E. coli,* e.g., lactate dehydrogenase.

A well-known by-product in yeast fermentation is glycerol. During the formation of biomass, there is a net conversion of the cytosolic cofactor NAD^+ to NADH. Since the respiratory chain is nonfunctional under anaerobic conditions, the only route to reconvert the cofactor to NAD^+ is through glycerol formation. Thus, glyceraldehyde-3-P is converted to dihydroxyacetone-P to glycerol-3-P and further to glycerol. There are two genes, *GPD1* and *GPD2*, encoding glyceraldehyde-3-phosphate dehydrogenase, the enzyme that regenerates NAD^+ from NADH while converting dihydroxyacetone-P to glycerol-3-P, but Gpd2 is the most important for glycerol formation (Nissen et al. 2000). To overcome the various inhibitory substances, metabolic, genetic, evolutionary, and gene disruption strategies were used. For example, a *GPD2* mutant of *S. cerevisiae*, grown under anaerobic conditions, had a 40% reduction in glycerol levels (relative to the amount of substrate consumed) and 8% higher ethanol yield than the unmodified strain. Succinate is another by-product generated in ethanol production. In order to eliminate succinate formation in ethanologenic *E. coli* KO4 and consequently increase the ethanol yield, the fumarate reductase gene (*frd*) was deleted generating *E. coli* strain KO11. In this strain, the channeling of a small fraction of phosphoenolpyruvate toward the formation of succinate was avoided (Ohta et al. 1991).

For low-value products such as ethanol, a product concentration as high as possible is essential for the process economy. What normally occurs is that, as the ethanol concentration in the broth increases, most microorganisms begin to experience some impairment of membrane integrity. According to Dombek and Ingram (1986), the 24 h response to ethanol stress correlates with the type of lipids in the cellular membrane. In fact, the two well-known ethanologens, *S. cerevisiae* and *Z. mobilis*, display peculiar membrane structures. Thus, the membrane of *S. cerevisiae* is rich in sterols, whereas the membrane of *Z. mobilis* is exceptionally rich in the fatty acid *cis* vaccenic acid, as well as in compounds known as hopanoids (analogous to sterols). *S. cerevisiae* tolerates up to 21% (w/v) ethanol (Walker 1998), whereas *Z. mobilis* tolerates up to 12% (w/v) ethanol (Rogers et al. 1996). Besides the cell membrane composition, factors such as the activity of plasma membrane ATPase and superoxide dismutase and the capacity of a strain to produce trehalose contribute to the ethanol tolerance trait in yeasts (Jeffries and Jin 2000). Some candidate proteins involved in the expression of stress-related genes like the zinc finger protein (MacPherson et al. 2006) and alcohol sensitive ring/PHD finger 1 protein (Asr1p) (Betz et al. 2004) also play a role in ethanol tolerance in *Saccharomyces cerevisiae*. Lastly, the global transcription machinery engineering (gTME) technology can reprogram gene transcription and then improve glucose/ethanol tolerance of yeast cells.

16.7 Future Prospects

With industrial development growing rapidly and increasing demand for energy, there is an urgent need for environmentally friendly energy sources. Bioethanol is considered as an important renewable fuel to replace fossil fuels. Lignocellulosic bioethanol is a potential pathway for the global producers which provide renewable fuels. Lignocellulosic biomass represents as a promising candidate for ethanol production, including their output, input energy ratio, and their huge availability in both tropical and temperate regions.

In recent years, using metabolic engineering along with random mutagenesis techniques, advancement in terms of the enhancement of microorganism capabilities by adding/modifying traits such as tolerance to ethanol and inhibitors, hydrolysis of cellulose/hemicellulose, thermotolerance, reduced need nutrient supplementation, and improvement of sugar transport is underway. Further, improving pretreatment method and identifying metabolic pathways through genetic engineering for pentose fermentation, genomic sequencing, environmental genomics, and/or metagenomic technologies may assist to make bioethanol production more economical, practical, and commercially feasible.

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Third-Generation Biofuel: Algal Biofuels as a Sustainable Energy Source

17

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Abstract

Algal biofuels are third-generation biofuels which do not require agricultural land and potable water resources. Recently, culturing of microalgae as an alternative feedstock for biofuel production has received a lot of attention due to their fast growth rate and ability to accumulate high quantity of lipid and carbohydrate inside their cells for biodiesel and bioethanol production, respectively. Algae can grow in brackish, marine, and wastewater mostly unsuitable for cultivating of all of the traditional crops and a variety of climatic conditions. It can also grow in municipal, animal, and even industrial runoff and help in their purification. Autotrophic algae grow through photosynthesis – by converting plentiful available sunlight, CO_2, and available nutrients, including nitrogen, potash and phosphorous, magnesium, iron, calcium, and sodium into the vital biomaterial known as the green biomass. Most algae can grow or can be made to grow in the dark using fermentable simple sugars and the complex starch as "heterotrophic" growth or even in combine of both growth modes through the process called the "mixotrophic" growth. Attempts are made in this review to list some of the recent advances on algal biofuel production theory and practice citing various examples of establishments. The authors acknowledge the works of various companies cited in his paper with purely academic intention of providing wider perspective to readers.

Keywords

Algal biomass • Autotrophic • Biofuels • Fermentation • Lipids • Proteins

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17.1 Introduction

ALGAE (Alien to Light Green Antenna Entity) are one of the ever-prevailing but not utilized natural solutions to fuels as energy, economic, and climate challenges besides usage as food, feed, and pharmacy of global demands. Algae are very diverse micro- to macro-plants and found almost everywhere on the planet Earth. They play important role in many ecosystems to provide food, feed, and vital nutrients and also supply up to 60% of needed oxygen to all living beings vital for their survival. Algae as a promising everlasting source of fuel and other valuable products can double every few hours, can be harvested at will, and have potential to produce biomass and biofuel many times greater of most productive crops. Algae can be cultivated to produce a variety of products for large to small markets: biopolymers, bio-plastics, chemical feedstocks, lubricants, fertilizers, and even the cosmetics (http://tryforgood.com/members/dr-randhir-singh-gajraj/buddyblog/). Algae have been extensively studied for phototrophic synthesis of microbial oils using CO_2 as a carbon source. Fungi and yeast are emerging microbial oil producers because of their ability to use a variety of carbon sources (lactose, xylose, fatty acids, etc.) and relatively high growth rate. The algal biofuels are considered technically viable alternative energy source of third-generation biofuels (Rasala et al. 2010; Hallmann 2015; Singh et al. 2016; Rizza et al. 2017).

Diatoms; the green, red, and brown algae; and the cyanobacteria grow fast, do not compete with the agricultural lands and resources, and efficiently convert excessive amounts of *CO_2 into biomass*, thus participating in both *carbon fixation and organic chemical production* (Lam and Lee 2012).

For the past few years, vast research has been conducted on ethanol production from seaweeds using various yeasts and bacteria for hydrolysis and using enzymes for direct conversion of seaweeds to fermentable substrate (Lee and Lee 2016). Microalgae biomass production lies between 15 and 25 ton/ha/year. With an assumption of 30% lipid content in microalgae cells (without optimizing the growth condition), this is equivalent to a lipid production of 4.5–7.5 ton/ha/year (Tsukahara and Sawayama 2005). This amount is higher as compared to the production of oil from soybean (0.4 ton/ha/year), rapeseed (0.68 ton/ha/year), oil palm (3.62 ton/ha/year), and *Jatropha* (4.14 ton/ha/year) (Chisti 2007; Lam and Lee 2011). Thus, culturing microalgae for biodiesel production requires the least land area and holds an important key feature for effective and efficient land utilization (Lam and Lee 2012; Muniraj et al. 2013, 2015; Huang et al. 2013). Among potential biorefinery products, bioethanol from nonfood biomass could be used as a renewable fuel in the automotive sector (Tan and Lee 2016).

El-Dalatony et al. (2016) studied separate hydrolysis fermentation (SHF) and simultaneous saccharification fermentation (SSF) processes for bioethanol production from microalgal biomass. SSF was selected as an efficient process to enhance the bioethanol yield through repeated batches using immobilized yeast cells. Combined sonication and enzymatic hydrolysis of green alga *Chlamydomonas mexicana* generated 10.5 and 8.48 g/L of ethanol in SSF and SHF, respectively.

17.1.1 Algal Feedstocks

Algae are simple plants ranging from microscopic (microalgae) to large seaweeds (macroalgae), from small green dots to larger cyanobacteria, and the tinsel diatoms. There are nine major groups of algae which are cyanobacteria (*Cyanophyceae*), green algae (Chlorophyceae), diatoms (Bacillariophyceae), yellow-green algae (Xanthophyceae), golden algae (Chrysophyceae), red algae (Rhodophyceae), brown algae (Phaeophyceae), dinoflagellates (Dinophyceae), and "picoplankton" (Prasinophyceae and Eustigmatophyceae) (Hu et al. 2008). Among these nine groups, the green algae are the largest taxonomic group. Microalgae have been known to survive under a wide range of conditions. Under optimal algal culture and growth conditions, lipid content can be increased from 20% to 50% on fresh weight, and 5%, to 20% on dry weight basis (Hu et al. 2008).

Algal feedstocks are considered one of the most promising nonfood feedstocks for biofuels. Nearly 7.5–8 million tons of wet seaweeds are harvested worldwide per year (SubbaRao and Mantri 2006) The macroalgae Phaeophyta (brown algae), Rhodophyta (red weight, and algae), and Chlorophyta (green algae) are distributed worldwide and have high biomass productivity (Yanagisawa et al. 2011). Brown algae such as sea mustard (*Undaria pinnatifida*) and kelp (*Saccharina japonica*) are one of the promising biomass for biofuel production because cultivation productivity based on area is the highest among three types of macroalgae (Aizawa et al. 2007; Jung et al. 2013). They yield approximately 40 kg wet biomass/m^2 of gulfweed (*Sargassum muticum*) compared to 2.3 and 6.6 kg/m^2 of green laver (*Ulva lactuca*) and agar weed (*Gelidium amansii*), respectively (Lee and Lee 2016). According to Lee and Lee (2016), the large-scale cultivation of brown algae is already practiced in several countries including Korea, China, and Japan. Compared to microalgae, brown algae have a higher sugar and lower oil contents and are more suitable feedstock for bioethanol production than biodiesel. Saccharification of brown algae biomass is relatively easy due to the absence of lignin. Mannitol and glucose from laminarin (a form of glucan in brown algae) are normal sugars that are efficiently used for bioethanol fermentation (Horn et al. 2000; Lee et al. 2013). According to Lee and Lee (2016), metabolic engineering of brown algae for production of carbohydrates and conversion of those sugars to bioethanol will play a key role in the commercialization of bioethanol production from brown algae. These metabolically engineered microbes will be able to efficiently utilize alginate. *Gracilaria verrucosa*, a red alga from phylum Rhodophyta, contains lignocellulosic biomass comprised of rigid cellulose-based cell walls which can be hydrolyzed to sugars for subsequent fermentation to ethanol and/or biochemicals (Goh and Lee 2010). Shukla et al. (2016) collected the algal biomass of different species of *Gracilaria* and reported that *G. verrucosa* from coasts of Orissa and Tamil Nadu, India, is better in terms of total fermentable carbohydrate content (56.65%).

Microalgae are recognized as one of the oldest living microorganisms on Earth (Song et al. 2008). Microalgae like *Spirulina*, *Chlorella*, *Dunaliella*, and *Haematococcus* are currently cultivated commercially to produce

photosynthetically grown biomass from a few tons to several hundred tons annually. They grow at an exceptional fast rate, 100 times faster than terrestrial plants, and they can double their biomass in less than 1 day (Tredici 2010). Hence, they are the very promising renewable bio-resources for bioethanol production.

17.1.1.1 Cyanobacteria

Cyanobacteria are part of marine and freshwater phytoplankton. Sarsekeyeva et al. (2015) reviewed the recent achievements in the developments and production of cyanofuels—biofuels produced from cyanobacterial biomass. Many naturally occurring cyanobacterial species synthesize a vast range of fatty acid structures. Cyanobacterial fatty acids (FAs) are mainly represented by C16 and C18 species with 0–3 double bonds: 16:0, 16:1, 18:1, 18:2, and 18:3 (Los and Mironov 2015). Ideally, for the production of cyanodiesel, a cyanobacterial strain should be reaching C12–C18 medium-chain saturated and monounsaturated FAs. Some species, however, may have predominant C14 and C16 saturated and monounsaturated FAs (Sarsekeyeva et al. 2014). Such cyanofuel, composed of C14 and C16 saturated and monounsaturated FFAs, will yield high cetane number and low iodine values. Iodine values are the indicators of proper ignition and combustion quality, low deposit formation, and reduced lubricant degradation of a biofuel (Hoekman et al. 2012). The FA profiles of engineered *Synechococcus* (Ruffing and Jones 2012) or naturally occurring *Cyanobacterium* sp. (Sarsekeyeva et al. 2014) look quite appropriate for conversion to cyanodiesel.

17.1.1.2 Algal Genomics

The metabolic engineering strategies enable efficient biofuel production platforms, including the evaluation of thermodynamic feasibility of pathways, carbon flux redirection, manipulation of cellular energetics, use of waste carbon sources, engineering substrate uptake mechanism, removal of final products from culture broth to alleviate end-product inhibition and cell toxicity, optimization of process parameters, etc. (Caspeta and Nielsen 2013). Genetic modifications of cyanobacterial cells allow for conversion of the fixed atmospheric carbon not just into a crude biomass but also into desired end products, which are most suitable for the production of biofuels (Quintana et al. 2011). These genetically engineered cyanobacteria can secrete metabolic end products, alkanes, or free fatty acids (FFAs), into the culture medium. Thus, costly stages of cell collection and disruption to extract the products may be skipped (Peralta-Yahya et al. 2012).

17.1.1.3 Idea of Algal Fuel

The idea of using algae as a source of energy goes back more than half a century. They integrate the merits of microbes including rapid growth and ease of culture with those of higher plants in performing posttranslational modification and photosynthesis (Franklin and Mayfield 2005; Potvin and Zhang 2010; Specht et al. 2010). Use of transgenic microalgae for production of recombinant proteins has lagged behind other microorganisms, such as bacteria and yeasts (Specht et al. 2010), but

recently there has been a surge of interest in microalgae as a viable bioproduction system (Rasala et al. 2010). The majority of current work is performed with the well-characterized green unicellular alga, *Chlamydomonas reinhardtii* (Potvin and Zhang 2010; Rosenberg et al. 2008; Rasala et al. 2010).

17.1.1.4 Algal Production Systems

Autotrophic microalgae are cultivated mostly on land in large open ponds, or in enclosed so-called photobioreactors, using enriched CO_2 and other needed vital nutrients. The CO_2 can come in the form of flue gases or the greenhouse gas from power plants or be obtained from other fossil fuel combustion or the biological processes like the biogas plants, etc. Heterotrophic microalgae are grown in enclosed fermenters using sugar or starch, similar to the corn and sugarcane or beetroot ethanol fermentation already providing almost 5–20 % of liquid transportation fuels globally. Algae cultivation uses mostly the barren lands that in many cases are unsuitable for all of the traditional agriculture, as well as water sources that are not useable for other crops, such as sea, brackish, and wastewater. Algae thrive in nutrient-rich waters like municipal wastewaters (sewage), animal wastes, and many an industrial effluents and at the same time purify wastewaters while producing a biomass suitable for biofuels and protein-rich feed production. With more than 100 start-ups and large corporations, along with the US government, investing billions in this new industry, the USA is the leader in advancing algae-based fuels. Now algae biofuels are also being researched around the world in developing nations in Asia, Africa, and elsewhere.

Heterotrophic microalgae are grown in enclosed fermenters using sugar or starch, similar to the corn and sugarcane or beetroot ethanol fermentation already providing almost 5–20 % of liquid transportation fuels globally.

17.1.1.5 Green Alga *Chlorella* Cultivation

The choice of algae species should address specific characteristics that allow the use of flue gas as the CO_2 source. Much research has been done on the tolerance of different species to flue gases. Several species were found to be suitable for the growth of algae using flue gas. One of these many species is *Chlorella* species. Hanagata et al. (1992) found that *Chlorella* is tolerant to CO_2 concentrations of up to 40% by volume. Sung et al. (1999) reported that *Chlorella* grew in conditions of up to 40°C. These results indicate that *Chlorella* is a good choice for this study. The oil content of *Chlorella* typically ranges between 28% and 32% dry weight (Chisti 2007) but can reach 46% dry weight under stress conditions (Hu et al. 2008) and 55% dry weight when grown heterotrophically (Miao and Wu 2006). Information about the fatty acid compositions of various microalgae (namely, the green algae in the classes Chlorophyceae and Prasinophyceae) was published in 1992 (Dunstan et al. 1992). *Chlorella* is in the class Chlorophyceae, and the fatty acid compositions of three *Chlorella* species were listed. *Chlorella* sp. (CS-195) was used in this analysis because of its potential ease for use in simulation. It is interesting to note that the *Chlorella*

protothecoides (CS-41) composition includes the same fatty acids present in the *Chlorella* sp. chosen (Dunstan et al. 1992) but in slightly different proportions. Another reason why *Chlorella* sp. was chosen is the availability of information about its growth, harvesting, and extraction.

17.1.1.6 Production Process of Biodiesel from Alage

Miao and Wu (2006) have shown that a species of *Chlorella* (*Chlorella protothecoides*) can be used to produce biodiesel that meets ASTM standards. The microalgae were grown heterotrophically to increase the oil content from 14.6% dry weight to 55.2% dry weight. Acid transesterification was used since the acid value for the algal oil was reported as 8.97 mg KOH/g. The biodiesel yield was approximately 70% at 50 °C and conditions of 60% H_2SO_4 catalyst, 5-h reaction time, 160 rpm, 9.12 g microalgal oil, and 30:1 methanol-to-oil ratio (Miao and Wu 2006).

17.1.1.7 Algal Bioreactors

Algae production technologies in their growth and underdevelopment phases harbor open ponds to closed and complex photobioreactors, the simple fermenters to high-tech hybrid systems, to some that combine these various needs and methods of their mass production today. The idea of using algae as a source of energy goes back more than half a century. From 1980 to 1996, the US Department of Energy supported the Aquatic Species Program (ASP), a relatively small effort (about $25 million over almost 20 years) with the specific goal of producing oil from microalgae. Granata (2017) reviewed algal production rates for biofuels and commercial products. They reported open bioreactors, such as ponds and raceway flumes, and closed bioreactors, such as vertical, horizontal, and helical tubes, flat plates, and other unique designs. Obtaining the high productivities required for algal biodiesel production at low cost is likely only to be possible in open raceway reactor systems (Greenwell et al. 2010). Although closed photobioreactor systems offer advantages (avoidance of contamination, high algal concentrations and productivities, better control of culture conditions), only a very limited number of commercial systems have been developed. For biofuel applications, they may provide a contaminant-free inoculum for raceway systems (Greenwell et al. 2010; Malcata 2011). Operation of these systems is function of their location and desired end product.

17.1.1.8 Harvesting Method

The harvesting method can be manual to machine oriented to get biomass for food, feed, biopolymers, bio-pigments, or the nutraceuticals or biofuel makings. Open pond systems for the production of biofuels are already under development. For example, Sapphire Energy is developing a large commercial-scale open pond production facility in the southern New Mexico and is aiming to produce millions of gallons of fuel annually. Similar in zone is the Algenol and the Solazyme in the USA.

17.1.1.9 Fermentation Bases

Alternative approach to growing algae besides using sunlight is growing them in the dark on simple or complex but broken down sugars, in the so-called "heterotrophic" fermentation. Algae convert these simple sugars to oil and biomass, which can be converted into biofuels and other vital bioproducts of immense usage in many industries globally. Algenol and Solazyme produce renewable ethanol and vital oils for the chemicals, nutrition, and skin and personal care space utilizing today's existing industrial-scale fermentation capacity very closely knit to corn- and molasses-based ethanol units in the USA and Brazil. The "hybrid" systems and processes that combine two or more of the above methods maximize the individual advantages of each process. A company named Pycal, with operations in Hawaii, and the new entrant from India, Sabran Bioenttri, use innovative H-CAB (hydrogen-chemicals and algal biofuel) type of integrated algal growth module systems to cultivate and use algae for food, feed, and biofuels. In hybrid algal systems, algae can treat wastewater by absorbing waste nutrients and waste marble stone-based CO_2, breaking down and removing toxic materials (www.sabranbioenttri.com).

H-CAB is an innovative first of its kind Sāmbhar Lake algae-based bio-module, which ably and efficiently can use and consume all the alkali and acid and other inorganic minerals as used in the process of scouring and resizing of cotton fabric used later to get bloc prints, and thus it releases harmful high COD and BOD laded wastewater at the Sanganer and Bagru bloc print industries near pink city Jaipur. Sabran Bioenttri Pvt. Ltd. which modulated H-CAB system can generate hydrogen gas as clean energy source with other biochemical, natural pigments, and algal biomass as feedstock to make biodiesel, bioethanol, Green Fodder Forever, the Cattlact feed, bio-ink from algae, and biomanure for agro-usage. H-CAB work is in progress to set up its prototype at the banks of Sāmbhar Lake in the state of Rajasthan, India.

17.1.1.10 Extraction

The lipids of algae are located within the cell; the exception is *Botryococcus braunii*, which secretes the lipids through the cell wall but has a low productivity. Lipids cannot be recovered effectively from algae using methods designed for oil extrusion from crops such as soybeans, due to the small size of the algae and the mechanical strength of their walls. The intracellular location of the lipids requires that the algae be economically harvested and the oil recovered by breakage of the algal cell wall. These are two of the most significant issues in commercializing algal biodiesel production. Typical separation methods such as centrifugation, filtration, and chemical flocculation are expensive or introduce Al^{3+} as a contaminant (Lee et al. 2010a, b). A recent review of extraction methods indicates that mechanical methods are optimal, but these have a high specific energy consumption, which exceeds the energy content available from the extracted lipid (Lee et al. 2012).

17.1.1.11 Fuel Milking

Many companies work to modify algae to produce biofuels through "excretion," thus making algal growth and biofuel happening in a parallel mode to save time and energy and get valuable biofuels quite rapidly. This approach has the advantage of

requiring only an initial amount of algal biomass that would continually produce oils, avoiding the need for harvesting and processing which can add value to the overall cost. Ethanol, butanol, fatty acids, hydrocarbons, gaseous fuels, etc., can be excreted by modifying the algae via GMO route. Algenol is a global leader, and the Synthetic Genomics is working with ExxonMobil on this type of approach.

17.1.1.12 Protein Biofuels

Microalgae also offer potential for large-scale and cost-effective production of recombinant proteins. Huo et al. (2011) demonstrated the feasibility of using proteins as raw material for biorefining by applying a nitrogen-centric metabolic engineering strategy. They integrate the merits of microbes including rapid growth and ease of culture with those of higher plants in performing posttranslational modification and photosynthesis (Xu et al. 2012).

The large-scale protein production using microalgae, natural selection under frequent or continuous harvesting conditions will favor fast-growing and robust microorganisms, which generally contain high-protein contents and are fully adapted to the local environment. Biofuels are currently produced from carbohydrates and lipids in feedstock. Proteins, in contrast, have not been used to synthesize fuels because of the difficulties of deaminating protein hydrolysates. Recombinant strategies could also be used to produce enzymes of biofuel production in large scale. Huo et al. (2011) applied metabolic engineering to generate *Escherichia coli* that can deaminate protein hydrolysates, enabling the cells to convert proteins to C4 and C5 alcohols at 56% of the theoretical yield. They introduced three exogenous transamination and deamination cycles, which provided irreversible metabolic force to drive deamination reactions to completion.

Huo et al. (2011) demonstrated that *Saccharomyces cerevisiae*, *E. coli*, *Bacillus subtilis*, and microalgae can be used as protein sources, producing up to 4035 mg/l of alcohols from biomass containing ~22 g/l of amino acids. These results show the feasibility of using proteins for biorefineries, for which high-protein microalgae could be used as a feedstock with a possibility of maximizing algal growth and total CO_2 fixation.

To test the feasibility of using algal and bacterial proteins as a feedstock, Huo et al. (2011) grew green algae *Chlorella vulgaris*, red algae *Porphyridium purpureum*, green-blue algae *Spirulina platensis*, and cyanobacterium *Synechococcus elongatus*, as well as *E. coli* and *B. subtilis*, in 1-L flasks or 30-L fish tanks. Biomass was collected, digested with protease, and used as feedstock for biofuel production with the engineered *E. coli* strain YH83. In all experiments, the protein concentration was adjusted to 21.6 g/l, which is equivalent to the amount of protein in 4% yeast extract.

17.1.1.13 Hybrid Algal Systems

Algae can treat wastewater by absorbing nutrients and CO_2, breaking down and removing toxic materials. The algal biomass produced during wastewater treatment processes can then be used to generate methane, produce fertilizers and animal and

aqua feed, and yield oil and other liquid fuels. By removing nutrients, algae efficiently check algal blooms in lakes and coastal waters solving a major environmental issue. Companies like the BioProcess Algae and Accelergy Corporation have their proprietary biological carbon capture and recycle technology, which passes CO_2 (from ethanol making or a coal power plant) through photobioreactors for growing concentrated algae for varied usages (https://www.eia.gov).

17.1.1.14 Biodiesel from Algae

Biodiesel is an alternative fuel, produced from algal renewable resources that can be produced via transesterification process. It contains methyl esters and no petroleum, thus can be blended at any level with petroleum diesel to create a biodiesel blend. Biodiesel is simple to use, biodegradable, nontoxic, and essentially free of sulfur and aromatics and can be blended with regular diesel fuel in almost any proportion. By-product glycerine is usually sold to be used in soaps and other products.

Algae are a logical source from which to make biodiesel, as the oil found inside algal cells is similar to other vegetable oils like rapeseed, soy, and canola and can easily be transformed into biodiesel. Algae grow much faster and yields more oils as well in a well sunlit area.

17.1.1.15 Green Gasoline

Algae are a feedstock for gasoline alternative, referred to as "green gasoline." Green gasoline chemically mimics the properties of petroleum-based gasoline. As a result, green gasoline could be used at any blend level, in any gasoline engine.

17.1.1.16 Alcohol Fuels

Algae produce ethanol, currently produced in the USA, India, and Brazil from corn and molasses, with billions of liters produced so far. Most ethanol is blended into gasoline and then sold at a 5 to 10 to 20% level referred to as E5, E10, and E20.

E85 a blend of 85 % ethanol and 15% traditional gasoline is an alternative fuel designed for use in flexible-fuel vehicles (FFVs). There are more than 8.5 million FFVs on North and South America's roads today.

Algenol Biofuels in the USA with GMO algae and on a 36-acre integrated biorefinery in Florida produces up to 100,000 gallons of algae-based ethanol each year. Another approach is to grow algae that produce high amounts of starch that could be fermented into ethanol (similar to how corn-based ethanol is produced).

Algae-based alcohol fuel butanol is a brainchild of DuPont and Bio Architecture Lab, in California, under an $8.8 million Department of Energy USA grant.

Hydrogen production from algae has been studied for many years and could be successful with continued research and development. Microalgae are used to produce hydrogen now and in the future, avoiding the need for the production of sugars by traditional crops. All in all, the potential of algae to produce a variety of fuels is yet to be fully explored. Sabran Bioenttri from India is in its R&D phase to give shape and impetus to hydrogen, bioethanol, and biodiesel making via their proposed

H-CAB (hydrogen-chemicals and algal biofuels) from algal sources by using waste chemicals and waters of bloc print Sanganer-based industries.

The focus of today's algae industry is on bringing the advances of science and technology for the production of algae products into the marketplace. These efforts are proceeding quickly, primarily driven by those that recognize algae's high per-acre yield and its suitability for making a variety of different products, from the small-volume high-value to the large-volume commodities such as fuels and feeds.

In recent years, overall investments into this space, private and public, exceed billions of dollars. This growth trend is now playing out globally. Many companies believe they are only a few years away from commercial production.

17.1.1.17 Methane

The algal biomass produced during wastewater treatment processes can then be used to generate methane, to produce fertilizers and animal and aqua feed, and to yield oil and other liquid fuels. By removing nutrients, algae efficiently check algal blooms in lakes and coastal waters solving a major environmental issue. Companies like the BioProcess Algae and Accelergy Corporation have their proprietary biological carbon capture and recycle technology, which passes CO_2 (from ethanol making or a coal power plant) through photobioreactors for growing concentrated algae for varied usages.

Production of glycerol and methane gas from algae was proposed in the early 1950s and received a big impetus during the energy crisis of the 1970s, when projects were initiated to produce gaseous fuels (hydrogen and methane). From 1980 to 1996, the US Department of Energy supported the Aquatic Species Program (ASP), a relatively small effort (about $25 million over almost 20 years) with the specific goal of producing oil from microalgae. The ASP researchers grew algae in open ponds, making initial contributions to our understanding of growing algae for fuel. Different species were isolated and tested, the impacts of different organic and inorganic nutrient and CO_2 concentrations were done and documented, and engineering challenges of mass-producing algae were addressed. A solid foundation of algae fuel research was built. To stop dependency on imported fuels and curb greenhouse gas emission, advances in biotechnology, such as the ability to genetically engineer algae to produce more oils and convert solar energy more efficiently, have opened new possibilities not feasible in earlier ASP days. Most of the activity in algae research and commercial production has been in the USA, Australia, and Europe. With more than 100 start-ups and large corporations, along with the US government, investing billions in this new industry, the USA is the leader in advancing algae-based fuels (www.grants.gov). Now algae biofuels are also being researched around the world in developing nations in Asia, Africa, and elsewhere. One of the key advantages of using algae as a feedstock for biofuels is that they can be used to produce many different types of fuel. Whether it's biodiesel, ethanol, biojet fuel,

green gasoline, or others, algae have the ability to meet our transportation fuel needs now or in the future, here on Earth or in space explorations on Mars, etc.

17.1.1.18 Growing Interest

Algae biofuel production is increasingly attracting the attention of large corporations. ExxonMobil and Reliance industries from India are perhaps the best example as they have allocated up to 800 plus million dollars for algae biofuel research and development over an initial 5-year time period.

Governments are also increasingly involved, as they too recognize the economic, environmental, and national security benefits of homegrown energy. The US Federal Government and European Union support, in the form of R&D, grants, and loan guarantees, now approaches billions of dollars. State and local governments have added several hundred million dollars to help establish algal production projects.

The Indian government is also ahead in this path via BIRAC and DBT and CSIR channels in grant and soft loan funding. At the federal and government levels, there is mounting support for policies that promote clean energy overall, without favoring one technology over another (www.birac.nic.in; www.dst.gov.in; and www.dbtindia.nic.in).

The future of algae production will be determined by the size of the market it serves. Several initiatives are underway to support a strong marketplace for algal fuels in the hope that this will accelerate true commercial production.

One of the earliest large markets for algae biofuels are the military and space explorations of various countries. Besides algal biofuel, demand for other algae-derived products is growing. In 2010, only 9–13 % of chemical sales were bio-based, a segment that is expected to grow to 22–28% of total chemical sales by 2025 that translates into a market size of $483–$614 billion in the USA alone (Figs. 17.1, 17.2, 17.3, 17.4, and 17.5).

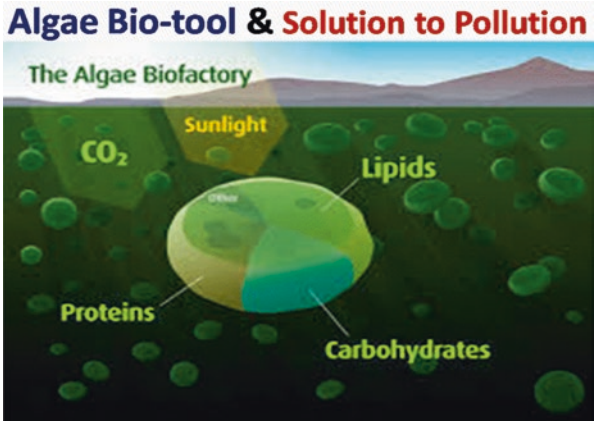

Fig. 17.1 Glimpse of usage of algae as tool to solve pollution issues

Fig. 17.2 Algal products and algae tech module H-CAB of Sabran Bioenttri, Mumbai

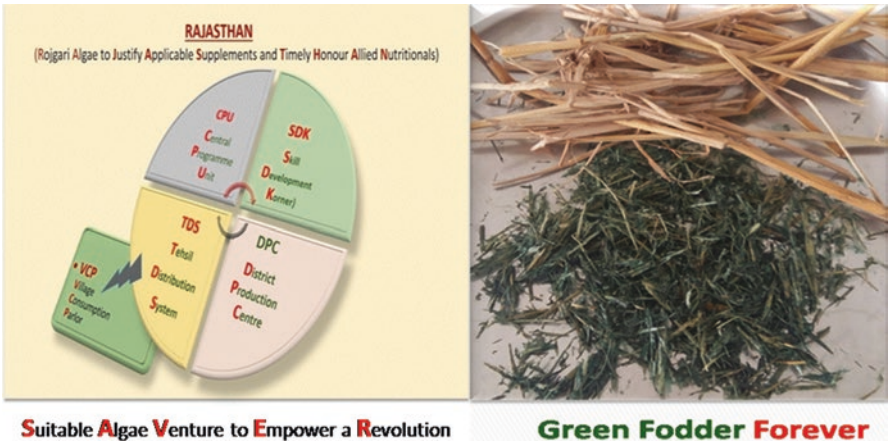

Fig. 17.3 H-CAB based and ideated RAJSTHAN venture and Green Fodder Forever

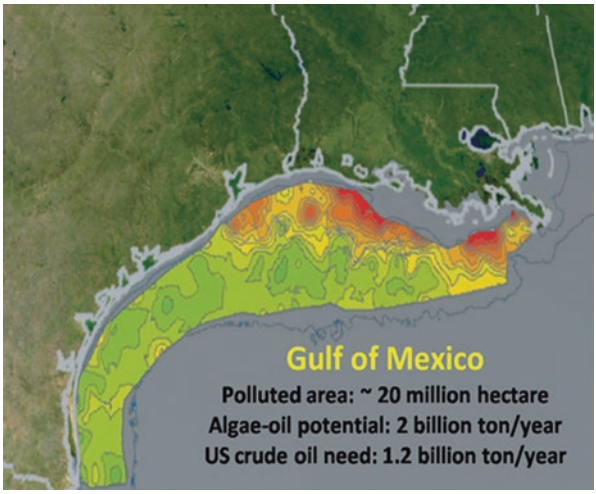

Fig. 17.4 An overview of the potential of algae as source of oil by wastewater usage in the USA

Fig. 17.5 Algae-based innovative inroads and path adopted by Sabran Bioenttri Pvt. Ltd

17.2 Discussion

Microalgal oils represent another attractive option for biodiesel production because microalgae require less arable land than plant-based feedstocks. According to Liu et al. (2011), oleaginous algae are most popular in the microbial biofuel field because they have the ability to produce substantial amounts of triacylglycerols (TAG) as a storage lipid. However, production of TAG by microalgae requires environmental stresses, which makes the process complicated and costly (Hu et al. 2008). Algae can be grown in many ways—in freshwater, saltwater, or wastewater and in closed photobioreactors or open ponds. Although one key advantage of algae is that its cultivation does not require cropland but other resources are needed, the amounts of these resources vary widely from one algae production pathway to another (Razeghifard 2013).

The use of microalgae as a source of biofuel raw materials and for wastewater treatment has recently gained enormous research interest. This is due to several reasons: microalgae are able to convert CO_2 into biofuel stocks, as well as food, feeds, and high-value products (Chisti 2007; Chen and Dixon 2007; Klein-Marcuschamer et al. 2013). Bilad et al. (2014) suggested that membrane process optimizations are still required when they are applied to enable microalgae biomass bulk production to become competitive as a raw material for biofuel production.

Besides microalgae-based carbohydrates occur primarily in the form of starch and cellulose without lignin, which are much easier to convert to monosaccharides units, offering a comprehensive advantage over other biomass feedstock such as lignocellulosic materials (Singh et al. 2016).

However, ethanol production from algal biomass also requires pretreatment to reduce substrate recalcitrance and enhance accessibility of starch to fermentative microorganisms for bioethanol production (Gallego et al. 2015). Various pretreatment processes like sonication and enzymatic hydrolysis have been extensively utilized for cell lysis and subsequent conversion of residual cellulose to monomeric sugars (Kumar and Sharma 2017).

El-Dalatony et al. (2016) studied separate hydrolysis fermentation (SHF) and simultaneous saccharification fermentation (SSF) processes for bioethanol production from microalgal biomass. SSF was selected as an efficient process to enhance the bioethanol yield through repeated batches using immobilized yeast cells. Immobilized yeast cells enabled repetitive production of ethanol for cycles displaying a fermentation efficiency up to 79% for five consecutive cycles. Combined sonication and enzymatic hydrolysis of *Chlamydomonas mexicana* generated 10.5 and 8.48 g/L of ethanol in SSF and SHF, respectively. A total energy recovery of 85.81% was achieved from microalgal biomass in the form of bioethanol.

Besides as a source of food, feed, pharmacy-based product and many by-product deliveries, algae contain high levels of oils, carbohydrates, and simple to complex sugars that can be used to produce renewable fuels in line of biodiesel, ethanol, gasoline, methane gas, and butanol. In fact, algae, both micro- and macroalgae (seaweeds), are already widely used in the growing energy and biofuel needs of the world.

Microalgae can be a source of lipids (including triglycerides and fatty acids) for conversion to biodiesel. After oil extraction, the remaining algal biomass can be dried and "pelletized" and used as fuel that is burned in industrial boilers and other power generation means. Extraction of intracellular lipids from intact algae is difficult as the lipids are bound within cell membranes and cell disruption is required to maximize lipid recovery (Lee et al. 2010a, b). Obtaining the high productivities required for algal biodiesel production at low cost is likely only to be possible in open raceway reactor systems (Greenwell et al. 2010). A real yield of algal lipids can be higher than terrestrial oil crops (Griffiths and Harrison 2009) (e.g., 2650 gal/acre/year for *Pleurochrysis carterae* (Moheimani and Borowitzka 2006) compared with palm oil at 400–600 gal/acre/year and soybean at 40 gal/acre/year). Algae can power ecosystems, houses, factories, and vehicles, can recycle CO_2 and wasteful nutrients besides providing nutrition and products for animals and people, are also assisting in creating jobs for millions, and shall save Earth's climate too by sequestration of the PRIME greenhouse gas, the CO_2. Food and energy needs in outer space are to be met by algae sooner or later on the Moon, Mars, etc. Algae are emerging to be one of the most promising long-term, sustainable sources of green biomass and oils for fuel and other coproducts. What makes them so very attractive are the large number and wide variety of benefits associated with them. Nearly all these benefits stem from the fact that these plants have evolved over 3.2 billions of years to produce and store energy in the form of oil, and they do this more efficiently than any other known natural or bio-engineered process.

According to Sheridan (2013), algae-based biofuels coming along biofuels derived from photosynthetic algae are at an earlier stage of development, although several firms, including Cellana, Joule Unlimited, Sapphire Energy, and S. San Francisco, California-based Solazyme, are still advancing their respective technologies. Synthetic Genomics and ExxonMobil are still engaged in basic research program involving the application of synthetic genomic technologies to improve the production characteristics of algae.

The protein biomass could also be used by anaerobic digestion for biogas production or thermochemical treatment for heat production, but existing processes are not able to produce liquid fuels, bulk chemicals, or pharmaceutical intermediates, specifically without high-pressure and high-temperature conditions. Perhaps biorefining scheme can bypass the need for expensive photobioreactors or the lignocellulose recalcitrance problem by using protein biomass from algal cultures, waste biotreatment, and the fermentation industry as a long-term, sustainable protein source. However, several challenges remain to be addressed, including large-scale algal production, harvesting, product purification, and nitrogen recycling. Besides energy and economic factors associated with the above challenges will need to be considered as the technology moves into practical application. The potential advantages of using proteins as raw material for biorefining will stimulate development in this direction.

Current biorefinery schemes are suboptimal for several reasons. First, schemes based on algae have limited efficiency because cultures used for biofuel production must be starved so that they produce lipid feedstocks, resulting in less cell growth and less total CO_2 fixation. Second, all existing schemes, including sugar-based or cellulosic biorefining, lead to the accumulation of protein by-products, but there are no strategies to convert these by-products into liquid fuels. These protein by-products are typically used as animal feed. But despite the current profitability of animal feed, the feed market has a limited ability to absorb the increasing protein by-products from the fast-expanding biorefinery industry. Third, in all existing schemes, reduced nitrogen is not recycled, resulting in a net loss of reduced nitrogen (Miller 2010) and an increase in nitrous oxide production, which is a greenhouse gas almost 300 times more potent than CO_2 (Crutzen et al. 2008). Moreover, the lost reduced nitrogen must be replaced by supplementing future crops with reduced fertilizer nitrogen, which is produced by the energy-intensive and environmentally unfriendly Haber-Bosch process (Huo et al. 2014).

17.3 Summary

Billions of dollars are being injected in research and development of algae-based biotechnologies in replacing fossil fuels: “old algae” with “new algae” advance biofuels. Nearly all these benefits stem from the fact that these plants have evolved over 3.2 billions of years to produce and store energy in the form of oil, and they do this

more efficiently than any other known natural or bio-engineered process. Algae replicate and reproduce quickly and need only sunlight (or another form of energy, like fermentable sugars), water, carbon dioxide, and inorganic nutrients to grow and sustain their growth and accumulate oil, carbohydrates, natural pigments, vitamins, minerals, the very vital proteins, and omega-3 and omega-6 fatty acids. After oil extraction, the remaining algal biomass can be dried and "pelletized" and used as fuel that is burned in industrial boilers and other power generation means. Halophytes and algae are fast becoming biomass plant matter of interest in aviation fueling studies. The potentially high oil yields of algae are up to 150 times that of soybeans. If marine Alage are grown along with halophytes on coastal areas (see Chap. 13 this volume) it conserves both arable land and fresh water and produces significant amount of biomass for biofuel production. Developments for synthetic and biomass terrestrial fueling are applicable to space missions where very harsh environments will require processing and reprocessing of wastes as sources of energy. Algae-based food and feed and energy-based need can be summarized as national energy security in terms of own customized biofuel as energy source; economic security in the form of food, feed, fertilizer, pharmacy product, and jobs; and climate change restrain by sequestering greenhouse gases for the good of all. However, there is much work to be done on how to use algae, halophytes, and symbiotic bacteria (e.g., cyanobacteria) on space missions. However, alga-based production is challenging with regard to obtaining productivities and separation processes that can meet the requirement for an economically viable process.

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Recent Progress in the Genetic Engineering of Biofuel Crops

18

Kinya Akashi and Yoshihiko Nanasato

Abstract

The utilization of biomass energy is increasingly considered as a promising means for the sustainable supply of energy and for long-term conservation of the global environment. In order to achieve the effective production of biomass-based energy, a key challenge will be the breeding of biofuel crops that enable high and stable biomass production. In this context, genetic engineering to optimize metabolism, create value-added biomass production, and enable environmental adaptability for growth on marginal land will be instrumental for establishing the next generation of biofuel crops. This review focuses on recent progress in the development of dedicated biofuel crops by means of genetic engineering, particularly switchgrass for lignocellulosic feedstock and jatropha and camelina for biodiesel feedstock.

Keywords

Arid region • Biofuel crop • Biomass production • Genetic engineering • Transgenic plants

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18.1 Introduction

Global energy demand has increased rapidly in the last decade, and securing a sustainable energy supply for the future constitutes an urgent challenge both in developed and developing countries. Owing to its abundance, accessibility, versatility, and renewability, plant biomass energy has been recognized as a potential solution for this issue (Yuan et al. 2008). Bioenergy from various biofuel crops is already making an important contribution to meet global energy demand, in the form of heat, electricity, and transport fuels (Dudley 2014). Based on the type of principal chemical compound used for energy productions, biofuel crops can be classified into four groups:

- Sugar-producing crops, e.g., sugarcane (*Saccharum officinarum*) and sweet sorghum (*Sorghum bicolor*)
- Starch-producing crops, e.g., maize (*Zea mays*) and cassava (*Manihot esculenta*)
- Lignocellulosic biomass crops, e.g., switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus giganteus*), and poplar (*Populus* spp.)
- Oilseed crops for biodiesel, e.g., soybean (*Glycine max*), canola (*Brassica napus*), camelina (*Camelina sativa*), and jatropha (*Jatropha curcas*)

Currently, a large proportion of biofuel feedstock is derived from sugar- or starch-producing edible crops, which could potentially cause competition with food production over resources such as lands and water. In contrast, bioethanol production from lignocellulose, which is a more abundant resource, remains to be fully developed. This is partly because of the recalcitrant nature of lignocellulose, which requires harsh physicochemical pretreatments and/or enzymatic degradation before ethanol fermentation and, therefore, a higher associated cost. Technical innovation to overcome these limitations has been one of the central issues in biofuel research. Other challenges in biofuel development include the generation of more productive biofuel crops and the expansion of cultivation areas to unused arid lands. The development of biofuel crops with higher yields and better stress resistance is expected to play a central role in this process of innovation. Together with fundamental research into germplasm exploration and conventional breeding, genetic engineering has been increasingly recognized as an alternative approach for the improvement of agronomical traits in biofuel crops (Fig. 18.1). In this chapter, we focus on technological innovations in the genetic engineering of biofuel crops that have been applied to improve various aspects of their agronomic traits (Table 18.1).

18.2 Development for Genetic Engineering in Biofuel Crops

To explore the potential of genetic engineering for biofuel crops, the establishment of efficient transformation protocols in a given plant species is of crucial importance. Plant transformation procedures are composed of several elaborate steps

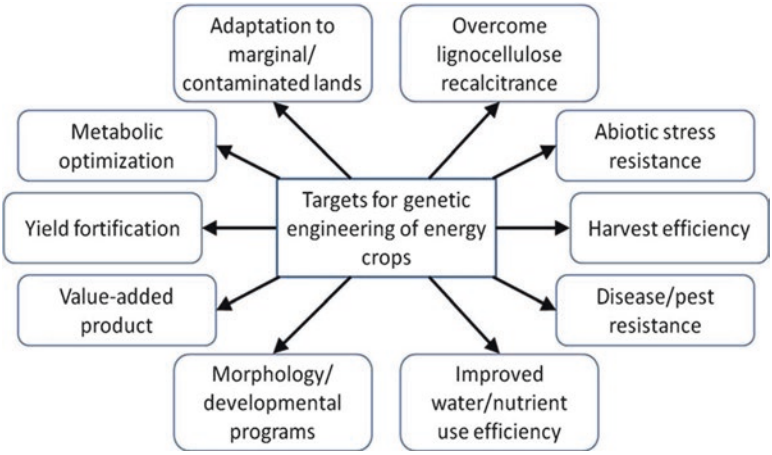

Fig. 18.1 Targets for genetic engineering of energy crops. Sustainable and profitable production of energy crops depends on the availability of varieties with suitable agronomic traits, and genetic engineering potentially contributes to the enhancement of existing genotypes to maximize the productivity of large-scale biofuel production

including design of foreign gene vector constructs, gene delivery into the selected plant tissues, regeneration of the transformed cell/tissues, and the selection of stable transgenic plants with the desired traits. Because the establishment of a transformation procedure requires extensive effort to optimize the experimental conditions for each step, it is not surprising that transformation technologies are relatively more advanced in traditional edible crops with longer research histories, such as maize (Rhodes et al. 1988; Que et al. 2014), soybean (Chee et al. 1989; Homrich et al. 2012), sorghum (Howe et al. 2006; Girijashankar and Swathisree 2009), and sugarcane (Manickavasagam et al. 2004; Mayavan et al. 2013).

Encouragingly, in most of the newly proposed biofuel crops, major progress has been made recently in the development and optimization of transformation protocols. Recently established examples include those for switchgrass (Somleva et al. 2002; King et al. 2014), miscanthus (Wang et al. 2011), camelina (Lu and Kang 2008), and jatropha (Li et al. 2008; Kajikawa et al. 2012). In most cases, tissue culture techniques and/or plant regeneration protocols are prerequisite for the establishment of genetic transformation protocols. Methods of choice for gene delivery into plant cells can vary depending on the plant species and associated culture conditions: In miscanthus, foreign gene delivery was performed using a particle bombardment-mediated transformation system (Wang et al. 2011). For jatropha (Li et al. 2008; Kajikawa et al. 2012) and camelina (Lu and Kang 2008), many research groups have successfully employed *Agrobacterium*-mediated transformation. In camelina, a vacuum-infiltration-assisted floral dip transformation protocol has been developed (Lu and King 2008), which allowed high-throughput generation of transgenic plants in this species. In switchgrass, both *Agrobacterium*-mediated (Somleva

Table 18.1 Examples of genetic engineering in representative energy crops

Crop	Target gene	Engineering type	Target traits	References
Switchgrass				
PHB biosynthetic genes (*phaABC*)		Overexpression	Bioplastic production	Somleva et al. (2008)
Cinnamyl alcohol dehydrogenase (*CAD*)		RNAi	Lignin modification	Saathoff et al. (2011)
Caffeic acid *O*-methyltransferase (*COMT*)		RNAi	Lignin modification	Fu et al. (2011)
4-Coumarate-CoA ligase (*4CL*)		RNAi	Lignin modification	Xu et al. (2011)
miR156		Overexpression	Plant development	Fu et al. (2012)
LONG VEGETATIVE PHASE 1 (*LOV1*)		Overexpression	Plant development	Xu et al. (2012)
Jatropha				
Delta 12-desaturase (*FAD2*)		RNAi	Lipid composition	Qu et al. (2012)
Vacuolar Na^+/H^+ antiporter (*NHX1*)		Overexpression	Salinity resistance	Jha et al. (2013)
Curcin		RNAi	Toxin reduction	Patade et al. (2014)
FLOWERING LOCUS T (*FT*)		Overexpression	Early flowering	Li et al. (2014)
SUGAR-DEPENDENT 1 (*SDP1*)		RNAi	Lipid yield	Kim et al. (2014)
Bt endotoxin Cry1Ab/1Ac		Overexpression	Insect resistance	Gu et al. (2014)
Camelina				
Purple acid phosphatase 2 (*PAP2*)		Overexpression	Lipid yield	Zhang et al. (2012)
P_{1B}-ATPase (*HMA*)		Overexpression	Metal tolerance	Park et al. (2014)
MYB96		Overexpression	Wax fortification	Lee et al. (2014)
Gγ-subunit of G-protein (*AGG3*)		Overexpression	Lipid yield	Choudhury et al. (2014)
Fatty acid elongase (*KCS3*)		Overexpression	HFA production	Snapp et al. (2014)

The table depicts transgenic plants reported in dedicated biofuel crops (switchgrass, jatropha, and camelina), type of genetic engineering, and their target agronomic traits. For more detailed descriptions, see text

et al. 2002) and particle bombardment-mediated transformation (King et al. 2014) approaches have been reported.

Although *Agrobacterium*-mediated stable genetic transformation is well established in model plants, it remains less efficient and more technically demanding for most biofuel crops. One of the drawbacks of *Agrobacterium*-mediated gene transfer is production of the plant stress hormone ethylene during infection, which impairs transformation efficiency by repressing vir gene expression (Nonaka et al. 2008). To

improve the transformation efficiency, a gene for 1-aminocyclopropane-1-carboxylate (ACC) deaminase was introduced into *Agrobacterium* cells for the purpose of ethylene decomposition. Consequently, gene transfer efficiency was improved in recalcitrant energy crops including canola (Hao et al. 2010) and a cellulosic biofuel crop *Erianthus ravennae* (Someya et al. 2013), the latter represents a wild relative of sugarcane and shows very high dry matter production.

In addition to the development of efficient genetic transformation methods, a series of molecular tools are required to establish transgenic plant varieties for practical use. A reliable gene promoter that directs transgene expression in a desired tissue with sufficient strength is a necessity. Conventional gene promoters such as cauliflower mosaic virus (CaMV) 35S and rice ubiquitin 2 (*ubi2*) promoters often fail to deliver sufficiently high transgene expression levels in transgenic biofuel crops. To overcome this limitation, polyubiquitin gene promoters were isolated and analyzed using a series of promoter-GUS fusion constructs in transgenic switchgrass, which led to the characterization of a new promoter element that directed strong transgene expression in this energy crop (Mann et al. 2011). Increasing the repertoire of these molecular tools will further increase the versatility of genetic engineering in biofuel crops.

For the commercial production of transgenic biofuel crops in the field, technical development and practical guidelines are needed to prevent the dispersal of transgenic pollen into the environment. Technological innovations to control pollen-mediated gene flow from transgenic plants have made significant progress in recent years (Sang et al. 2013), and these technological concepts are now being applied to various biofuel crops. The Bxb1-*att* recombination system is composed of a Bxb1 enzyme and its recognition sequences *attP* and *attB*, and these components execute unidirectional site-specific recombination without the need for cofactors (Yau et al. 2011). This system was introduced into switchgrass using pollen-specific promoters, and the specific removal of marker genes from switchgrass pollen and generation of transgene-excised progeny was demonstrated (Somleva et al. 2014). In other studies, a Cre-*lox*-mediated recombination system was introduced into transgenic jatropha for the purpose of marker gene excision (Qu et al. 2012), and marker-free transgenic jatropha expressing Bt-endotoxin Cry1Ab/1Ac protein for lepidopteran insect resistance was generated using this technique (Gu et al. 2014).

18.3 Metabolic Engineering

Biodiesel in the form of fatty acid methyl esters is produced by the transesterification of triacylglycerols (TAGs) with methanol in the presence of acid or alkali. TAGs are abundant in plant seeds, which serve as excellent sources of biodiesel feedstock (Durrett et al. 2008). The quality of biodiesel is highly dependent on the composition of fatty acids and other ingredients in seed storage organs. To optimize biodiesel production, not only increasing the oil yield in plant seeds but also

engineering the plant metabolism for the optimal composition of fatty acids and other ingredients are required.

In plants, the carbon source for seed TAG biosynthesis is derived from sucrose, a product of photosynthesis in the leaves. TAG biosynthesis starts in the seed plastids where fatty acid chains are elongated, and the intermediates are transported to the endoplasmic reticulum where acyl-CoAs are converted to diacylglycerols (DAGs) (Durrett et al. 2008). DAGs are then converted to TAGs by diacylglycerol acyltransferase (DGAT), a committing step for TAG biosynthesis from the membrane lipid biosynthetic pathway. Knowledge obtained from research in model plants such as *Arabidopsis* is now being applied to metabolic engineering in practical crop cultivars used for biofuels. For example, overexpression of a fungal DGAT2 enzyme resulted in a 1.5% increase in oil content in soybean seeds (Lardizabal et al. 2008). The molecular properties of DGATs were investigated in jatropha, which showed increased seed oil levels when expressed in transgenic *Arabidopsis* (Misra et al. 2013).

TAGs contain three fatty acid chains per molecule, each of which is esterified to a glycerol backbone. Thus, the supply of glycerol-3-phosphate influences TAG biosynthesis. Indeed, overexpression of the yeast glycerol-3-phosphate dehydrogenase (*ghpd1*) gene increased the lipid content in canola seeds by 40% (Vigeolas et al. 2007), suggesting that carbon flux into the glycerol backbone is also important for increasing oil accumulation in seeds.

An alternative approach for increasing TAG abundance in seeds is to suppress its degradation. *Sugar-dependent 1* (SDP1) is a specific lipase that regulates the first step of TAG catabolism (Eastmond 2006). Expression of a *SDP1* homolog was suppressed in oilseed rape (*Brassica napus*), which resulted in an increase in oil yield in these transgenic plants (Kelly et al. 2013). A similar strategy was undertaken for a jatropha homolog, *JcSDP1*, by RNA interference (RNAi) technology, which resulted in a 13–30% increase in the total seed lipid content, at the expense of a 7% decrease in protein content in transgenic jatropha seeds (Kim et al. 2014).

Optimization of the fatty acid composition with the aim of developing desirable physicochemical properties for biodiesel fuels is also an important research target (Durrett et al. 2008). A monounsaturated oleic acid (C18:1) is a preferred component as an acyl chain in TAGs, because of its high cetane value, low melting point, and resistance to oxidation. In soybean, modulation of the fatty acid composition was achieved by suppressing the expression of FATB, an acyl-ACP thioesterase, which led to the accumulation of oleic acid up to 85% compared to 18% in the wild type (Buhr et al. 2002). In jatropha, when the gene encoding delta 12-desaturase (*FAD2*), which catalyzes the conversion of oleic acid to linoleic acid (C18:2), was suppressed by RNAi, it achieved a significant increase in oleic acid up to 78% and a corresponding reduction in polyunsaturated fatty acids in the transgenic seeds (Qu et al. 2012).

In addition to usage as a fuel, there has been a growing interest in the use of biomass materials as other industrial feedstocks, including for plastics and chemicals. Polyhydroxyalkanoate (PHA) polymers occur in nature in some microbes as a storage reserve (Anderson and Dawes 1990), and PHA-based bioplastics have been

increasingly used in a various commercial products (Snell and Peoples 2009). Pathways for PHA production have been introduced into various crops by a transgenic approach (Suriyamongkol et al. 2007), including switchgrass (Somleva et al. 2008; McQualter et al. 2014).

18.4 Lignocellulose Engineering

Lignocellulose in plant cell walls is the most abundant biomaterial on earth. It is composed of three major polymers, the polysaccharides cellulose and hemicellulose and the phenolic polymer lignin. (Pauly and Keegstra 2008; Sticklen 2008; Zhao et al. 2012). Although technological innovation in the chemical and/or biological conversion of lignocellulose into liquid fuels has advanced considerably in recent years (Zhao et al. 2012), the processes still require harsh physicochemical pretreatments to extract monosaccharides from this polymer, and these are associated with higher capital and operating costs. To design biofuel crops with improved lignocellulosic characteristics for more efficient breakdown, research has focused on the fundamental aspects of biosynthesis and degradation of plant cell walls, and this new understanding is now being applied to the molecular breeding of biofuel crops.

Among lignocellulose components, lignin is recognized as one of the major factors responsible for cell wall recalcitrance for biofuel conversion (Weng et al. 2008). Lignin polymers are composed of several phenylalanine-derived lignin monomers (or monolignols), which are synthesized by the phenylpropanoid pathway in the cytoplasm. The monomers are transported across the plasma membrane to the cell wall, where they undergo oxidative coupling and crosslinking with polysaccharides components of the cell wall (Bonawitz and Chapple 2010). Initial attempts to lower the lignin level by transgenic approaches resulted in growth penalties in plants, suggesting the pivotal role of the phenylpropanoid pathway in plant development and productivity. Transgenic alfalfa, in which genes for the phenylpropanoid pathway downregulated, showed increased hydrolysis of cell wall polysaccharides, but this change was accompanied by dwarfism, low biomass yield, and severe growth defects (Chen and Dixon 2007). Interestingly, individual transgenic plants with different steps in the phenylpropanoid pathway blocked were phenotypically diverse and did not show a correlation with lignin accumulation levels (Bonawitz and Chapple 2013 for review). These observations indicated the pleiotropic roles of this metabolic pathway on plant development and biotic/abiotic stress resistance, which are often exerted in a plant species-specific manner. These studies highlighted the importance of the elaborate design required for lignin engineering and the need to achieve lignin modification without penalty in growth and productivity.

Genetic engineering of lignin modification has been attempted in practical biofuel crops, which gave rise to intriguing results. Caffeic acid *O*-methyltransferase (*COMT*) catalyzes *O*-methylation of the 5-hydroxyl groups of monolignol precursors, thereby functioning in the latter step of the lignin biosynthetic pathway. RNAi-mediated downregulation of was conducted in model plant species, leading to a decrease in the S/G lignin monomer ratio and total lignin content without

detrimental effects on plant growth (Guo et al. 2001; Jung et al. 2012). This strategy was introduced into switchgrass, and downregulation of *COMT* resulted in a 30–38% increase in ethanol yield per unit biomass compared with controls (Fu et al. 2011). Subsequently, the performance of *COMT*-downregulated transgenic switchgrass plants was analyzed in the field for two growing seasons, and higher sugar release and ethanol production with no apparent growth penalties were observed (Baxter et al. 2014). It was estimated that a 50% increase in liters of ethanol per hectare can be achieved using these transgenic plants, demonstrating the potential of lignin engineering to overcome the limitations of biofuel production with standard source crops.

18.5 Stress Resistance

Abiotic stresses such as water deficit and high salinity are recognized as one of the most influential factors for plant growth and crop productivity. Thus, improving the environmental resistance of biofuel crops represents a key challenge to expand their cultivation zones to drought-, heat-, and/or salinity-affected marginal land. To meet this challenge, a number of stress resistance genes have been tested, intransgenic biofuel crops. The vacuolar Na^+/H^+ antiporter (*NHX1*) functions in the sequestration of excess Na^+ into the vacuole, thereby contributing to Na^+ homeostasis and salinity stress resistance in plants (Apse et al. 1999; Blumwald 2000). A *Nhx1* gene from the extreme halophyte *Salicornia brachiate* was introduced into jatropha (Jha et al. 2013), and transgenic lines exhibited enhanced tolerance to 200 mM NaCl in the growth media, thereby demonstrating the effectiveness of this approach. In another study, transgenic jatropha plants with drought-resistance genes were generated, including phosphopantetheine adenylyltransferase (*PPAT*) for coenzyme A biosynthesis, the B subunit of the nuclear factor Y (*NF-YB*) transcription factor, and the *GSMT/DMT* genes for the biosynthesis of compatible solute glycine betaine (Tsuchimoto et al. 2012). Further physiological analysis of these transgenic plants will evaluate the effectiveness of the transgenes on drought stress resistance. The transcription factor MYB96 regulates foliar cuticle wax biosynthesis and is implicated in drought resistance (Seo et al. 2011). Overexpression of this gene was attempted in camelina, which resulted in fortification of foliar cuticle layer and improved drought resistance (Lee et al. 2014).

Soil contamination by heavy metals represents a serious environmental issue in many regions of the world and impairs plant growth and productivity. The P1B-ATPase (*HMA*) gene family is implicated in the homeostasis and detoxification of heavy metals in plants (Williams and Mills 2005). Genomic and transcription analyses have identified a heavy-metal-responsive *CsHMA3* gene from camelina, and *CsHMA3* overexpression in transgenic camelina showed improved heavy metal tolerance (Park et al. 2014).

18.6 Developmental Regulation

The morphological and developmental traits of plants, such as those for the aboveground canopy and root system architecture, vegetative/reproductive transition, and leaf/flower/fruit development, have strong impacts on the biomass yield of biofuel crops (Jakob et al. 2009). Knowledge obtained from model plants such as *Arabidopsis* is now being applied to practical energy crops, which has given rise to promising results. The *FLOWERING LOCUS T* (*FT*) gene is one of the central factors integrating flowering signals and plays a crucial role in the transition from vegetative phase to flowering (Wigge 2011). A homolog gene *JcFT* was isolated from jatropha, and transgenic jatropha overexpressing this gene exhibited a significant early flowering phenotype (Li et al. 2014). It is expected that such introduced traits will be helpful for improving the otherwise unreliable and poor flowering trait in this plant.

In contrast to the case of jatropha, in which its seeds are utilized for biodiesel production, a longer vegetative phase and late flowering would be a preferred trait in the case of switchgrass, because lignocellulose in the vegetative tissues is utilized for bioethanol production. *LONG VEGETATIVE PHASE 1* (*LOV1*) encodes a NAC-type transcription factor and contributes to cold resistance and a delayed flowering phenotype under long-day conditions in *Arabidopsis* (Yoo et al. 2007). Overexpression of the *Arabidopsis LOV1* gene led to pleiotropic effects in transgenic switchgrass, such as smaller leaf angle, altered lignin content and monolignol compositions in the cell wall, and delayed flowering (Xu et al. 2012), demonstrating the potential advantages of developmental engineering in biofuel crops.

In plants, microRNA-156 (miR156) is a member of a gene family of small, noncoding RNAs with a higher expression level in the juvenile phase of development (Wu et al. 2009). Expression of most members of the *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (*SPL*) transcription factor family, which are implicated in diverse developmental processes such as leaf development, shoot maturation, and flowering, is regulated by miR156 in a Dicer-like protein (DCL1)-dependent manner (Xing et al. 2010; Gou et al. 2011). In transgenic switchgrass, overexpression of endogenous miR156b resulted in diverse developmental changes such as altered apical dominance, later flowering, and increased biomass yield (Fu et al. 2012), suggesting the potential of morphological alteration for improving the biomass yield of energy crops.

18.7 Concluding Remarks

In recent decades, there have been significant developments in the technological aspects of genetic engineering of energy crops. Using these experimental systems, a number of transgenic energy crops have been generated with the aim of improving various aspects of their agronomic traits. These new plants have been subjected to evaluation by a range of criteria including technological effectiveness and stability, socioeconomic benefits, and environmental sustainability. Considering many of the

promising results presented in this chapter, it has been forecast that innovation in genetically engineered biofuel crops will accelerate further in the future. Moreover, in the next phase, pyramiding of multiple transgenes conferring combined traits in a single plant is expected. Furthermore, promotion of field studies using these genetically engineered biofuel crops, with the aim of commercial GMO-based biofuel production, is anticipated for the successful adaptation of plant biotechnology to the biofuel industry.

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Bioresources and Technologies That Accelerate Biomass Research

19

Yasuyo Himuro and Masatomo Kobayashi

Abstract

Recently, plant sciences have been dramatically advanced by the use of resources of the model plant, *Arabidopsis thaliana*. Scientists have characterized biological functions of *Arabidopsis* genes using mutants and full-length cDNA clones that are preserved in stock centers. Model plants can also play important roles in the promotion of biomass research. The RIKEN Biomass Engineering Program (BMEP) uses a model grass, *Brachypodium distachyon*, in breeding research in monocot plants for biomass production. Resources and technologies developed by the BMEP will be provided to the research community through the RIKEN BioResource Center (BRC). In this paper, *Arabidopsis* resources that are available from the RIKEN BRC and recent developments and perspectives of the BMEP are described and discussed.

Keywords

Arabidopsis • Brachypodium • Biological resource • Model plant • Resource • Stock center

19.1 Introduction

Recent technology development has provided biologists with sophisticated and comprehensive analytical tools. One of these is the next-generation sequencing technology, which enables researchers to characterize whole-genome sequences at

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Fig. 19.1 *Arabidopsis thaliana* (L.) Heynh. (Columbia) at flowering stage

low cost within a short period. Under such circumstances, standardization and preservation of biological resources are indispensable for obtaining reproducible data.

To promote life science research, the Japanese government launched the National BioResource Project (NBRP) in 2002. To date, the government has selected *Arabidopsis*, rice, wheat, barley, *Lotus*/*Glycine*, tomato, morning glory, and *Chrysanthemum* as target plant species for NBRP, and resources of these plants are preserved in and distributed from core facilities for each plant. Most of the resources in NBRP have been collected from the Japanese research community, and the total number of recorded resources is approximately 4,500,000 (Yamazaki et al. 2010).

Arabidopsis thaliana is a widely used plant in genomic and molecular studies (Fig. 19.1). RIKEN BioResource Center (BRC) was founded at the RIKEN Tsukuba Campus in 2001 and joined the NBRP in 2002. The Experimental Plant Division of

Fig. 19.2 *Brachypodium distachyon* (L.) Beauv. (*purple* false brome), community standard line Bd21, grown in 9-cm plant pot at 22 °C for 6 weeks after sowing. Bar=5 cm

RIKEN BRC collects, preserves, and distributes *Arabidopsis* seeds, cultured cells, and genetic materials. Plant materials of RIKEN BRC are well known to the research community for their high quality. They are utilized in various research fields, including biomass production.

Recently, RIKEN has started the Biomass Engineering Program (BMEP) to solve problems of global warming and shortage of petroleum. In the project, a model monocot plant, *Brachypodium distachyon*, is employed as a model grass and used for designing a biomass crop (Fig. 19.2). RIKEN BRC has joined the project and prepares resources and technologies of *Brachypodium* to promote studies on grass.

Table 19.1 Useful sites for *Arabidopsis* research

Name	Type	Country	URL
RIKEN BRC	Stock center	Japan	http://epd.brc.riken.jp/en/
ABRC	Stock center	USA	http://abrc.osu.edu/
NASC	Stock center	UK	http://Arabidopsis.info/
TAIR	Information center	USA	http://www.Arabidopsis.org/
Araport	Portal site of research information	USA	https://www.araport.org/
NBRP	Resource network	Japan	http://www.nbrp.jp/

The first half of this paper describes resources and technologies of *Arabidopsis* available for the plant research community. The second half summarizes recent developments in *Brachypodium* research.

19.1.1 Resources and Technologies for *Arabidopsis*

19.1.1.1 General Information

Arabidopsis is known as the first higher plant whose genomic sequence was fully determined in 2000. Because the features of *Arabidopsis*, such as small size and short life cycle, make it suitable for laboratory studies, *Arabidopsis* has been employed in many studies contributing to understanding plant growth. Genomic resources of *Arabidopsis* have been comprehensively prepared in the "*Arabidopsis* 2010 Project" conducted by the Multinational *Arabidopsis* Steering Committee (MASC) to elucidate the functions of all 27,000 *Arabidopsis* genes. The resources established by the project are distributed from three resource centers: the *Arabidopsis* Biological Resource Center (ABRC) in the USA, Nottingham *Arabidopsis* Stock Center (NASC) in England, and RIKEN BRC in Japan (Table 19.1). To date, insertion lines are available for more than 95% of *Arabidopsis* genes and full-length cDNA clones for more than 70% (The multinational Coordinated Arabidopsis thaliana Functional Genomics Project, http://www.arabidopsis.org/portals/masc/2010_MASC_Report.pdf).

19.1.1.2 How to Search Materials

There is a universal number for every *Arabidopsis* gene called "Arabidopsis Genome Initiative (AGI) code." The AGI code At1g10000 indicates that the gene is located on chromosome 1 and that its map position is 10,000 (numbered from top to bottom of chromosome). With the code, researchers can search for materials in each stock center. In addition, the *Arabidopsis* Information Resource (TAIR) provides a useful site for searching resources in all three major resource centers. Another useful site is *Arabidopsis* Information Portal (Araport) which was established in 2014. Araport provides community resources as well as gene structures of *Arabidopsis* and is designed to grow with the community.

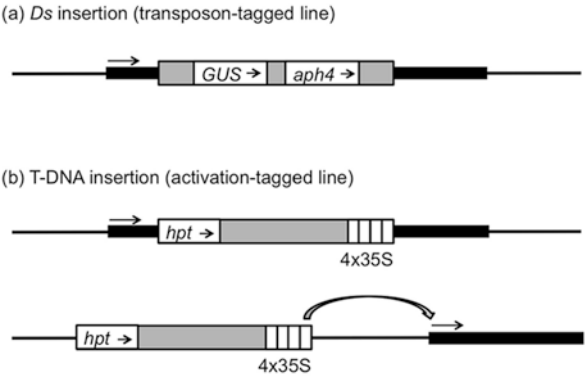

Fig. 19.3 Disruption and enhancement of gene functions by Ds and T-DNA insertions. (**a**) Schematic diagram of disruption of gene (*bold line*) by an insertion of *Ds* transposon (Modified from Tsugeki et al. 1996). (**b**) Schematic diagrams of disruption or enhancement of gene (*bold line*) by an insertion of T-DNA excised from pPCVICEn4HPT

19.1.1.3 Full-Length cDNA Clones

Full-length cDNAs are indispensable in life sciences because they are used in various experiments, such as creation of over-expressers and preparation of functional proteins. RIKEN *Arabidopsis* full-length (RAFL) cDNA clones are the world largest *Arabidopsis* cDNA resource, covering 70% of the genes annotated in the *Arabidopsis* genome. Approximately 21,000 RAFL clones have been fully sequenced, and 5′- and 3′-end sequences have been obtained from 230,000 clones (Seki et al. 2002; Sakurai et al. 2005). Researchers should note that not all of the RAFL clones have identical sequences as those annotated by computers. Alternative splicing, alternative transcription start/stop sites, and polymorphisms in the genome lead to discrepancies with the annotation.

19.1.1.4 Transposon-Tagged Lines

Transposable elements are known to cause various changes in the phenotype of plants, such as maize and morning glory. Application of the maize *Ac*/*Ds* system to *Arabidopsis* has enabled researchers to create useful mutant lines. A *Dissociation* (*Ds*) element in T-DNA was inserted into the *Arabidopsis* genome and transposed under the occurrence of *Activator* (*Ac*). Progeny plants harboring a transposition of the *Ds* element but lacking Ac were screened, and flanking sequences of the *Ds* element were characterized (Fig. 19.3). Establishment and characterization of more than 17,000 transposon-tagged lines have been reported by Ito et al. (2005) and Kuromori et al. (2004) and are available from RIKEN BRC.

As gene disruption mutants, numbers of T-DNA tagging lines are available from the stock centers. However, it is known that T-DNA transformation often causes mutations unlinked to the T-DNA insertion. Moreover, the average number of T-DNAs inserted into a single plant is usually 1.5–2. Thus, researchers who use T-DNA tagging lines should be careful to confirm the linkage of the T-DNA insertion with the phenotype of interest.

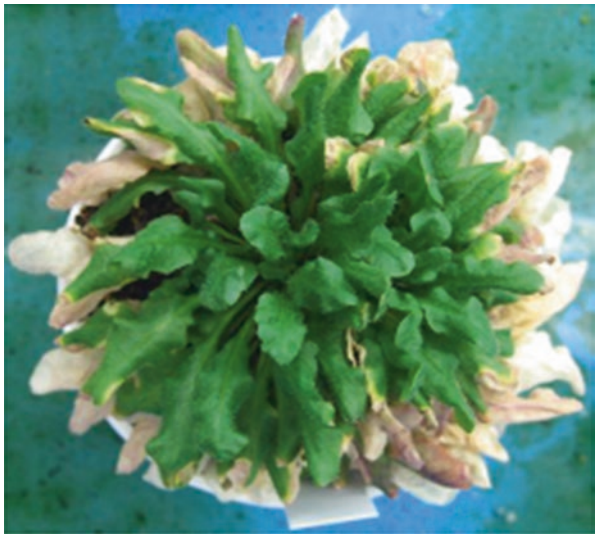

Fig. 19.4 Natural accessions of *Arabidopsis* (ecotype Li-3-3, RIKEN BRC # sja13700). Some of the natural accessions require a much longer period until flowering than Columbia

19.1.1.5 Activation-Tagged Lines

This resource was established using a modified T-DNA tagging vector pPCVI-CEn4HPT. In addition to the disruption of gene function by T-DNA insertion, the presence of four transcriptional enhancer units from the CaMV35S RNA promoter at the right border of the T-DNA can enhance the expression of neighboring genes. The numbers of lines have been established by Weigel et al. (2000) and Nakazawa et al. (2003) and are distributed from ABRC and RIKEN BRC, respectively.

19.1.1.6 FOX Hunting Lines

This unique resource was designed by Ichikawa et al. (2006) for comprehensively eliciting useful genes. *Arabidopsis* full-length cDNA clones were mixed, randomly cloned into the pBIG2113SF binary vector, and used for the transformation of *Arabidopsis* plants. The progeny seeds of the successively transformed plants were harvested and pooled for screening. The insert cDNA was over-expressed by the CaMV35S enhancer and promoter in pBIG2113SF. As with activation-tagged lines, users can expect dual effects of T-DNA insertion in screens.

19.1.1.7 Natural Accessions

Arabidopsis natural accessions (Fig. 19.4) are useful genetic resources for studying the responses of plants to environmental factors, such as cold, drought, microbial infection, and insect attack. They have been collected from various regions of the globe and preserved in stock centers. The word “ecotype” is used in manuscripts to identify natural accessions, although experimental lines such as Columbia (Col) and Landsberg erecta (Ler) are also categorized as ecotypes.

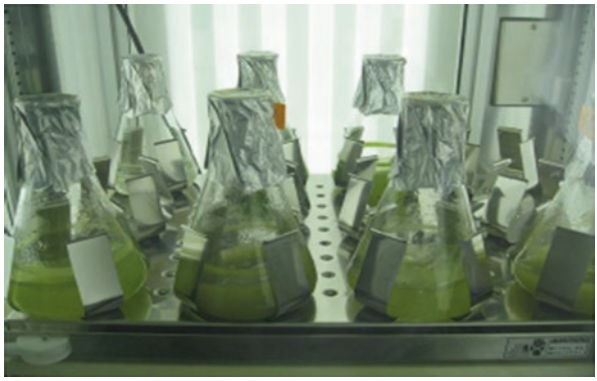

Fig. 19.5 *Arabidopsis* T87 cells cultured in a chamber. Nakamichi et al. (2003) used the cells in elucidating signal transduction of circadian clock

It must be noted that polymorphisms are also present in each ecotype, given that most of the ecotype resources are maintained as a mixture of seeds from more than several individual plants. Thus, it is possible that the genetic backgrounds of the same ecotype maintained in two stock centers are different. For this reason, users are advised to describe the source of the ecotype in the manuscript.

19.1.1.8 *Arabidopsis* T87 Cell Line

Cultured plant cells are useful tools for studying cell biology processes such as signal transduction. They are useful because researchers can prepare a large number of cells within a short period. The *Arabidopsis* T87 cell line was developed by Axelos et al. (1992) and has become well known worldwide (Fig. 19.5). RIKEN BRC preserves T87 cells and distributes them not only to domestic but to overseas institutions.

Cultured plant cells are also useful for manufacturing valuable materials such as medicinal compounds. The number of cell lines of medicinal plants has been developed and used for studies of biosynthesis of various compounds. Recently, transformation technology has been successfully applied to cultured plant cells, allowing researchers to produce medicinal peptides using transformed cells. For this purpose, the tobacco BY-2 cell line, also available from RIKEN BRC, is quite useful because of its flourishing growth.

19.1.1.9 Transformation Technology

Transformation is not only a key tool for elucidating gene function but also an indispensable technology for producing useful materials in living organisms. For example, industries use genetically modified microorganisms for the production of various pharmacological substances.

The soil bacterium *Agrobacterium tumefaciens*, now renamed as *Rhizobium radiobacter*, is commonly used for genetic manipulation of *Arabidopsis*. The protocol of transformation is exceptionally simple and easy for *Arabidopsis*, because high efficiency is obtained just by dipping flowering stems into a culture of *Agrobacterium* that harbors the T-DNA plasmid with a transgene of interest (Clough and Bent 1998).

19.1.1.10 Utilization of *Arabidopsis* Resources in Biomass Research

Although these genomic resources have been prepared to promote *Arabidopsis* research employing reverse genetics approaches, they are also helpful for eliciting genes that are useful for breeding research. One example is the *DREB* gene family, which plays a key role in responding to various abiotic stresses, such as drought, salinity, and cold. This knowledge is useful for cultivating biomass crops under severe environments. Yamaguchi-Shinozaki and Shinozaki (2006) have reviewed the feature and potential value of *DREB*. In this study, we introduce our recent work on the *Arabidopsis STOP1*, which plays a key role in responding to acid and aluminum stresses.

Arabidopsis STOP1 was first characterized and reported by Iuchi et al. (2007). They screened *Arabidopsis* EMS-mutagenized seeds grown on acidic agar medium to identify a mutant that was sensitive to acid stress (Fig. 19.6). The mutated gene (At1g34370) encodes a Cys2/His2-type zinc-finger protein and was named *STOP1*. They also found that *STOP1* is required for growth in soil enriched with aluminum and reported that the expression of *AtALMT1*, a key gene for response to aluminum stress, is regulated by *STOP1*.

With the wide distribution of acidic soil in the world, the utilization of the *STOP1* gene will provide a solution to urgent global problems concerning food and environment. Studies on *STOP1* homologs in various plants have been performed. The *STOP1* gene is conserved in various plants, including monocots, suggesting that its role in responding to acid and aluminum stresses is common in higher plants (Garcia-Oliveira et al. 2013; Ohyama et al. 2013; Sawaki et al. 2014).

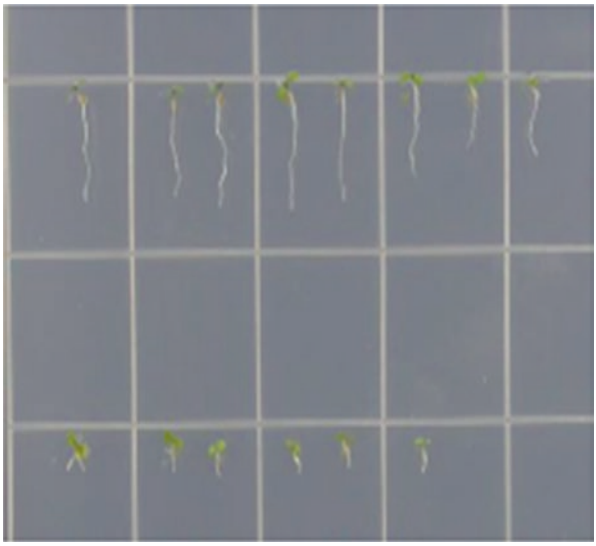

Fig. 19.6 Growth of wild-type *Arabidopsis* (*top*) and *STOP1* mutant (*bottom*) in hydroponic culture at pH 4.7 (Iuchi et al. 2007)

19.2 Development of Research Infrastructure for *Brachypodium*

19.2.1 *Brachypodium* as a Model for Biomass Research

Arabidopsis research has provided breakthroughs in plant science since the 1980s (Somerville and Koornneef 2002). The success of *Arabidopsis* research demonstrates the importance of model organisms and their resources. *Brachypodium* was first proposed by Draper et al. (2001) as a model monocot plant for understanding the genetics and molecular biology of cereal crops and temperate grasses (Fig. 19.2). *Brachypodium* is a grass species belonging to the subfamily Pooideae, which includes major crops such as wheat and barley. This species has physical and genetic features (small plant size, short life cycle, simple growth habit, self-fertility, and small genome size) that make it suitable for laboratory work like *Arabidopsis* (Fig. 19.7; Opanowicz et al. 2008; Vogel and Bragg 2009). In addition, the cell wall of *Brachypodium* has the typical structure and composition of grasses, and thus, *Brachypodium* is also expected to be a model for biomass research.

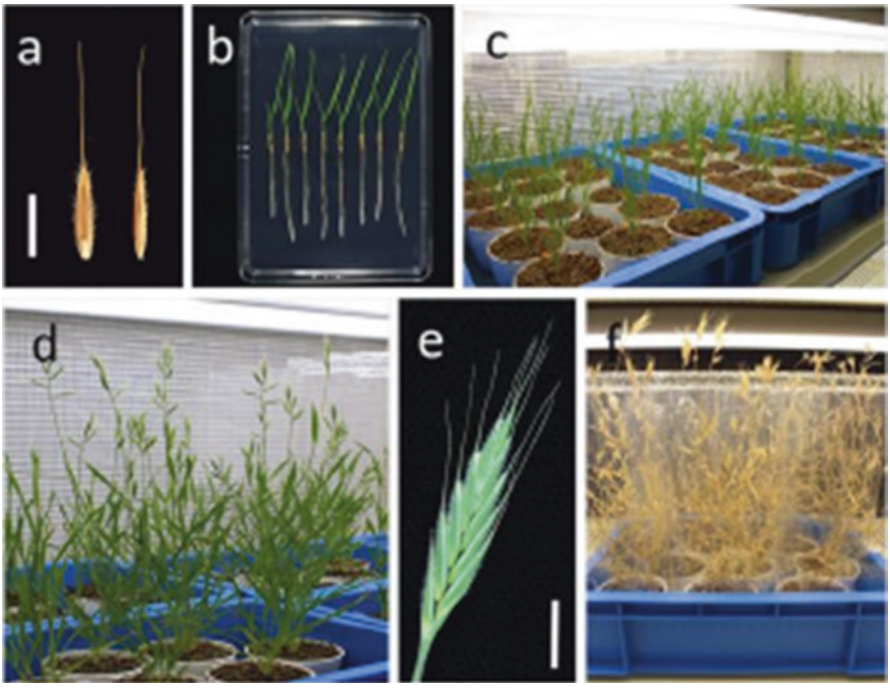

Fig. 19.7 Growth habit of *Brachypodium distachyon* Bd21. (**a**) Seed morphology; (**b**) Seven-day-old seedlings incubated on MS medium in the growth chamber (16 h light at 25 °C); (**c**) Plants grown under controlled condition (20 h light at 22 °C) for 3 weeks after transfer from plate to soil; (**d**) Heading plants 6 weeks after transfer; (**e**) Close-up of spikelet; (**f**) Twelve-week-old plants after transfer. Bar=5 mm

19.3 Trend of *Brachypodium* Research Community

The International *Brachypodium* Initiative held its first meeting on genomics at the 14th Plant and Animal Genome conference in 2006. Four years later, the whole-genome sequence of Bd21, the community standard line of *Brachypodium*, was released (International *Brachypodium* Initiative 2010, http://www.Brachypodium.org). The sequence indicates that the *Brachypodium* genus is more closely related to wheat, barley, and forage grasses than to rice. In 2011, the first European *Brachypodium* workshop was held in France and attracted approximately 200 researchers. In 2013, the first international *Brachypodium* conference was held in Italy. The conference featured seven topics, including "*Brachypodium* as a model for biomass crops." In 2015, the second international *Brachypodium* conference was held in the USA, and they decided to build a new committee for *Brachypodium* research, which is now named as International *Brachypodium* Steering Committee (IBSC).

19.4 *Brachypodium* Resources

A large number of research resources such as germplasm collections (Garvin et al. 2008), genetic markers (Vogel et al. 2009), a genetic linkage map (Garvin et al. 2009), bacterial artificial chromosome (BAC) libraries (Huo et al. 2006, 2008), and microarrays (http://www.affymetrix.com) have been developed with the rapid advances by the research community in *Brachypodium* (Brkljacic et al. 2011). Germplasm collections were gathered from various regions in the middle and near east many years ago. Inbred lines were created from those accessions and have been distributed to the community by the USDA National Plant Germplasm System (www.ars-grin.gov/npgs). Moreover, accessions (>200) were gathered by researchers from many locations in Turkey for increasing the utility of collections. The natural accessions show many variations in generation time, plant size, number of tillers, days until heading, and pathogen resistance (Opanowicz et al. 2008; Filiz et al. 2009). Fifty-four diverse accessions of the collection have been re-sequenced (unpublished). The diversity of accessions is one of the attractions of *Brachypodium* resources. Databases of *Brachypodium* resources have already been developed to support the research community, and their website URLs have been listed in previous reports (Opanowicz et al. 2008; Brkljacic et al. 2011; Mochida and Shinozaki 2013). An important collection of 23,649 T-DNA insertion mutants is available from Joint Genome Institute (JGI, http://jgi.doe.gov/our-science/science-programs/plant-genomics/brachypodium/). Mutant lines and natural accessions including RIL populations are generated in INRA (https://www6.versailles-grignon.inra.fr/ijpb-brachypodium_eng/The-NaN3-mutant-collection-from-Versailles).

Table 19.2 Previous reports of genetic transformation in *Brachypodium*

Genotype	Approach	Selectable gene	Transformation efficiency[a]	References
ABR100	Particle bombardment	*HPT*	2.3 plant lines/g of callus (3 exp.)	Draper et al. (2001)
BDR018	Particle bombardment	*BAR*	5.3% (1 exp.)	Christiansen et al. (2005)
Bd21-3	*Agrobacterium*	*HPT*	3.2% (3 exp.)	Vogel et al. (2006)
Bd21-3	*Agrobacterium*	*HPT*	22% (6 exp.)	Vogel and Hill (2008)
BDR018	*Agrobacterium*	*HPT*	55% (2 exp.)	Păcurar et al. (2008)
Bd21	*Agrobacterium*	*HPT*	17% (6 exp.)	Vain et al. (2008)
Bd21	*Agrobacterium*	*HPT*	20% (53 exp.)	Alves et al. (2009)
Bd21	Particle bombardment	*HPT*	3.4% (3 exp.)	Himuro et al. (2014)

[a]Percentage of the number of transgenic plant lines generated per embryogenic calli used as target for transformation. Frequency of transformation experiments for calculation of average value is indicated in parentheses

19.5 Transformation Technologies for *Brachypodium*

Genetic transformation technologies are essential for any model system. An efficient transformation system, the floral dip method for *Arabidopsis*, is one of the key factors that has established its status as a dicot model plant. Biolistic- and *Agrobacterium*-mediated transformation systems have been developed for some of *Brachypodium* genotypes (Table 19.2). To date, the efficient *Agrobacterium*-mediated transformation method has been used for Bd21 among the research community (Fig. 19.8). Using this efficient transformation system, the generation and characterization of T-DNA insertion lines have been initiated. Since 2008, the BrachyTAG project at the John Innes Centre has generated T-DNA mutants and distributed more than 1000 lines (Thole et al. 2010). Then the Western Regional Research Center has also collected approximately 8491 T-DNA insertion lines (Bragg et al. 2012), which are now provided from JGI. Recently, An et al. (2016) created 7000 T-DNA insertion lines of *Brachypodium* and applied them for screening genes that are involved in the non-host resistance to wheat stripe rust. The use of *Brachypodium* as a model system for crop researches will open a possibility for accelerating agricultural science.

19.6 RIKEN BMEP

RIKEN started a cross-organizational research program, BMEP, in 2010. This project aims to develop innovative technologies that contribute to the shift from a fossil fuel-based society to a sustainable society with recyclable plant biomass utilization. BMEP employs *Brachypodium* in breeding research in monocot plants for biomass production and develops research infrastructure such as full-length cDNAs,

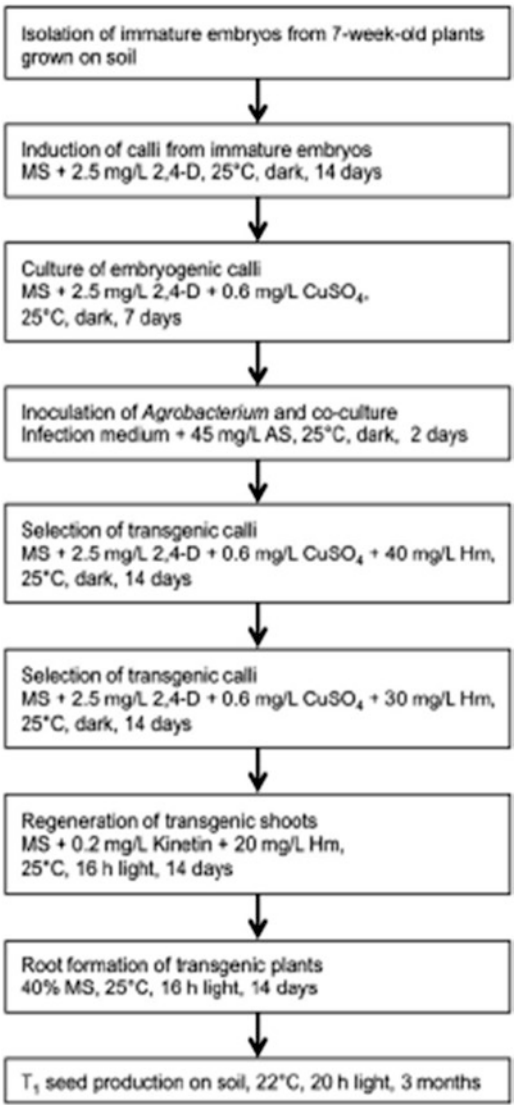

Fig. 19.8 Flow chart of transformation method for *Brachypodium distachyon* Bd21. *MS* Murashige and Skoog medium, *2,4-D*, 2,4-dichlorophenoxy-acetic acid, *AS* acetosyringone, *Hm* hygromycin

assembly databases, and omics data analysis. In particular, a large collection of full-length cDNAs is an essential genomic resource for annotation and functional analysis of genes. BMEP accordingly developed *Brachypodium* full-length cDNA clones ahead of the other resources (Mochida et al. 2013, http://brachy.bmep.riken.jp/ver.1/index.pl). More than 16,000 full-length cDNA clones have been fully sequenced to support approximately 10,000 nonredundant gene models. The *Brachypodium* full-length cDNA clones are available from RIKEN BRC (http://epd.brc.riken.jp/en/) and will be helpful in comprehensive screening of useful genes for biomass production.

Fig. 19.9 Napier grass (*Pennisetum purpureum*) suitable for biomass production. It is also known as elephant grass because of its fast growth, height (over 4 m), and high yield potential (80 t/ha/year) in tropical areas (Photo material courtesy of Dr. Y. Ishii and Dr. T. Gondo, University of Miyazaki)

RIKEN BRC has also joined the project and developed an infrastructure of resources and transformation technologies for *Brachypodium*. Recently, we established a particle bombardment-mediated transformation method for Bd21 (Himuro et al. 2014). Particle bombardment-mediated transformation often triggers problems, such as gene silencing, in contrast to the *Agrobacterium* method. However, the method has several advantages, including simultaneous co-transformation. Particle bombardment-mediated transient expression of reporter genes is a powerful tool for promoter analysis (Wang et al. 2000; Hernandez-Garcia et al. 2010; Jose-Estanyol and Puigdomènech 2012) and identification of localization signals in proteins (Takemoto and Jones 2014). We introduced *Arabidopsis thaliana* galactinol synthase 2 (*AtGolS2*), a gene known to improve stress tolerance, into *Brachypodium* by particle bombardment. *AtGolS2* is a key enzyme in the biosynthesis of the raffinose family of oligosaccharides and is expressed under drought stress (Taji et al. 2002).Transgenic *Brachypodium* overexpressing *AtGolS2* showed significantly improved drought tolerance compared with control plants (Himuro et al. 2014). The research results obtained using resources and technologies of *Brachypodium* are applicable to candidate biomass crops, such as switchgrass and napier grass (Fig. 19.9). Development of drought-tolerant biomass crops may enable stable biomass production under severe environments such as arid lands. The research infrastructure for stress response of *Brachypodium* will provide benefits not only for breeding biomass crops but for improving cereal crops and pasture grasses.

19.7 Conclusion

It is well understood that the global issues on food, health, and environment are urgent and unavoidable subjects to be solved in the twenty-first century. Plant science can contribute greatly to the measures against the issues, and thus strategic promotion of plant research is anticipated. We believe that the utilization of the infrastructure of model plants described here in both basic and applied researches holds the key to sustainable development of human society.

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Biotechnological Research in *Cryptomeria japonica*

20

Toru Taniguchi

Abstract

Cryptomeria japonica (common name is sugi or Japanese cedar) is the most important forest tree species in Japan, and its wood is used for house construction, wooden ships, wooden barrels, and many articles for daily use. Artificial plantation of this species is thought to begin more than 500 years ago, and sugi currently covers 44% of Japanese artificial forest. Though discarded wood in forest by thinning or after harvesting is a valuable biomass, most of the discarded wood is left at forest, not used for materials of biomass energy. A traditional breeding program for sugi was started in the 1950s, and ca. 3600 plus trees (healthy individuals with superior growth performance) had been selected mainly in artificial forests. A current problem due to sugi is allergic reactions to pollen (pollinosis). A nationwide epidemiological survey showed that 26.5% of the Japanese population suffers from pollinosis due to sugi pollen. To address this problem, individuals with low male flower setting have been selected from plus trees. Male-sterile mutants were also discovered, and artificial crosses between the mutants and plus trees have been attempted. This review summarizes sugi breeding, concentrating on biotechnological research (DNA marker and genetic transformation). Genetic improvement in artificial forests increases their forest economic value, leading to sustainable forest management. Sustainably managed forests accumulate continuously renewable carbon in their trees, contributing to mitigation of global warming.

Keywords

Cryptomeria japonica • Somatic embryogenesis • Genetic transformation • Cryopreservation

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20.1 Introduction

Cryptomeria japonica D. Don (common name is sugi, or Japanese cedar) is a native conifer in Japan. Tsumura (2011) describes that *C. japonica* has been classified as belonging to the family Taxodiaceae, but recent molecular studies have revealed that it actually belongs to the Cupressaceae (sensu lato; Gadek et al. 2000; Kusumi et al. 2000). This species is currently naturally distributed from Aomori Prefecture (40_ 42° N, 140_ 12° E) to Yakushima Island (30_ 15° N, 130_ 30° E) in Japan (Honshu, Sikoku, Kyushu Islands) (Hayashi 1960), but the distribution is at discontinuous and covers small areas (Ohba 1993). Yakushima Island (the southern limit of the natural distribution and a UNESCO World Heritage natural site) is famous for its huge *C. japonica* trees that are more than 1000 years old (Ohba 1993). *Cryptomeria fortunei* (*C. japonica* var. *sinensis*) is described from China (Tsumura 2011), but there are no differences in cpDNA sequences between *C. japonica* and *C. fortunei* (Kusumi et al. 2000).

As *C. japonica* has a straight bole with soft wood, which makes it easy to process, the Japanese people have traditionally used it for construction of houses, wooden ships, construction in paddy rice, wooden barrels, and many articles for daily use. As a result, natural forests of *C. japonica* have been extensively exploited during the past 1000 years, resulting in a patchy current natural distribution (Ohba 1993). Today, *C. japonica* wood is also very important as the past times, and *C. japonica* is the most important afforestation tree species in Japan. Artificial plantation of this species is believed to have begun more than 500 years ago (Ohba 1993), though the species had been planted earlier near temples and shrines. Because of the establishment of artificial plantation methods and the adaptability of artificial forests to the environment in Japan, the extent of artificial forests of *C. japonica* increased. Though clonal forests were famous on Kyushu Island, southwest of Japan, seedlings were used for plantation in other areas. Around 1950, to address the shortage of wood during the recovery from destruction due to World War II, *C. japonica* was extensively planted, replacing forests of broad-leaved tree species.

Currently, *C. japonica* covers 44% of Japanese artificial forests. However, the disease Japanese cedar pollinosis, which is mainly caused by pollen dispersed from *C. japonica* forests, is a serious social problem in Japan. A nationwide epidemiological survey showed that 26.5% of the Japanese population suffers from pollinosis (Nakae and Baba 2010). As a countermeasure, one of the most important objectives in sugi breeding is reduction or elimination of pollen disposal from *C. japonica* trees.

The wood of *C. japonica* is mainly used for lumber. Besides its use as timber, the wood can be a sustainable source of energy, a valuable renewable alternative to finite fossil fuels (Kojima 2009). “The master plan for promotion of biomass utilization” established by the Japanese government in 2010 describes the following: “Most lumber wood residues from sawmills are used for pulping and biomass energy. Construction wood wastes are also recycled for pulp material, board material, material for livestock bedding, and biomass energy. However, most of the huge amount of forest wood residues including from forest thinning is wasted at the

forest. This lignocellulosic waste must be effectively used." In order to confront this issue, Bardant et al. (2010) reported an improvement of saccharification of pulp derived from *C. japonica*, supposing the production of biofuels from the forest wood residues. Kojima (2009) reviewed the development of utilization methods of woody biomass and the problems related to their use in Japan.

This review summarizes *C. japonica* breeding, concentrating on biotechnological research (DNA marker and genetic transformation). Genetic improvement of artificial forests increases the forests' economic value, leading to sustainable forest management. Sustainable forest management will continuously accumulate renewable carbon in trees, contributing to mitigation of global warming.

20.2 Conventional Breeding in *Cryptomeria japonica*

C. japonica is the most important plantation species in Japan. The development of clonal forestry began in the eighteenth century in the Kyushu area of Japan, and it is said that there are more than 200 clonal cultivars in southwestern Japan (Ohba 1993). The *C. japonica* breeding project started in the early1950s with the main objective of growth trait improvement. Tree breeding for *C. japonica* started with plus tree selection, as for other plantation conifers. The plus trees were selected mainly from artificial forests. Clonal seed orchards or scion gardens were established using the plus tree clones, followed by progeny testing at several different sites (Ohba and Katsuta 1991). Characteristics of the plus trees, mainly, growth performance and stem straightness, were evaluated by progeny testing. Wood properties of plus tree clones were also evaluated at clonal breeding stock gardens (Hirakawa et al. 2003). After progeny testing, breeding populations were established using the offspring of plus trees with good traits. Candidates for second-generation plus trees were selected mainly in breeding populations, and now, in total, 146 second generation plus trees were selected, based on the evaluation of tree traits, i.e., growth performance, wood properties, stem straightness, and amount of male flowers (Forest Tree Breeding Center 2013). From the latest statistics, 53% of planting stock used for artificial plantation of *C. japonica* came from seed orchards or scion gardens established with bred trees (first-generation plus trees) (Forest Tree Breeding Center 2013). Although, clonal cultivars have been preferred for plantation in the southwestern area (Kyusyu), in the other areas of Japan, most of the planting stock is derived from seed orchards.

A recent important objective of *C. japonica* breeding is a countermeasure to pollinosis, because pollinosis due to *C. japonica* pollen is a serious allergic disease in Japan. The concentrations of the major allergen, Cry j 1 of *C. japonica* pollen, were measured in eight clones growing at four sites, and the results suggested that the Cry j 1 concentration is controlled primarily by genetic factors (Goto et al. 2004). The concentrations of allergens (Cry j 1 and Cry j 2) were investigated in 146 plus tree clones, and the concentration varied up to ten times among clones (Goto-Fukuda et al. 2007). Clonal variation of male flower production of over 700 plus trees selected from the Kanto Breeding Region of Japan was also investigated (Senda and

Kondo 1998; Tsubomura et al. 2013), and data on full-sib families indicated that male flower production is highly controlled by genetic factors with high general combining ability (GCA) and negligible specific combining ability (SCA) (Tsubomura et al. 2012). Recently, planting stocks derived from seed orchards established using plus trees with low flower production traits have been used for forestation to reduce pollen release and mitigate pollinosis. A male-sterile *C. japonica* mutant was first found in Toyama Prefecture (Taira et al. 1993). Taira et al. (1999) suggested that the male-sterile trait of this mutant is controlled by a pair of recessive gene. After the first find, several other mutants were discovered (Takahashi et al. 2007). In order to breed male-sterile *C. japonica* possessing superior growth performance, the mutants have been crossed with plus tree clones (Saito and Taira 2005; Saito 2009).

20.3 Application of DNA Markers to *Cryptomeria* Breeding

Many molecular markers have been developed for *C. japonica* (summarized by Tsumura 2011). Microsatellite (simple sequence repeat, SSR) markers (Moriguchi et al. 2005) are used for genotyping of breeding materials or breeding stocks and for gene flow analysis. More than 55,000 expressed sequence tags (ESTs) were constructed in *C. japonica*, and using EST information, STS, CAPS, and SNP markers have been developed for mapping and population analyses (Tsumura 2011). High-density linkage maps mainly using SNPs have enabled developing marker-assisted selection (MAS) and genome-wide association studies (GWAS) for accelerating conventional breeding programs.

20.3.1 Genotyping and Paternal Analysis

The planting stock used for artificial plantation is mainly derived from seed orchards established by bred *C. japonica*. Therefore, evaluation of the quality of the seeds collected from the seed orchards is very important for genetically improved plant supply for plantation. Moriguchi et al. (2005) elucidated the pollen contamination rates from sources outside the seed orchards, the degree of self-fertilization, and the proportion of paternal contributions from constituent clones in five seed orchards of *C. japonica* by genotyping and paternal analysis using SSR marker. Contamination rates were found to vary both among ramets within seed orchards (10.0–76.7%) and among seed orchards (35.0–65.8%). Among ramets, there were significant negative correlations between pollen contamination rate and distance from the orchard edge; among seed orchards, there were significant positive correlations between the pollen contamination rate and the area of nearby *C. japonica* forest. The self-fertilization rate in each seed orchard was 1.4–4.4%, and there were significant positive correlations between self-fertilization rate of each clone (0–20%) and the number of ramets per clone in seed orchard. The paternal contribution tendency was similar for all seed orchards (i.e., about 20% of the clones accounted for about 60% of the total

gene flow, and about 30% of total clones made no contribution), in spite of differences in the type of seed orchard (common and miniature) and their locations. All of these factors affect seed quality produced in the seed orchards; therefore, this information will be useful for management of seed orchards. SSR makers are also valuable for genotyping of clonal breeding stock or seed orchards to eliminate misplanting or mislabeling that may have occurred during establishment and management of the seed orchards (Moriguchi et al. 2005, 2011).

20.3.2 Marker-Assisted Selection

Marker-assisted selection (MAS) of superior trees within a family has great potential for use in woody plants, including conifers (O'Malley and McKeand 1994), because of the hardness in trait assessment caused by their long reproductive period, long life cycles, and large tree size of woody plant. Quantitative trait loci (QTLs) affecting several characters in *C. japonica* were detected in genetic linkage maps. Yoshimaru et al. (1998) mapped QTLs in linkage map with 85 genetic markers (72 RFLP, 11 RAPD, 1 isozyme, and 1 morphological loci) published by Mukai et al. (1995) to investigate the genetic control of juvenile growth, male and female flowering, and rooting ability in *C. japonica*. QTLs for wood strength and wood density were detected in linkage maps with 177 RAPD markers that covered 62/40% of the total genome (Kuramoto et al. 2000). Ujino-Ihara et al. (2012) reported that a stable QTL associated with male flower abundance under natural conditions was detected in a region homologous to a QTL that had previously been identified in a study involving the artificial induction of male flowers by a phytohormone, gibberellic acid, in a different genetic background (Yoshimaru et al. 1998). Moriguchi et al. (2012) constructed a high-density linkage map of 1262 markers (mean distance between two adjacent markers was 1.1 cM) mainly using 968 SNPs and located a major recessive male-sterile gene (*ms1*) on the ninth linkage group. The map distance between the *ms1* gene and the closest marker was only 0.5 cM, and this will enable MAS of male-sterile trees in particular pedigree of *C. japonica*. A male-sterile gene (*ms2*) possessed in other pedigree was located on the fifth linkage group, and the map distance between the *ms2* gene and the closest marker was1.6 cM (Moriguchi et al. 2014).

20.3.3 Genome-Wide Association Studies

Although the use of MAS has continued to increase in plant breeding, in general, most applications have been constrained to simple, monogenic traits (Heffner et al. 2009; Iwata et al. 2011). Two primary limitations to MAS are that (1) results of biparental QTL mapping studies do not readily translate to breeding applications and (2) the statistical methods used to identify target loci and to implement MAS have been inadequate for improving polygenic traits and have therefore constrained the range of the application of MAS to traits controlled by a few large-effect genes

rather than many small-effect genes (Heffner et al. 2009; Iwata et al. 2011). In order to achieve further improvement in many traits such as growth rate, stem straightness, wood properties (density, strength, and water content), and male flower production, information from GWAS between genotypes and traits will be useful (Tsumura 2011). GWAS is also an alternative to biparental QTL mapping in long-lived perennials, such as trees (Uchiyama et al. 2013). Iwata et al. (2011) simulated a *C. japonica* breeding program with genomic selection (GS) based on GWAS and showed that GS can be useful in *C. japonica* breeding with a realistic number of DNA markers (e.g., one in every 1 cM). Uchiyama et al. (2013) examined the potential of GWAS in conifers using 367 unrelated plus trees of *C. japonica* and tried to detect significant associations between traits (i.e., wood property obtained by Mishima et al. (2011) and male flower abundance given in Tsubomura et al. (2013)) and 1032 SNPs. As a result, in total, six SNPs were found to have significant associations with variations in phenotype, suggesting that GWAS has potential for use in future breeding programs for *C. japonica* (Uchiyama et al. 2013).

20.4 Genetic Engineering in *Cryptomeria*

Genetic engineering of organisms including forest trees have potential for adding highly desirable or novel traits, which would be unreasonable or difficult to realize by conventional breeding, to the host genotype in a short time. In *C. japonica*, foreign genes are introduced into embryogenic cells mainly by *Agrobacterium*-based methods, and transgenic *C. japonica* is regenerated via somatic embryogenesis. Our main effort for genetic engineering of *C. japonica* is production of male-sterile trees to address Japanese cedar pollinosis. Male sterility is also useful to mitigate the possibility of transgene flow from transgenic trees to wild populations.

20.4.1 Somatic Embryogenesis

Embryogenic cells of conifer are suitable targets of genetic transformation because of their high efficiency for genetic transformation and plant regeneration (Tang and Newton 2003). Therefore, development of methods for somatic embryogenesis and plant regeneration in *C. japonica* have been attempted, and Maruyama et al. (2000) first reported somatic embryogenesis and plant regeneration of this species. Since then, some protocols for plant regeneration of *C. japonica* through somatic embryos were reported (Igasaki et al. 2003a, b; Maruyama and Hosoi 2007; Taniguchi et al. 2008). Although, as for *Pinus* species (Klimaszewska et al. 2007), *C. japonica* somatic embryos can be formed only from embryogenic cells induced from megagametophyte explants containing immature zygotic embryos, some attempts at efficient somatic embryogenesis were reported (Igasaki et al. 2003a; Maruyama and Hosoi 2007). Phytosulfokine (PSK), a small sulfated peptide, was identified as a plant growth factor (Matsubayashi and Sakagami 1996). Igasaki et al. (2003a) reported that PSK stimulates somatic embryogenesis in *C. japonica* as in other

plants, for instance, carrot (Kobayashi et al. 1999). Igasaki et al. (2003a) showed that addition of synthesized PSK to embryo induction medium stimulated the formation of somatic embryos of *C. japonica* and approximately 80% of somatic embryos induced on PSK containing medium germinated, with synchronous sprouting of cotyledons, hypocotyls, and roots. Maruyama and Hosoi (2007) found higher effectiveness of polyethylene glycol (PEG) 6000 (av. Mol. Wt.: 7500) as the osmoticum, instead of PEG 4000 (av. Mol. Wt.: 3000), which is commonly used for conifer somatic embryogenesis (Jain et al. 1995; Stasolla and Yeung 2003).

In our laboratory, a somatic embryogenesis protocol was developed (Taniguchi et al. 2008) with some modifications of medium components from previous reports (Maruyama et al. 2000; Igasaki et al. 2003b). Using this protocol, megagametophyte explants containing immature zygotes removed from immature open-pollinated seeds of 20 clones of *C. japonica* plus trees were cultured on embryogenic cell induction medium. Induction rates of the embryogenic cells varied greatly from below 10% to approximately 80%, depending on the mother tree clone, with an average initiation rate of 45.6% (Taniguchi and Kondo 2000). When embryogenic cells were induced from explants collected from immature artificially crossed seeds of 16 seed families, the induction rate of each family also varied from 10% to 80%, with an average of 46.6% (Table 1) (Taniguchi et al. 2012a, b), resembling the rate of open-pollinated seed families (Taniguchi and Kondo 2000). Mature somatic embryos with cotyledon primordia were formed from 227 out of 351 embryogenic cell lines of the 16 artificially crossed seed families. The average number of somatic embryos that formed from the embryogenic cell lines in each seed family varied greatly from 0.1 per dish to 48.1 per dish (Fig. 20.1) (Taniguchi et al. 2012a, b), indicating that somatic embryo formation capacity in *C. japonica* would be genetically controlled as it is for other conifers such as *Picea glauca* (Park et al. 1993), *Pinus sylvestris* (Niskanen et al. 2004), and *Pinus taeda* (MacKay et al. 2006).

20.4.2 Cryopreservation of Embryogenic Cells

Embryogenic cells have high potential for plant regeneration through somatic embryogenesis. However, somatic embryogenesis in conifers including *C. japonica* has some troublesome issues: (1) In *C. japonica*, embryogenic cells can be induced only from immature seeds collected in limited season (early July). (2) Subculture of embryogenic cells is very laborious and has a risk of microbial contamination. (3) Embryogenic potential is lost during long-term subculture. In order to solve these problems and to ready embryogenic cells for genetic transformation, a cryopreservation method for embryogenic cells must be developed.

Cryopreservation of *C. japonica* embryogenic cells has only been reported by Maruyama et al. (2000), who showed the possibility of cryopreservation of two cell lines. Ogawa et al. (2012) reported a high-throughput, simple, and reliable cryopreservation method for cells of five plant species, *Arabidopsis thaliana*, *Daucus carota*, *Lotus japonicus*, *Nicotiana tabacum*, and *Oryza sativa*. In Ogawa's method, suspension cells were incubated in cryoprotectant solution (2 M glycerol, 0.4 M

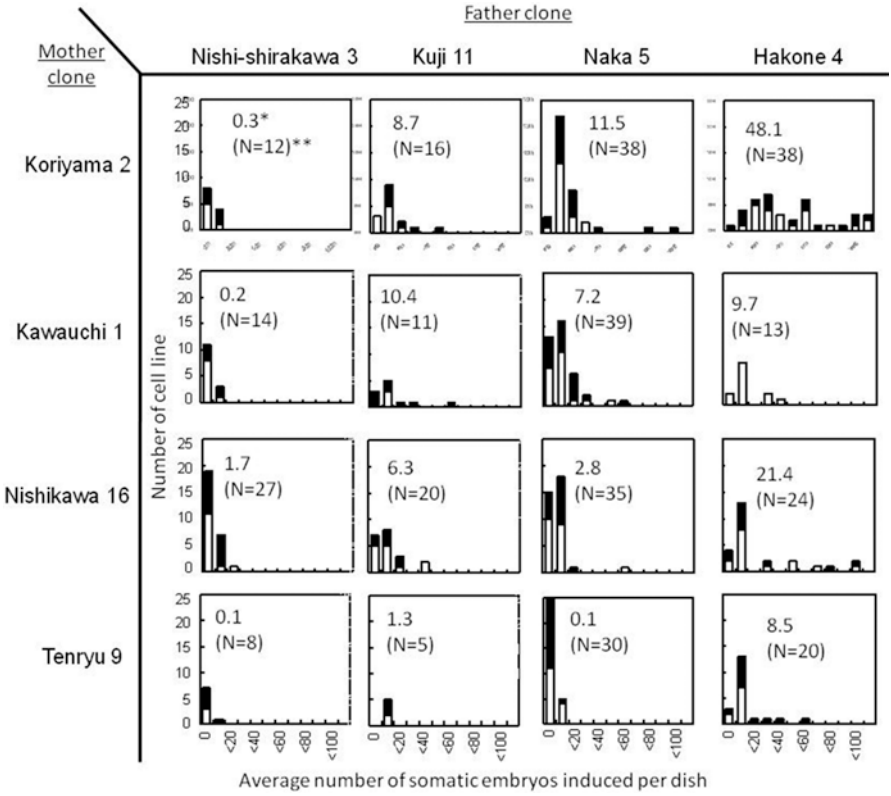

Fig. 20.1 Frequency distribution of a number of somatic embryos induced from embryogenic cells in each of 16 artificially crossed seed families in *C. japonica* (Modified from Taniguchi et al. 2012). Five clumps of ca. 50 mg of embryogenic cells per dish were cultured on embryo induction medium (Taniguchi et al. 2008), and after 1.5 months of culture, the number of somatic embryos was counted. Three dishes were used for each cell line. * Numbers in figure indicate average number of somatic embryos of each cell line within a family. ** *N* indicates number of cell lines tested in each family. *Open bars*, cell lines derived from explants collected on July 3, 2010; *filled bars*, cell lines derived from explants collected on July 13, 2010

sucrose, and 86.9 mM proline) with shaking at 60 rpm at room temperature. After incubation, the cell suspension was dispensed into 1.2 ml cryovials. Cryovials containing the cell suspension were placed in expanded polystyrene (EPS) tube containers (50-well EPS Tube Rack HS4283, Heathrow Scientific, or the Tube Holder SD-14, Maruemu Works Co. Ltd.), transferred to a −30 °C freezer, and then prefrozen for 1–8 h. After prefreezing, vials were immediately plunged into liquid nitrogen and cryopreserved. Thawed cells were regrown with high viability in all five of the tested plant species. Based on this method, using the 50-well Tube Holder SD-14, embryogenic cells of *C. japonica* were successfully cryopreserved (unpublished data). Figure 20.2a shows an example of the results of cryopreservation of *C. japonica* embryogenic cells which subcultured on gelled medium. Twenty-eight

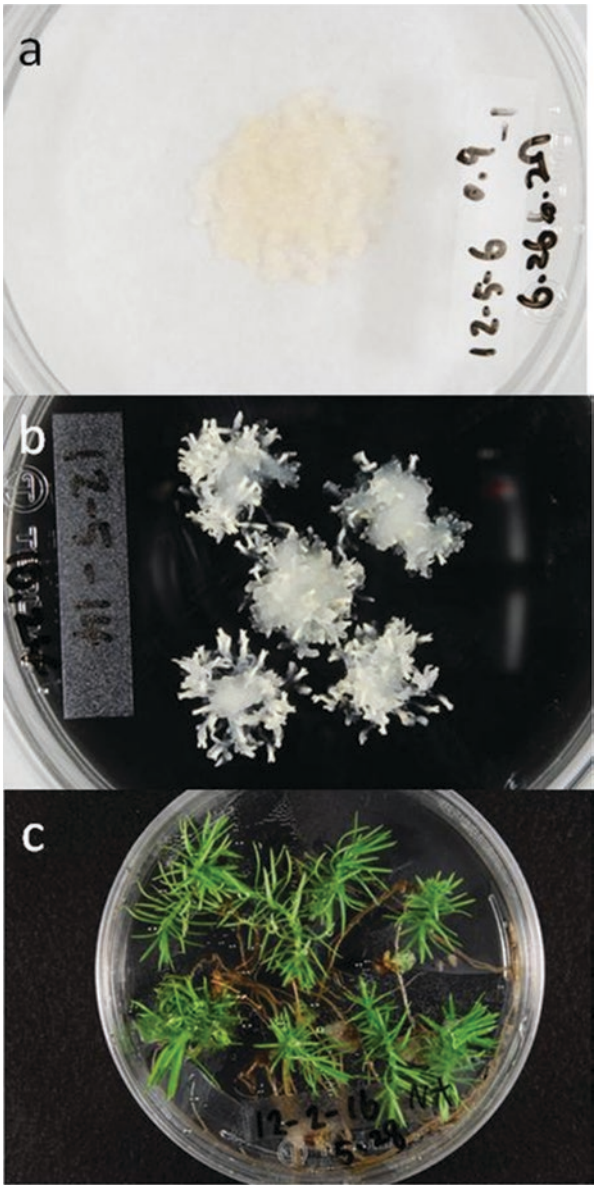

Fig. 20.2 Plant regeneration from cryopreserved embryogenic cells of *C. japonica*. (**a**) Regrowth of cryopreserved cells on filter paper over gelled medium 4 weeks after thawing. (**b**) Somatic embryo induction from cryopreserved cells after 6 weeks of culture on embryo induction medium. (**c**) Plants regenerated from the somatic embryos after 3 months of culture on embryo germination medium. Dish diameter was 9 cm (**a**–**c**)

cell lines of ten artificially crossed seed families were cryopreserved. Regrowth of cryopreserved cells was recognizable within 1–3 weeks after thawing, depending on cell line. At 4 weeks after thawing, regrowth was recognized in 27 out of 28 tested lines (Fig. 20.3). The fresh weight of regrown cells varied (Fig. 20.3), and higher fresh weight tended to be observed in the cell lines with growth restored faster. Regrowth of one remaining cell line was recognized when it was subcultured to

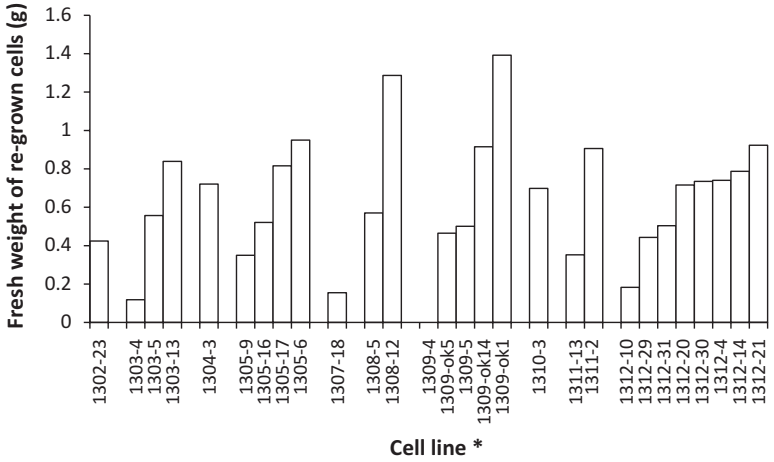

Fig. 20.3 Fresh weight of cells regrown on filter paper over a gelled medium 4 weeks after thawing 28 cryopreserved cell lines derived from *C. japonica* embryogenic cells induced from 10 artificially crossed seed families. * Cell line numbering format indicates "family name"-"line name"

fresh medium 4 weeks after thawing. The success of cryopreservation was affected by both duration of treatment with cryoprotectant solution and duration of prefreezing. The cell mass spread on the filter paper over gelled medium after thawing also affected regrowth of cryopreserved cells of *C. japonica*. Cryopreserved cells grew on maintenance medium, as did non-cryopreserved cells. Somatic embryos were induced from cryopreserved cells (Fig. 20.2b), and plantlets were regenerated normally from embryos on germination medium (Fig. 20.2c). This cryopreservation method promises to advance research on genetic transformation of *C. japonica*.

20.4.3 Genetic Transformation Procedure for *C. japonica*

Gene delivery to zygotic embryos of *C. japonica* using a particle gun has been reported (Mohri et al. 2000; Maruyama et al. 2000; Taniguchi et al. 2004), showing transient expression of marker genes (*LUC*, *GUS*, and *GFP*). Transgenic *C. japonica* was regenerated via embryogenesis after gene delivery to embryogenic cells by particle bombardment (Maruyama E, personal communication). Regeneration of transgenic *C. japonica* plants after *Agrobacterium tumefaciens* mediated genetic transformation of embryogenic cells was first reported by Taniguchi et al. (2008), showing *GFP* gene integration into the genome by PCR and Southern blotting and GPF expression by observation of GFP fluorescence. The transformation efficiency was greatly improved by Konagaya et al. (2013b). They evaluated the effect of culture support during cocultivation, showing that the cocultivation of embryogenic tissues and *Agrobacterium* on filter paper wicks moistened with cocultivation liquid medium which prevented the excess growth of *Agrobacterium* compared with that observed on solid medium, leading to an increased number of gene-transferred

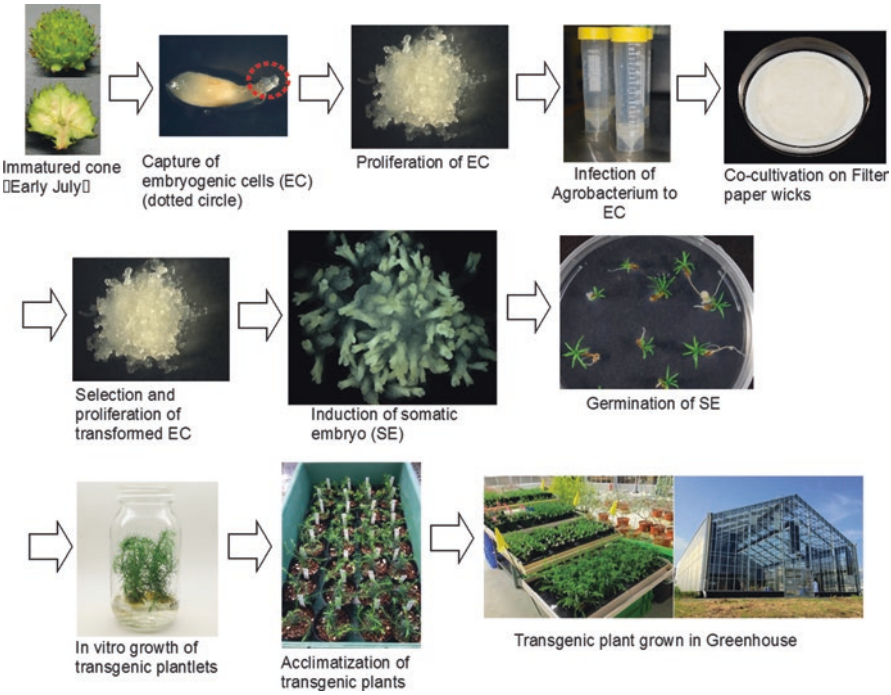

Fig. 20.4 Outline of genetic transformation method for *C. japonica* (Taniguchi et al. 2008; Konagaya et al. 2013a, b)

cells. Comparing antibiotics used for *Agrobacterium* elimination, meropenem was more efficient for genetic transformation than carbenicillin. By these two improvements (filter paper wicks during cocultivation and meropenem used for *Agrobacterium* elimination), the transformation efficiency of *C. japonica* was increased approximately 30-fold higher than in previous method (Taniguchi et al. 2008). Figure 20.4 shows an outline of the genetic transformation method for *C. japonica* in our laboratory. Transgenic *C. japonica* plants available for acclimatization in the greenhouse can be acquired approximately 1 year after infection of *Agrobacterium* to embryogenic cells.

20.4.4 Application of Genetic Transformation to Male Sterility

As described in the Introduction, allergic reaction to *C. japonica* pollen (Japanese cedar pollinosis) is a severe problem in the Japanese population. In order to address this problem, construction of male-sterile *C. japonica* trees by genetic engineering has been attempted. Control of pollen release by male sterility is also required to mitigate the possibility of transgene flow from transgenic trees to the wild population, when transgenic trees are released to field trials and commercial deployment.

Therefore, a transgenic approach to sterility was undertaken in trees (reviewed by Brunner et al. 2007). One of the promising strategies for male sterility is the ablation of reproductive organ by expressing a cytotoxic gene in plants, including tree species. Barnase is a lethal cytotoxic ribonuclease from *Bacillus amyloliquefaciens* (Hartley 1988), which degrades RNAs in cell and therefore prevents protein synthesis, being lethal to the cell. In silver birch (*Betula pendula*), barnase was expressed under the control of a flower-specific promoter of the birch gene *BpMADS1* (Lemmetyinen et al. 2004a). *BpMADS1* is similar to *SEPALLATA3*, one of the MADS box gene family, and is expressed in the inflorescence meristem at a very early stage, as well as in the developing stamens and carpels, but not in the tepals or scales (Lemmetyinen et al. 2004b). In greenhouse-grown transgenic birch expressing barnase under control of the *BpBADS1* promoter, flowering was prevented, but some side effects (e.g., growth disruption and abnormal branching) were also observed, indicating that ectopic expression of barnase likely occurred due to the homeotic promoter driving barnase. The *LEAFY* promoter of poplar and the *BpFULLI* (*Arabidopsis FRUITFULL* homolog) promoter of birch also showed side effect in poplar and birch, respectively (Wei et al. 2007; Lännenpää et al. 2005). In order to attenuate the side effects of barnase, several efforts were attempted. A specific inhibitor of barnase, barstar (Hartley 1988), driven by a constitutive promoter, was co-introduced to poplar with barnase driven by the poplar *LEAFY* promoter (Wei et al. 2007). Attenuation of barnase by constitutive expression of barstar elevated the transformation efficiency compared with the absence of attenuation. However, transgenic events with low expression ratios of barstar to barnase determined by real-time PCR tend to lead to abnormal growth and morphology in the greenhouse and field. A barnase mutant (*barnaseH102E*; His at position 102 replaced by Glu) with reduced ribonuclease activity was used to avoid the side effects of barnase (Zhang et al. 2012). *BarnaseH102E* was driven by the *PrMC2* promoter, a male cone-specific promoter from *Pinus radiate*. The *PrMC2* gene was expressed only in the tapetum of male cones of *Pinus radiate*, as analyzed by in site hybridization (Walden et al. 1999). Transgenic hybrid pine (*Pinus rigida x P. taeda*) and *Eucalyptus occidentalis* that contained *barnaseH102E* driven by the *PrMC2* promoter showed complete prevention of pollen production in greater than 95% of independently transformed events in large-scale and multiple-year field tests (Zhang et al. 2012). They reported that the transgenic pine and *Eucalyptus* grew similarly to control trees.

20.4.5 Gene Analysis in Male Cone Development in *C. japonica*

Some studies have reported genes expressed in male cones of *C. japonica*. The expression pattern of two B function genes (*CjMADS1* and *CjMADS2*) of the MADS box gene family isolated from *C. japonica* was analyzed spatiotemporally by Northern blotting (Fukui et al. 2001). Shiokawa et al. (2008) isolated and functionally analyzed *CjNdly*, a homolog in *C. japonica* of the *LEAFY* gene. Expressed

sequence tags (ESTs) were comprehensively isolated from cDNA library of several developmental stages of *C. japonica* male cones (Futamura et al. 2008).

In order to identify the genes associated with the formation and development of the male cone of *C. japonica*, and to use the information for male-sterile transgenic *C. japonica* production, we established spatiotemporal gene expression profiles associated with male cone development in *C. japonica* by suppression subtractive hybridization (Kurita et al. 2011). From these gene profiles, we discovered a male cone-specific gene that was expressed abundantly in male cones at the tetrad stage of microsporogenesis and analyzed it by RT-PCR. The gene, named *CjMALE1*, has sequence homology to the *A9* gene from *Arabidopsis thaliana* (Paul et al. 1992) and *Brassica napus* (Scott et al. 1991), which is specifically expressed in the tapetum. The *A9* homolog of *Pinus radiata* (*PrMC2*) is also expressed in the tapetum (Walden et al. 1999). *GUS* driven by the *CjMALE1* promoter (CjMALE1::GUS) was introduced into *A. thaliana*, and histochemical analysis revealed that GUS activity was detected only in the anther, not in the sepal, petal, carpel, stem, silique, seed, leaf, trichome, or root (Kurita et al. 2013). The CjMALE1::GUS construct was also introduced into *C. japonica*, and male cones were induced on transgenic *C. japonica* by GA_3 treatment of leaves (male cone of *C. japonica* can be easily induced by GA_3 treatment even in 1-year-old seedlings). GUS expression was detected in the tapetum (Fig. 20.5) and microspore mother cells of meiosis and tetrad in the *C. japonica*, as same as in *A. thaliana* (Kurita et al. 2013).

20.4.6 Production of Male-Sterile Transgenic *C. japonica*

We attempted to introduce barnase genes driven by a male cone-specific promoter into embryogenic cells. However, we either failed to obtain transformants or the transformants showed extreme growth inhibition (Konagaya et al. 2013a). These problems could be caused by ectopic expression of barnase, as observed in poplar (Wei et al. 2007). In order to inhibit ectopic activity of barnase, a constitutive promoter (NOS promoter)-driven barstar gene was ligated into barnase cassette-containing vectors and transformed into *C. japonica*. We succeeded in obtaining transformants efficiently, and transgenic *C. japonica* with the CjMALE1::barnase-NOS::barstar had no pollen in male cones. The growth and other aspects of the phenotype of the transgenic *C. japonica* were equivalent to wild type in the greenhouse (Konagaya et al. 2013a).

20.5 Conclusion

C. japonica is the most adaptive and useful forest tree in Japan. However, genetic improvement in *C. japonica* as in other forest trees is restricted by its long life cycle, their huge tree size, outbreeding character, and difficulty in assessing traits. Conventional breeding of this species is expected to accelerate due to application of molecular markers, particularly in genome-wide association studies. Recently,

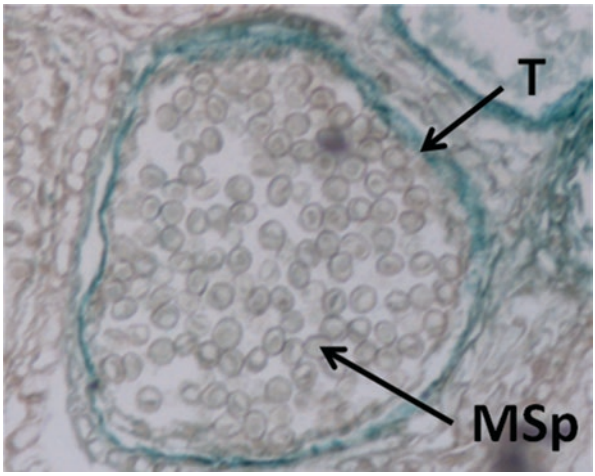

Fig. 20.5 Histochemical analysis of GUS activity in pollen sac sections of *C. japonica*. CjMALE1::GUS was introduced to embryogenic cells of *C. japonica* by *Agrobacterium*-based methods. GUS-stained male cones of the transgenic *C. japonica* were sectioned by a cryomicrotome. GUS activity was detected in the tapetum. GUS activity was also detected in tapetum and microspore at earlier stages of microsporogenesis (meiosis and tetrad stage) (Kurita et al. 2013). *T* tapetum, *MSp* microspore

extensive ESTs were collected from the cambial zone and differentiating xylem of *C. japonica* (Mishima et al. 2014). This achievement will help molecular marker application to genetic improvement of wood properties, combined with high-throughput phenotyping.

Genetic engineering can add highly desirable traits to elite genotypes fabricated through a breeding program using conventional methods or using methods fortified by application of molecular markers. In order to clarify the true traits of transgenic trees and side effects of transgenes introduced by genetic engineering, field trials of transgenic trees in the natural environment are very important. In Japan, field trials of transgenic trees are very limited (Taniguchi et al. 2012a; Yu et al. 2013). We plan a field trial of transgenic *C. japonica*, which would assess their male sterility in greenhouse for 2 years, after obtaining permission according to domestic laws.

Genetic improvement of trees in artificial forests by combining conventional breeding methods with a biotechnological approach will accelerate increasing forest economic value. Artificial forests with higher economic value generate traction for sustainable forest management. Sustainable management of healthy forests that continuously accumulate renewable carbon can contribute to mitigation of global warming.

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Cinnamyl Alcohol Dehydrogenase Deficiency Causes the *Brown Midrib* Phenotype in Rice

21

Toshiaki Umezawa, Masahiro Sakamoto, and Taichi Koshiba

Abstract

Because lignin encrusts lignocellulose polysaccharides, it presents obstacles to chemical pulping, forage digestion, and enzymatic hydrolysis of plant cell wall polysaccharides for biorefining. Hence, it would be beneficial for plant materials to either contain less lignin or to have lignin that is easier to remove for these processes. Grass mutants known as *brown midrib* (*bm*) mutants generally show a reduced lignin content and higher in vitro digestibility compared with wild-type plants. Several *bm* mutants have been isolated only from the C4 grasses, maize, sorghum, and pearl millet, but have not been detected in C3 grasses including rice (*Oryza sativa*). Recently, the *cad2* (*cinnamyl alcohol dehydrogenase 2*) null mutant isolated from retrotransposon *Tos17* insertion lines of *O. sativa* ssp. *japonica* cv. Nipponbare was observed to exhibit brown-colored midribs in addition to hulls and internodes, clearly showing both *bm* and *gold hull and internode* (*gh*) phenotypes. In addition, chemical analysis of the mutant indicated that the coloration was probably due to the accumulation of cinnamaldehyde-related structures in the lignin. The lignin content of the *cad2* null mutant was lower than that of the control plants, while the enzymatic saccharification efficiency in the culm of *cad2* null mutant was increased compared with that of the control plants. This mutation could be applied to breed forage paddy rice cultivars and other grass biomass plants that are suitable for use as fodder and industrial feedstock.

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21.1 Introduction

Lignocellulosic biomass accounts for the highest proportion of land biomass on Earth. Its annual production worldwide is estimated as 80,000 million tons. This is about 20 times the annual global oil consumption. About 2.2% of the annual lignocellulose biomass production, which equates to about 3500 million m^3, is exploited. About half of this amount (1870 million m^3) was consumed for wood fuel and 1657 million m^3 for industrial round wood in 2012 (FAO forest products statistics, http://www.fao.org/forestry/statistics/80938/en/). Wood production from artificial forests was 1400 million m^3 in 2005, which was mostly consumed for pulp and wood products (Carle and Holmgren 2008). This indicates that huge areas of natural forests are still being exploited mainly for wood fuels. From an environmental conservation perspective, this should be reduced by, for example, sustainable utilization of unused land such as *Imperata cylindrica* grassland as well as increasing the productivity of the existing artificial forests and biomass crops. In addition, the unused land utilization and the increase of the biomass productivity could be further accelerated by the additional demand for biomass as the raw material for future biomass refinering. Moreover, the efficient use of the biomass by improving utilization characteristics is necessary to enhance biomass utilization economy.

Many plant biotechnology strategies can contribute largely to the increase in the productivity of biomass plants and the improvement of the biomass utilization characteristics. Among these approaches, in this chapter, we describe improvement of the utilization characteristics of biomass plants, with emphasis on the utilization of lignocellulosic polysaccharides by lignin reduction and structural modification.

Lignin is a complex phenylpropanoid polymer that is biosynthesized via oxidative coupling of *p*-hydroxycinnamyl alcohols (monolignols) and related compounds that are formed in the cinnamate/monolignol pathway (Umezawa 2010). Lignin fills the spaces between cell wall polysaccharides and confers mechanical strength and imperviousness to the cell wall (Boerjan et al. 2003). Lignin accounts for about 20–30% of total lignocellulosic biomass. Therefore, enhanced production of lignin through activation of its biosynthesis and high value-added utilization of lignin using organic chemical approaches are important and challenging topics. On the other hand, because lignin encrusts lignocellulosic polysaccharides, lignin represents obstacles to chemical pulping, forage digestion, and enzymatic hydrolysis of plant cell wall polysaccharides for biorefining. For these processes, it is beneficial for plant materials either to contain less lignin or to contain lignin that is easier to remove. Plant mutants in which genes encoding lignin biosynthetic enzymes are defected are generally expected to show lower lignin content and higher enzymatic

saccharification efficiency (Novaes et al. 2010). For these reasons, lignin metabolic engineering and mutants defective for lignin biosynthesis have received much attention for many years (Chiang 2006; Dixon and Reddy 2003; Vanholme et al. 2008; Weng et al. 2008; Sattler et al. 2010).

21.2 Grass Mutants with Lower Lignin Contents

Grass *brown midrib* (*bm* or *bmr* for sorghum) mutants show reddish-brown pigmentation in the leaf midrib and internodes. They generally have reduced lignin content and higher in vitro digestibility compared with wild-type plants. Moreover, because these mutants are not classified as genetically modified organisms, their deployment does not involve the costly regulatory hurdles that antisense or RNA interference (RNAi) lines clear. Hence, these mutants are of considerable interest not only for silage purposes (Barrière et al. 2004; Cherney et al. 1991; Sattler et al. 2010) but also for biofuel production (Sattler et al. 2010). In addition, rice straw, together with other inedible lignocellulosic biomass products such as corn stover, sugarcane bagasse, and wheat straw, is expected to be a promising feedstock as an industrial fermentation substrate (Park et al. 2011). In Japan, annual domestic production of rice straw accounts for about 9.6 Mt. However, more than 60% of rice straw is deposited on rice fields (Park et al. 2011). In addition, rice is an important model plant for large-sized graminaceous biomass plants, such as *Erianthus*, napier grass, sorghum, and switch grass (Yamamura et al. 2013). Hence, bioconversion of rice straw to fermentable carbohydrates is a focus of many ongoing research projects in Japan (Park et al. 2011). In this bioconversion process, enzymatic saccharification of lignocellulosic materials is the key step and is affected largely by the amount and structure of lignins.

According to Jorgenson (1931), the first example of *bm* maize plant appeared in 1924. Subsequently, several *bm* (*bmr*) mutants had been isolated by 2012 in maize (*Zea mays*), sorghum (*Sorghum bicolor*), and pearl millet (*Pennisetum glaucum*) as a result of either spontaneous or chemical mutagenesis (Barrière and Argillier 1993; Barrière et al. 2004; Cherney et al. 1991; Sattler et al. 2010). In maize, six *bm* (*bm1* to *bm6*) loci have been identified (Ali et al. 2010; Sattler et al. 2010). It was concluded that *bm1* is not a null mutation of *cinnamyl alcohol dehydrogenase2* (*ZmCAD2*), but affects expression of this gene, possibly through alteration in upstream or downstream noncoding regions (Halpin et al. 1998). Later, Guillaumie et al. (2007) reported that the *bm1* mutation was probably located in a gene that regulates the expression of the *ZmCAD* gene family. On the other hand, the *bm3* mutation was found to occur in the gene encoding caffeic acid *O*-methyltransferase (ZmCAOMT) (Morrow et al. 1997; Vignols et al. 1995). In addition, down-regulation of CAOMT in maize using sorghum CAOMT resulted in a *bm* phenotype (He et al. 2003). In sorghum, at least four independent *bm* loci were identified, which were designated *bmr2*, *bmr6*, *bmr12*, and *bmr19* (Saballos et al. 2008). The abbreviation *bmr* was adopted to distinguish it from *bm*, which was already in use for the sorghum *bloomless* mutants (Saballos et al. 2009). *SbCAD2* was found to be

responsible for the phenotype of the *bmr6* mutants (Saballos et al. 2009; Sattler et al. 2009), while the *bmr12* mutation was found to be located in the gene encoding SbCAOMT (Bout and Vermerris 2003).

Interestingly, all these grasses that gave rise to *bm* (or *bmr*) mutants were C4 and diploid plants; no *bm* mutants were identified or described for the C3 grasses such as rice (*Oryza sativa*) (Sattler et al. 2010). The mechanisms responsible for the brown midrib coloration in the mutants have not been fully elucidated. However, the red-purple coloration of the CAD-down-regulated tobacco has been attributed to the incorporation of hydroxycinnamaldehydes into lignin (Hibino et al. 1995), because a synthetic lignin, a dehydrogenation polymer, derived from coniferaldehyde, exhibited a red-purple, wine-red-like coloration (Higuchi et al. 1994), and because hydroxycinnamaldehyde contents in the mutants were significantly elevated compared with those of the corresponding wild-type plants (Barrière et al. 2004; Sattler et al. 2009). In addition to the grasses, similar reddish-brown coloration was observed in a gymnosperm tree; a mutant pine (*Pinus taeda*) deficient in *cad-n1* showed brown coloration in the wood (MacKay et al. 1997). Furthermore, some transgenic plants in which genes encoding enzymes in the cinnamate/monolignol pathway were down-regulated showed unusual red, brown, or orange coloration, which was not observed in the corresponding wild-type plants. For example, *CAOMT* down-regulation in poplar (*Populus tremula* × *Populus alba*) (Van Doorsselaere et al. 1995) and in aspen (*Populus tremuloides*) (Tsai et al. 1998) resulted in xylem tissues with pale-rose and reddish-brown coloration, respectively. The red-brown coloration in the transgenic aspen was ascribed to a higher amount of coniferaldehyde residues in the transgenic line (Tsai et al. 1998). Down-regulation of *CAD* in alfalfa (*Medicago sativa*) (Baucher et al. 1996), tobacco (*Nicotiana tabacum*) (Chabannes et al. 2001; Halpin et al. 1994; Hibino et al. 1995), and poplar (*P. tremula* × *P. alba*) (Baucher et al. 1999) resulted in a variety of red, brown, and pink colorations of the xylem. With cinnamoyl-CoA reductase down-regulation, brown (Chabannes et al. 2001) and orange-brown (Piquemal et al. 1998) colorations were observed in the xylem of transgenic tobacco plants (*N. tabacum*). In addition, brown coloration in the xylem of 4-hydroxycinnamate-CoA ligase (4CL)-down-regulated tobacco (*N. tabacum*) (Kajita et al. 1996) and orange coloration of xylem of caffeoyl-CoA *O*-methyltransferase (CCoAOMT)-down-regulated poplar (*P. tremula* × *P. alba*) (Zhong et al. 2000) were reported. Taken altogether, CAD-deficient mutants of C3 grasses likely show *bm* phenotypes as a consequence of coniferaldehyde structures incorporated in the mutant lignin.

Even though, however, CAD-deficient rice mutants (Ookawa et al. 2008; Zhang et al. 2006) and CAD-down-regulated rice (Shiba et al. 2007) were described, no *bm* mutants were reported. Point mutation of the *CAD2* (*GH2*) gene in *O. sativa* ssp. *indica* cv. Zhefu 802 (hereafter referred to as Zhefu 802) caused an obvious reddish-brown pigmentation in the internode and the base of the leaf sheath at the heading stage and golden-yellow coloration of the hull. Consequently, this mutant was designated as the *gold hull and internode* (*gh*) 2 mutant (Zhang et al. 2006). An additional rice *gh2* mutant of an unknown background showed decreased *OsCAD2* gene expression and lignin content compared with a control rice plant (Ookawa et al.

2008). In addition, Oryzabase (http://www.shigen.nig.ac.jp/rice/oryzabaseV4/) lists a number of rice *gh* mutants (*gh1*, *gh2*, and *gh3*). For example, a *gh2* mutant of *O. sativa* L. ssp. *japonica* cv. Miyazaki No.1 was reported by Iwata and Omura (1971), and recently a *gh1* mutant defective for a chalcone isomerase gene (*OsCHI*, Os03g0819600) involved in the flavonoid biosynthesis was reported (Hong et al. 2012). However, none of the *gh* mutants were reported to show the *bm* phenotype.

21.3 Rice *BM* Mutant

In this context, recently we characterized a rice mutant that harbored a retrotransposon *Tos17* insertion in the *CAD2* (*GH2*) gene and observed that the mutant exhibited higher enzymatic saccharification efficiency and the *bm* phenotype as well as the *gh2* phenotype (Koshiba et al. 2013). The null *gh2* mutant was isolated from the *Tos17* mutant panel population of *O. sativa* spp. *japonica* cv. Nipponbare (hereafter Nipponbare), which is the mutant lines induced by insertion of rice retrotransposon *Tos17* (Kumar and Hirochika 2001; Miyao et al. 2003; Hirochika 2010). The CAD2-deficient *gh2* mutant isolated harbors a retrotransposon insertion in the second exon of *CAD2*. The homozygous *gh2* mutant exhibits obvious reddish-brown coloration in the panicles (hulls), internodes, and nodes at the heading stage (Fig. 21.1), in which a relatively high level of *OsCAD2* (*GH2*) expression was observed (Fig. 21.2). These phenotypes were typical of the *gh2* mutant of Zhefu802 (Zhang et al. 2006). Except for the reddish-brown coloration in these specific tissues, the *gh2* mutant of Zhefu802 showed similar development to the wild type, and the *bm* phenotype was not shown (Zhang et al. 2006). In sharp contrast, the Nipponbare *gh2* mutant clearly showed the *bm* phenotype, as indicated in Fig. 21.1 (arrowhead). The reddish-brown pigment was observed in the sclerenchyma and vascular bundle cell walls in the leaf and culm (Fig. 21.3). In addition, histochemical staining with Wiesner reagent showed enhanced red-purple coloration in the leaf midribs and internodes of the *gh2* mutant compared with that of the control (*GH2*) plant (Fig. 21.3). Although each *Tos17* mutant line harbors a number of retrotransposon insertions (Kumar and Hirochika 2001; Miyao et al. 2003), a transgenic plant in which *OsCAD2* (*GH2*) gene expression was down-regulated by the RNAi technique also exhibited reddish-brown coloration in the midrib, i.e., the *bm* phenotype (Fig. 21.4). In all of the tissues tested, the *gh2* mutant contained 10–20% less lignin than that of the control plants (Fig. 21.5). Taken together, these results unequivocally show the occurrence of a *gh*/*bm* phenotype in *O. sativa* ssp. *japonica* cv. Nipponbare, and it was confirmed that this phenotype is due to the impaired *OsCAD2* expression.

Histochemical staining with Wiesner reagent indicated the red-purple coloration was greater in the *gh2* mutant than in the control plant, strongly suggesting the occurrence of cinnamaldehyde residues in the mutant, which is a typical characteristic of CAD-deficient mutants or transgenic plants (Barrière et al. 2004; Kim et al. 2003; Ralph et al. 2001; Sattler et al. 2010). This was further confirmed by the detection of a degradation fragment derived from the cinnamaldehyde structure; thioacidolysis of the *gh2*/*bm* mutant detected a cinnamaldehyde-specific

Fig. 21.1 The *gh2* mutant shows the *brown midrib* phenotype. (**a**) Culms of *GH2* (*left*) and *gh2* (*right*) plants at the heading stage. (**b**) Panicles of *GH2* (*left*) and *gh2* (*right*) plants at the heading stage. (**c**) Seeds of *GH2* (*left*) and *gh2* (*right*) plants at maturation. (**d**) Flag leaves of *GH2* (*left*) and *gh2* (*right*) plants at the heading stage. The *arrowhead* indicates the *reddish-brown* coloration of the midrib in the *gh2* mutant

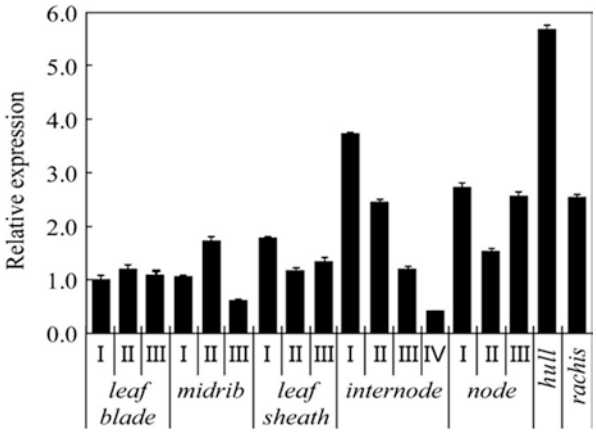

Fig. 21.2 Expression level of *OsCAD2* in different tissues of wild-type plants. The tissues comprise the leaf blades, midribs, leaf sheaths, internodes, nodes, hulls, and rachises at the heading stage. Numbers I, II, and III above the "leaf blade," "midrib," and "leaf sheath" indicate the flag leaf, second-youngest leaf, and third-youngest leaf, respectively. The numbers above the "internode" and "node" indicate the corresponding first, second, third, and fourth internode or node, respectively. The leaf blades did not include the midribs. Each value is the mean of three replicates ± SD and expressed relative to the expression in leaf blade no. 1

degradation product, i.e., the indene compound (Kim et al. 2002) (Fig. 21.6), from the hull, whereas the compound was not detected in the control plants. These results indicated the occurrence of coniferaldehyde residues in the lignin of the *gh2* mutant, which probably results in the reddish-brown coloration in the *gh*/*bm* phenotype.

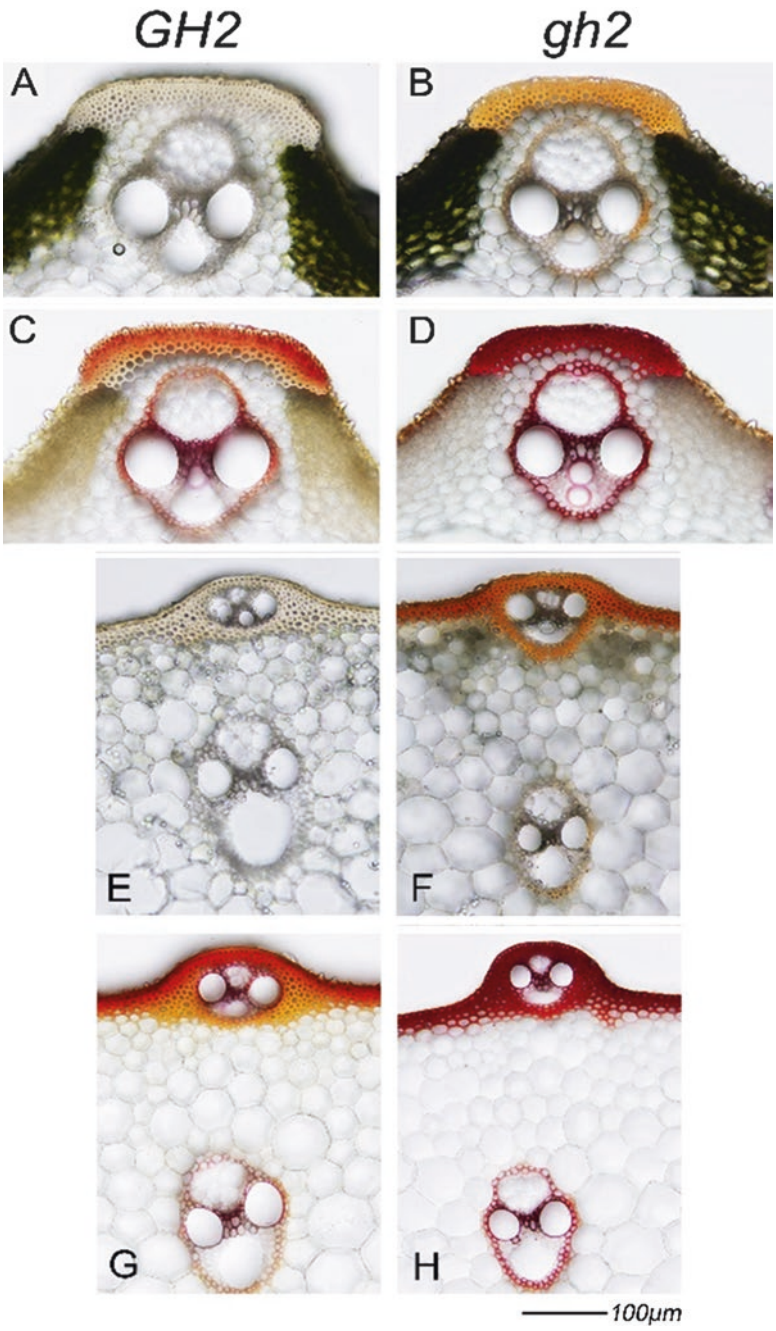

Fig. 21.3 Histochemical analysis of *GH2* and *gh2* plants. Transverse sections of flag leaves (**a**–**d**) and third internodes (**e**–**h**) from *GH2* (*left column*) and *gh2* (*right column*) plants were photographed under a bright field microscope. (**a**, **b**, **e**, **f**) Transverse sections without staining. (**c**, **d**, **g**, **h)** Transverse sections stained with Wiesner reagent. *Reddish-brown* pigment was deposited in the walls of sclerenchyma cells and vascular bundle cells of flag leaves (**b**) and internodes (**f**) of *gh2* plants. Enhanced staining with Wiesner reagent was observed in the regions in which the *reddish-brown* pigment was accumulated in *gh2* plants (**d**, **h**). The dots visible in the intracellular spaces in (**e**, **f**) were starch granules

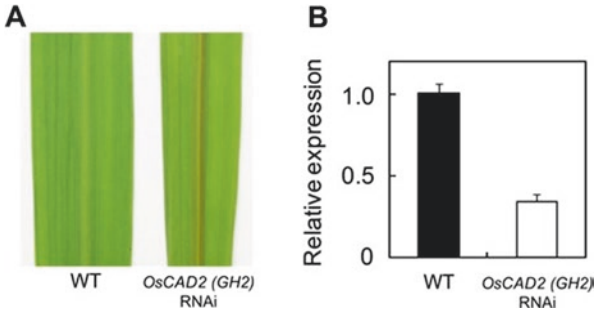

Fig. 21.4 Phenotype of transgenic *OsCAD2* (*GH2*)-knockdown (*OsCAD2* RNAi) plant. (**a**) Midribs of flag leaves in wild-type (*WT*) (*left*) and *OsCAD2* (*GH2*) RNAi (*right*) plants at the heading stage. (**b**) *OsCAD2* (*GH2*) expression levels of flag leaves in WT and *OsCAD2* (*GH2*) RNAi plants at the heading stage were measured by real-time RT-PCR. Each value is the mean of three replicates ± SD

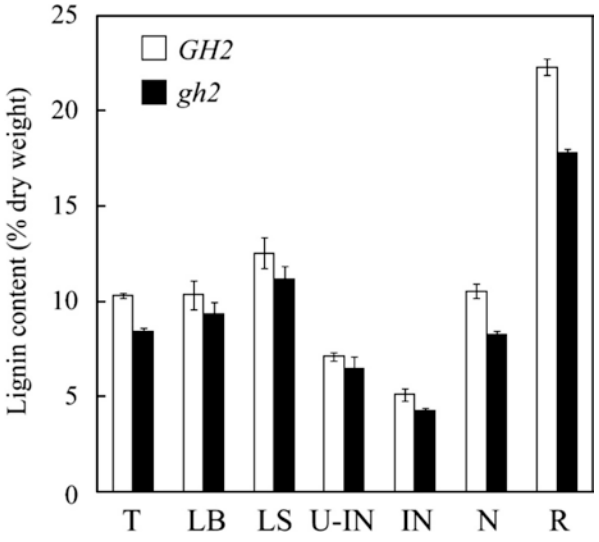

Fig. 21.5 Thioglycolic acid lignin content in tissues of *GH2* and *gh2* plants. Each value is the mean of three replicates ± SD. T, total fraction without spikelets; *LB* leaf blades, *LS* leaf sheaths, *U-IN* uppermost internodes, *IN* internodes without uppermost internodes, *N* tissues around ±5 mm from nodes; *R* rachises and panicle branches without spikelets

All of the tested tissues of the *gh2*/*bm* mutant of Nipponbare cultivar contained 10–20% less lignin than those of the control plants (Fig. 21.5). These reductions were higher than those previously reported for the *gh2* mutant of the Zhefu802 cultivar (*gh2* mutant, 14.2%; wild type, 15.0% in the internodes) (Zhang et al. 2006). The reduction in lignin levels was accompanied by the higher enzymatic saccharification efficiency of the lignocelluloses in the *gh2* mutant. After enzymatic incubation for 48 h, saccharification efficiency in the control plants reached 35.0% and that

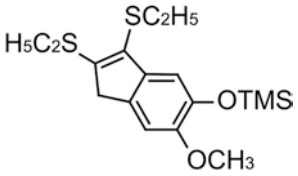

Fig. 21.6 Structure of an indene compound (2,3-bis-ethylsulfanyl-6-methoxy-1*H*-inden-5-ol) (trimethylsilyl ether). TMS, trimethylsilyl

of the *gh2* mutant was 40.6%, which is about 16.1% higher than that in the control plants. These results clearly indicate that rice *gh2*/*bm* mutants are promising for fodder and as a feedstock for industrial fermentation. Importantly, the mutation in the Nipponbare can be exploited to breed novel rice cultivars by cross-fertilization, especially forage paddy rice cultivars. In addition, the present indicates there is potential for the breeding of diploid grass biomass plants, other than maize, sorghum, and pearl millet, such as barley (*Hordeum vulgare*) and rye (*Secale cereale*), for improvement of enzymatic saccharification efficiency and digestibility by lignin reduction.

In conclusion, the *cad2* null mutant isolated from retrotransposon *Tos17* insertion lines of Nipponbare showed the *bm* phenotype in addition to the *gh* phenotype. This is the first report of a *bm* mutant from C3 grasses. The *gh2*/*bm* mutant showed higher enzymatic saccharification efficiency and lower lignin content compared with those of the control plant. This mutation could be applied to breed forage paddy rice cultivars and other grass biomass plants that are suitable for use as fodder and as industrial feedstock.

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The Distribution, Evolution, Structural Characteristics, and Functional Analysis of the *Mariner*-Like Elements in Bamboo

22

Ding-Qin Tang and Ming-Bing Zhou

Abstract

Most bamboo species are distinguished by the rapid growth, e.g., more than 100 cm/d in *Phyllostachys pubescens*. The latest research showed that the genome of *P. pubescens* is large and contains large and diverse families of transposable elements, which were assumed to effect on its morphogenesis and development. *Mariner*-like elements (MLEs) are class II transposable elements found in almost all eukaryotic genomes. We have characterized 82 amplification fragments and 79 full-length *mariner*-like transposases representing MLEs derived from 79 representative bamboo species from 38 genera within six subtribes of the Bambusoideae. Phylogenetic analysis of these MLE transposase sequences shows that MLEs are widespread, diverse, and abundant in the Bambusoideae. There is horizontal transfer between distantly related species or an ancestral MLE polymorphism followed by divergent evolution and stochastic loss. Two full-length MLEs were isolated with typical ITR consensus sequences of plant MLEs, intact DNA-binding motifs, and DD39D catalytic domain, and many residues are previously shown to be critical for transposase activity from *P. pubescens*, implying that both transposons are likely natively active. Transformation into *Arabidopsis thaliana* showed that the MLE transposons left the primary site, jump, and produce footprints in the *A. thaliana* genome. The active bamboo Tc1/*mariner* will provide a foundation for future comparative analyses of animal and plant elements, a new wide host range transposable element available for plant gene tagging.

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Keywords
Bamboo • Evolution • *Mariner*-like elements • *Phyllostachys pubescens* • Transposition

22.1 Introduction

Transposable elements (TEs) are "jumping" of a DNA segment to another place of the genome, which are divided into class I and class II according to mechanism of transposition. Class I elements (retrotransposons) transpose by means of an RNA intermediate in a reaction involving several enzymes of reverse transcriptase and integrase. In contrast, class II elements (DNA transposons) move directly via DNA, and the transposition reaction is catalyzed by a transposase encoded by autonomous DNA transposons (Feschotte et al. 2002). Many studies show that large genomes contain abundant TEs which are significantly responsible for the huge differences in eukaryotic genome size (Bennetzen 2002; Feschotte et al. 2002). For example, they account for at least 45% of the human genome (Lander et al. 2001), 85% of the maize genome (Schnable et al. 2009), and 59% of bamboo genome (Peng et al. 2013). TEs also are one of the propulsors of genome evolution. They cause both large-scale rearrangements and changes in the structure and expression of individual genes, through activities such as excision, integration, chromosome breakage, and ectopic recombination (Naito et al. 2009; Sinzelle et al. 2009).

Tc1/mariner-like elements are a diverse superfamily of class II transposable elements (Capy et al. 1998; Feschotte and Wessler 2002) that can be divided into three distinct monophyletic groups named after the best-characterized members: *Tc1*-like, *mariner*-like, and *pogo*-like (Doak et al. 1994; Plasterk et al. 1999). *Mariner*-like elements (MLEs) comprise a single open reading frame (ORF) encoding transposase, flanked by inverted terminal repeats (ITRs). The MLE transposase has two domains, the N-terminal domain containing a helix-turn-helix (HTH) motif that recognizes and binds to the ITRs and the C-terminal domain containing a common catalytic motif (DD34E/D for animals or DD39D for plants), comprising two aspartic acid residues separated from a glutamic acid residue (or a third aspartic acid residue) with characteristic spacing (Doak et al. 1994; Hartl et al. 1997a; Plasterk et al. 1999; Feschotte and Wessler 2002). A 150-amino acid domain surrounding the acidic triad is relatively well conserved and has therefore been used to establish the evolutionary relationships among *Tc1/mariner* elements (Capy et al. 1998; Plasterk et al. 1999; Feschotte and Wessler 2002). MLEs are widespread and diverse in fungi, insects, nematodes, fish, mammals, and plants (Capy et al. 1998; Feschotte and Wessler 2002). It has been suggested that the spread of MLEs across distantly related phyla may have occurred by "horizontal transfer," a process that involves transfer from one host to another followed by amplification (via repeated replicative transposition) in the recipient genome, resulting in insertional polymorphisms in the recipient population and ultimately the entire species (Lohe et al. 1995). However, because transposable elements are not positively selected, mutations may

accumulate over time resulting in partially or completely inactive copies, a process termed "vertical inactivation" (Hartl et al. 1997b). Therefore, although MLEs are widespread in nature, the vast majority is defective (Le et al. 2000; Bessereau et al. 2001; Feschotte et al. 2003; Holligan et al. 2006).

Bambusoideae is a subfamily of the grass family Poaceae and is further divided into nine subtribes comprising more than 80 bamboo genera and about 1400 species worldwide (Group 2012). There are economic, cultural, and ecological significance throughout the world for bamboo species, especially in the Asia-Pacific region. They not only have extensive utility as construction material, food source, and versatile raw products but also offer fodder resources and refuge to animal species, such as the giant panda (Group 2012). For example, *Phyllostachys pubescens* (synonym, *P. edulis*) account for over 3 million ha and approximately 2% of the total forest area because of its high economic value as bamboo wood and edible bamboo shoot (Fu 2001). The *P. pubescen*s genome is thought to be just over 2000 Mb in size (Gui et al. 2010), which are relatively large among the grass (Geilis et al. 1997: 4.17–5.3 pg DNA content in temperate bamboos and 2.34–3.23 pg DNA content in tropical ones). It is reasonable to assume that large and diverse families of repetitive sequences will be found in bamboo genomes.

Here we summarize (1) the distribution and evolution patterns of MLEs in bamboo, (2) the structure characteristics of full-length MLEs in *P. pubescen*s, and (3) the distribution and insertion polymorphism of potentially active MLEs, *Ppmar*1 and *Ppmar*2, in the *P. pubescens* genome.

22.2 The Distribution, Polymorphism, and Evolution Pattern of the *Mariner*-Like Elements in Bamboo

Feschotte and Wessler (2002) designed PCR primers for the amplification of MLE transposase genes from flowering plants. We therefore aligned the sequences of known bamboo MLE transposases from the species *Arundinaria simonii*, *Pariana radiciflora*, and *Lithachne humilis* (Feschotte and Wessler 2002) and designed a pair of simplified, bamboo-specific degenerate primers that flanked a fragment of the transposase gene located between the first two aspartic acid residues of the DD39D domain (covering ~128 amino acids). This strategy generated one ~380 bp distinct fragment in each of the species we tested (Fig. 22.1), providing a total of 82 products from the 44 species and 9 *P. pubescens* cultivars or forms (accession numbers DQ528658–DQ528739, Table 22.1).

All 82 MLE sequences we isolated were different. Pairwise comparisons showed 52.9–99.2% identity at the nucleotide sequence level and 44.9–100% identity for the corresponding amino acid sequences. In the intraspecies, pairwise identities of 39 *mariner*-like transposase fragments from 9 *P. pubescens* cultivars or forms were 54.2–100% for nucleotide sequences and 49.2–100% for amino acid sequences. Comparisons within each subtribe revealed a similar spectrum of diversity (55.1% identity among the Guaduinae, 55.1–98.4% among the Melocanninae, 57.1–96.9% among the Bambusinae, 54.3–97.6% among the Shibataeeae, and 45.7–98.4%

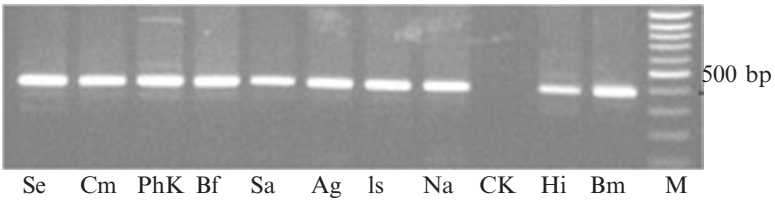

Fig. 22.1 Detection of MLE transposase genes in the bamboo subfamily using PCR with degenerate primers. Representative examples are shown from ten species (Abbreviations as shown in Table 22.1). CK: control with no genomic template DNA; M, 100-bp DNA ladder

among the Arundinarieae at amino acid level) indicating that evolutionary diversification has not matched speciation and that MLE transposases are highly polymorphic in the bamboo species tested. With regard to mutation of sequences, 39 MLE sequences contained insertions, deletions (1–11 bp), or substitutions that introduced stop codons or led to frameshifts. Considering partial sequences (about 1/3) of transposase gene were amplified here, it is most likely that a large proportion of bamboo MLEs contain inactive transposases. Thus, most of the bamboo MLE transposase genes may now be evolving as pseudo genes, accumulating mutations neutrally and thus rapidly within each species (Hartl 1997a, b; Robertson et al. 1998).

To analyze the phylogenetic relationship among the Bambusoideae MLEs, phylogenic trees were generated using nucleotide sequences from the 44 representative species that were selected to represent the different lineages within each subtribe with two animal MLEs as out-groups (Fig. 22.2a). The MLE tree indicated that distant species in different subtribes shared closely related MLEs. For example, Sv-1 MLE (*Sasa veitchii*) showed 96.6% nucleotide identity to Qt-1 MLE (*Qiongzhuea tumidinoda*), but the species represented the diverse subtribes Shibataeeae and Arundinarieae. The reciprocal situation was also observed, e.g., three diverse MLE subclades were present in the Melocanninae group. Sf-1 from subclade A1 shared only 56.9–98.7% identity with Pp-1, Mb-1, and Cp-1 from subclade A2 and 62.4% identity with Ms-1 from clade C. The incongruence between the ITS-based species phylogeny and the phylogeny of the MLEs within the same species indicates that bamboo MLEs have evolved independently of the host plant species. Summarily, all MLEs were widespread, abundant, and diverse in the Bambusoideae, and their distribution was "spotty," which meant that, among closely related species, a particular type of *mariner*-like transposase might be found in some species but not in others. There are four alternative hypotheses to explain the patterns of distribution including (1) horizontal transfer, (2) stochastic loss or vertical extinction, (3) ancestral polymorphism, and (4) evolution at different rates due to different constraint selection (Zhou et al. 2010).

To analyze the natural constraint selection on the MLE transposases from the same ancestor, 79 full-length *mariner*-like transposases were isolated from 79 representative bamboo species from 38 genera within six subtribes mainly found in China (HM101484-HM101562, Table 22.1). Pairwise comparisons of full-length *mariner*-like transposases showed 92.9–100% identity at nucleotide level and

Table 22.1 Bamboo species whose *mariner*-like transposase fragment and full-length sequences were analyzed in this study and GenBank accession numbers (AN)

Taxa	Abb.	Source	AN of ITS	AN of partial transposase	AN of full-length transposases
Subtribe Melocanninae					
Melocanna baccifera	Mb	Guangdong, China	GQ464827	DQ528681	HM101514
Cephalostachyum pergracile	Cp	Yunnan, China	GQ464810	DQ528664	HM101505
Monocladus saxatilis	Ms	Fujian, China	GQ464829	DQ528682	HM101526
Schizostachyum funghomii	Sf	Yunnan, China	GQ464842	DQ528734	HM101506
Pseudostachyum polymorphum	Pp	Yunnan, China	GQ464837	DQ528728	HM101515
Subtribe Bambusinae					
Bambusa multiplex	Bm	Yunnan, China	GQ464807	DQ528661	HM101525
B. chungii	Bc	Yunnan, China	GQ464806	DQ528660	HM101498
B. arundinacea	Ba	Ghana	GQ464805	DQ528659	HM101501
Dendrocalamopsis oldhamii	Do	Guangdong, China	GQ464815	DQ528669	HM101499
Gigantochloa levis	Gl	Fujian, China	GQ464820	DQ528674	HM101509
Thyrsostachys oliveri	To	Fujian, China	GQ464846	DQ528738	HM101513
Neosinocalamus affinis	Na	Yunnan, China	GQ464830	DQ528684	HM101508
Dendrocalamus minor	Dm	Zhejiang, China	GQ464816	DQ528670	HM101500
Melocalamus arrectus	Ma	Yunnan, China	GQ464826	DQ528680	HM101507
Subtribe Shibataeeae					
Hibanobambusa tranquillans	Ht	Zhejiang, China	GQ464822	DQ528676	HM101484
Indosasa shibataeoides	Is	Zhejiang, China	GQ464825	DQ528679	HM101485
Brachystachyum densiflorum	Bd	Zhejiang, China	GQ464809	DQ528663	HM101516
Shibataea chinensis	Sc	Zhejiang, China	GQ464844	DQ528736	HM101486
Semiarundinaria fastuosa	Se	Zhejiang, China	GQ464843	DQ528735	HM101487
Qiongzhuea tumidinoda	Qt	Zhejiang, China	GQ464838	DQ528730	HM101517
Sinobambusa tootsik	St	Zhejiang, China	GQ464845	DQ528737	HM101488

(continued)

Table 22.1 (continued)

Taxa	Abb.	Source	AN of ITS	AN of partial transposase	AN of full-length transposases
Chimonobambusa marmorea	Cm	Zhejiang, China	GQ464811	DQ528665	HM101510
Chimonobambusa quadrangularis	Cq	Zhejiang, China	GQ464812	DQ528666	HM101518
Phyllostachys nidularia	Phni	Zhejiang, China	–	–	HM101556
P. nidularia f. *farcta*	Phfa	Zhejiang, China	–	–	HM101559
f. *smoothsheath*	Phsm	Zhejiang, China	–	–	HM101560
P. sulphurea	Phsu	Zhejiang, China	–	–	HM101538
P. prominens	Phpro	Zhejiang, China	–	–	HM101539
P. viridis f. *houzeauana*	Phho	Zhejiang, China	–	–	HM101562
f. *youngii*	Phyo	Zhejiang, China	–	–	HM101561
P. praecox	Phpra	Zhejiang, China	–	–	HM101540
P. iridescens	PhI	Zhejiang, China	–	–	HM101541
P. glauca	Phglau	Zhejiang, China	–	–	HM101542
P. nigra	PhN	Zhejiang, China	–	–	HM101543
P. nigra var. henonis	Phhen	Zhejiang, China	–	–	HM101548
P. meyeri	PhM	Zhejiang, China	–	–	HM101554
P. aurita	Phauri	Zhejiang, China	–	–	HM101544
P. glauca var. variabilis	Phva	Zhejiang, China	–	–	HM101555
P. glabrata	Phglab	Zhejiang, China	–	–	HM101557
P. elegans	PhE	Zhejiang, China	–	–	HM101558
P. viridiglaucescens	Phvi	Zhejiang, China	–	–	HM101545
P. dulcis	PhD	Zhejiang, China	–	–	HM101546
P. aureosulcata f. spectabilis	Phsp	Zhejiang, China	–	–	HM101547
P. aureosulcata f. pekinensis	Phpe	Zhejiang, China	–	–	HM101550
P. yunhoensis	Phyu	Zhejiang, China	–	–	HM101549
P. flexuosa	PhF	Zhejiang, China	–	–	HM101551
P. aurea	Phaure	Zhejiang, China	–	–	HM101552

P. arcana	Phar	Zhejiang, China	–	–	HM101553
P. pubescens (seedling)	PhPs	Zhejiang, China	–	–	HM101528
P. pubescens	Ph	Zhejiang, China	GQ464833	DQ528692-DQ528695	HM101527
P. pubescens cv. Anjiensis	PhY	Zhejiang, China	–	DQ528702-DQ528705	HM101529
cv. Gracilis	PhG	Zhejiang, China	–	DQ528706-DQ528708	HM101530
cv. Heterocycla	PhP	Zhejiang, China	–	DQ528713-DQ528716; DQ528723-DQ528725	HM101531
cv. Luteosulcata	PhL	Zhejiang, China	–	DQ528699-DQ528701	HM101532
cv. Obliquinoda	PhO	Zhejiang, China	–	DQ528717-DQ528720	HM101533
cv. Tao Kiang	PhK	Zhejiang, China	–	DQ528696-DQ528698	HM101534
cv. Tubaeformis	PhT	Zhejiang, China	–	DQ528721-DQ528722	HM101535
cv. Ventricosa	PhV	Zhejiang, China	–	DQ528688-DQ528691	HM101536
cv. Viridisulcata	PhW	Zhejiang, China	–	DQ528709-DQ528712	HM101537
Subtribe Chusqueeae					
Chusquea coronalis	Ca	Yunnan, China	GQ464814	DQ528668	HM101511
Subtribe Arundinarieae					
Fargesia fungosa	Ff	Zhejiang, China	GQ464818	DQ528672	HM101512
Himalayacalamus intermedia	Hi	Shizuoka, Japan	GQ464823	DQ568677	HM101503
Yushania uniramosa	Yu	Zhejiang, China	GQ464847	DQ523739	HM101504
Pseudosasa japonica	Pj	Zhejiang, China	GQ464836	DQ528729	HM101519
Acidosasa gigantea	Ag	Zhejiang, China	GQ464804	DQ528658	HM101521
Pleioblastus gramineus	Pg	Zhejiang, China	GQ464835	DQ528727	HM101492
Pleioblastus chino	Pc	Zhejiang, China	GQ464834	DQ528726	HM101520
Bashania fargesii	Bf	Zhejiang, China	GQ464808	DQ528662	HM101493
Indocalamus latifolius	Il	Zhejiang, China	GQ464824	DQ528678	HM101494

(continued)

Table 22.1 (continued)

Taxa	Abb.	Source	AN of ITS	AN of partial transposase	AN of full-length transposases
Oligostachyum sulcatum	Os	Zhejiang, China	GQ464831	DQ528685	HM101497
Gelidocalamus annulatus	Ge	Zhejiang, China	GQ464819	DQ528673	HM101524
Menstruocalamus sichuanensis	Mo	Zhejiang, China	GQ464828	DQ528683	HM101489
Sasa fortunei	Sa	Zhejiang, China	GQ464839	DQ528731	HM101523
S. sinica	Ss	Zhejiang, China	GQ464840	DQ528732	HM101490
S. veitchii	Sv	Zhejiang, China	GQ464841	DQ528733	HM101522
Chimonocalamus pallens	Ch	Zhejiang, China	GQ464813	DQ528667	HM101495
Drepanostachyum luodianense	Dl	Zhejiang, China	GQ464817	DQ528671	HM101491
Subtribe Guaduinae					
Otatea acuminata	Oa	Mexico	GQ464832	DQ528686	HM101496
Guadua angustifolia	Ga	Ecuador	GQ464821	DQ528675	HM101502

96.2–100% identity for the corresponding amino acid sequences among the 44 tested species. Pairwise comparisons from six different subtribes were 92.9–100% identity for nucleotide sequences and 96.2–100% identity for amino acid sequences. Pairwise comparisons within each subtribe revealed a similar spectrum of diversity (99.4% identity among Guaduinae, 98–99.6% among Melocanninae, 96.9–99.6% among Bambusinae, 97.8–99.8% among Shibataeeae, and 97.6–100% among Arundinarieae at amino acid level). Among intra-genera, pairwise identities from 26 representative bamboo species of *Phyllostachys* genera were 99.4–100% for nucleotide sequences and 98.2–100% for amino acid sequences. Among intraspecies, pairwise identities from *P. pubescens* and its ten cultivars varied 97.9–100% for nucleotide sequences and 98.6–100% for amino acid sequences.

Up to date, at least six subfamilies of *mariner*-like transposases have been reported in flowering plant, i.e., clade A, clade B, clade C, clade D, clade Y, and clade Z (Feschotte and Wessler 2002). Several elements representative of the six subfamilies previously reported in Feschotte and Wessler (2002) were aligned together with all the above-characterized bamboo *mariner*-like transposases. Six *mariner*-like transposase clusters similar to the clades mentioned above are shown and are defined as the largest well-supported monophyletic group of sequences obtained from phylogenetic trees generated by four distinct methods (NJ, MP, ML, and BI bootstrap values >60%). All the *mariner*-like transposases from Bambusoideae subfamily were monophyletic and grouped into the subclade A2 (Fig. 22.3). These results show that all the identified *mariner*-like transposases are likely derived from the same ancestor.

To further analyze evolution patterns of *mariner*-like transposases and ITS, neutralist tests were performed. Overall Tajima's D (Tajima 1989) and Fu and Li's D* (Fu and Li 1993) of *mariner*-like transposase sequences are close to −2.684 ($P < 0.001$) and −5.323 ($P < 0.002$), respectively. In contrast, those of ITS are close to 1.618 ($P < 0.005$) and 0.325 ($P < 0.005$), respectively. The results show that selection patterns are different between *mariner*-like transposase and ITS sequences. ITS are under positive selection. In contrast, the *mariner*-like transposase might suffer the different constraint-selection pressure in different host genome which should be one of the causes of MLE "spotty" distribution in the Bambusoideae genome (Zhou et al. 2010).

For the identified 79 full-length transposases, most contain complete ORF. There also is not internal stop codon, indels, and frameshift mutations found in 78 full-length *mariner*-like transposases indicating all identified MLEs might be recent products of horizontal transmission. Many identified intact full-length *mariner*-like transposases could provide a foundation for future development of a new genetic tool based on MLEs available for the plant gene tagging (Zhou et al. 2011).

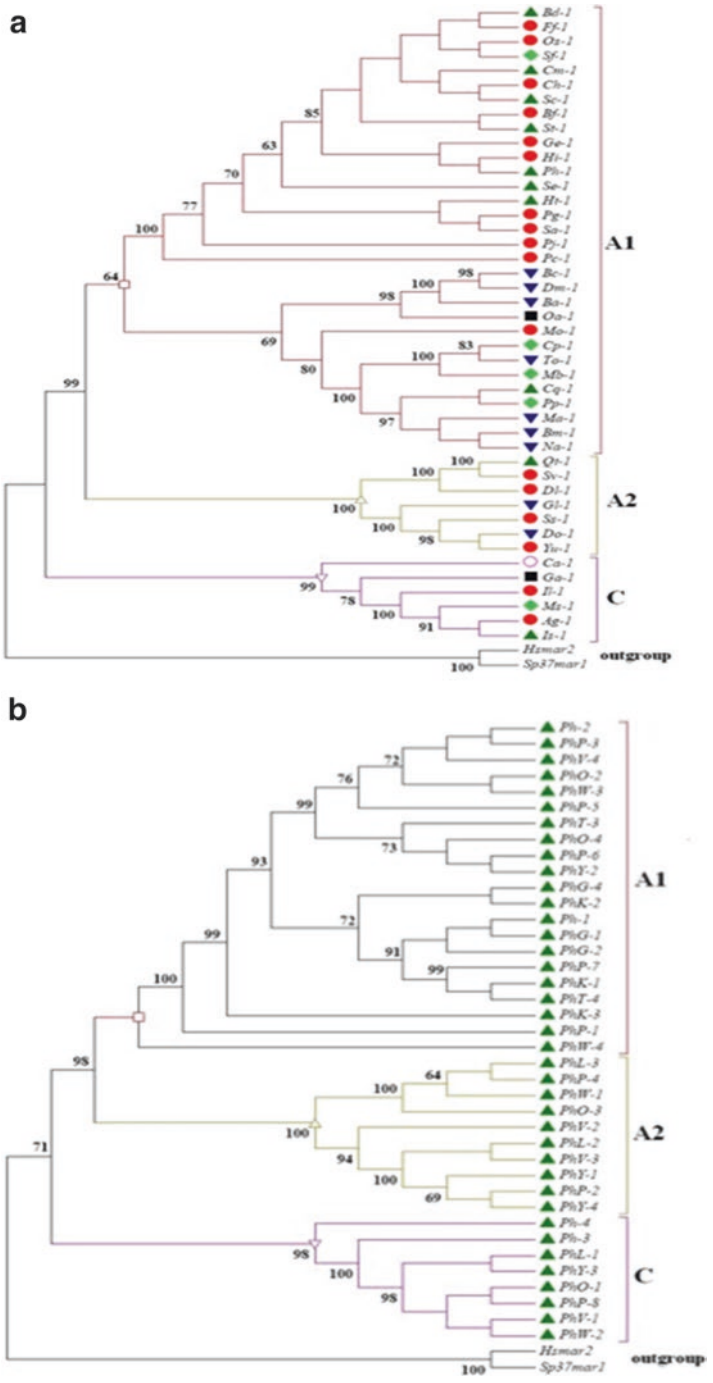

Fig. 22.2 Phylogenetic analysis of MLE transposase fragments and ITS sequences within the Bambusoideae subfamily. Naming of sequences was referred to the text for the details of MLE denomination. Main bootstrap values (1000 replicates) are shown. Groupings defining lineages

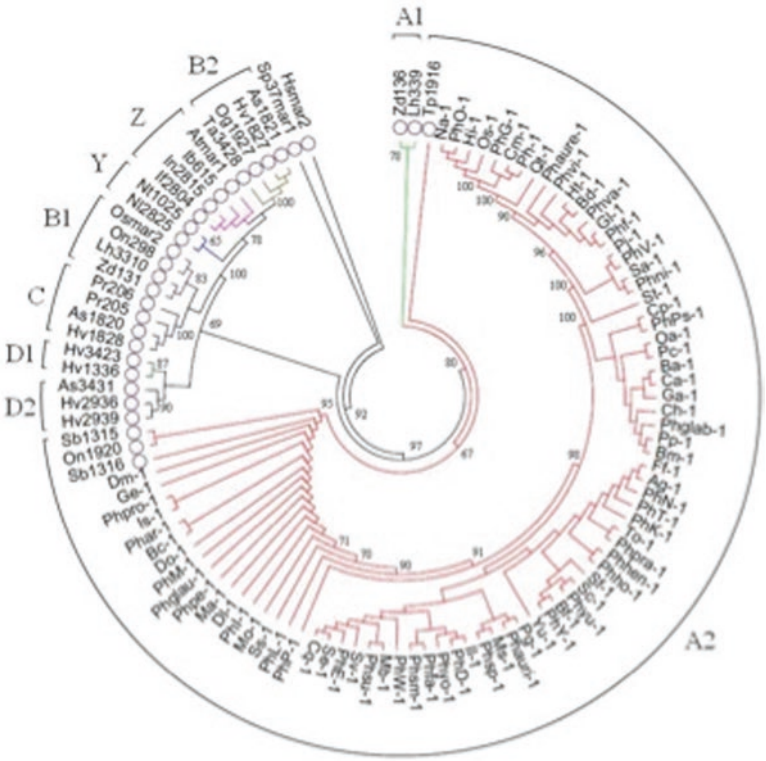

Fig. 22.3 Phylogenetic relationships of full-length transposases from bamboo and *mariner*-like sequences collected in data banks. The phylogenetic tree was generated by multiple alignments of 79 *mariner*-like transposases isolated by PCR and 31 *mariner*-like transposases obtained by database searches. The naming of bamboo sequences was referred to the text for the details of *mariner*-like element denomination. Other sequences were named according to the species initials followed by the number of the clone or the suffix *-mar*, following the nomenclature introduced by Robertson et al. (1998) and Feschotte and Wessler (2002). Main bootstrap values (1000 replicates) are shown. Groupings defining lineages and sublineages of plant MLE transposases are emphasized by capital letters and different colors

Fig. 22.2 (continued) and sublineages of bamboo MLE transposases are emphasized by capital letters and different colors. □: subclade A1; △, subclade A2; ▽, subclade C; ▲, Shibataeeae; ●, Arundinarieae; ◆, Melocanninae; ▼, Bambusinae; ■, Guaduinae; ○, Chusqueeae. (**a**) Phylogenetic relationships among bamboo MLE transposase fragments. The phylogenetic tree was generated by carrying out multiple alignments of 44 MLE transposase fragments obtained by PCR from the same number of representative bamboo species with two animal MLEs as out-group. (**b**) Phylogenetic relationships among 39 MLE transposase fragments isolated from *P. pubescens* and its 9 different cultivars or forms and obtained as above with two animal MLEs as out-group

22.3 The Characterization and Structure Analysis of Two Full-Length MLES Isolated in *P. pubescens*

Based on two conservative transposase sequences (HM101527 and DQ528699 in Table 22.1), we isolated two full-length MLEs (*Ppmar*1 and *Ppmar*2) from *P. pubescens* by chromosome walking using a modified magnetic enrichment procedure. Sequence analysis with GenScan (http://genes.mit.edu/GENSCAN.html) and Netgene2 (http://www.cbs.dtu.dk/services/NetGene2/) showed that *Ppmar*1 and *Ppmar*2 both contain complete ORFs encoding *mariner*-like transposases. In *Ppmar*1, the transposase ORF spans positions 1–1986, ends on a TAG codon, and contains four exons (*Ppmar*1-exon 1–4: 111, 69, 1104, and 216 bp in length, respectively) and three introns (*Ppmar*1-intron 1–3: 81, 327, and 78 bp in length, respectively). The *Ppmar*1 transposase coding sequence is therefore 1500 bp in length encoding a 499-amino acid product (Fig. 22.4a). In *Ppmar*2, the transposase ORF is relatively short, spanning positions 1–1877, ending on a TAG codon, and containing three exons (*Ppmar*2-exon 1–3: 111, 145, and 1092 bp in length, respectively) and two introns (*Ppmar*2-intron 1 and 2: 88 and 442 bp in length, respectively). The *Ppmar*2 transposase coding sequence is therefore 1350 bp in length, encoding a 449-amino acid product (Fig. 22.4b).

There were five *mariner*-like transposases (including *Osmar*1, *Osmar*5, *Osmar*9, *Osmar*17, and *Osmar*19) and two transposases repaired for frameshift mutations (including *Osmar*10 and *Osmar*14) that still could catalyze the transposition of MLE in rice (Yang et al. 2009). Here, we compared the *Ppmar*1 and *Ppmar*2 transposase sequences to the above seven *mariner*-like transposases in rice plus the transposase of the first identified *mariner*-like element in plant, *Soymar*1 from soybean (Fig. 22.5; Jarvik and Lark 1998). The *Ppmar*1 and *Ppmar*2 sequences were only distantly related, sharing 38.9% nucleotide sequence identity and 39.6% amino acid sequence identity. *Ppmar*1 was most closely related to *Osmar*19, with 71.6% nucleotide sequence identity and 66% amino acid sequence identity, whereas *Ppmar2* was most closely related to *Osmar*10, with 54.3% nucleotide sequence identity and 58.1% amino acid sequence identity.

Both the *Ppmar*1 and *Ppmar*2 transposases were shown to contain intact catalytic and DNA-binding domains, with canonical DD39D and HTH motifs. The C-terminal domain contained the typical catalytic motif (DD39D), comprising two aspartic acid residues separated from a third aspartic acid residue with 39-amino acid spacing. N-terminal domain was predicted to contain a helix-turn-helix (HTH) motif by the HELIX-TURN-HELIX MOTIF PREDICTION software (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hth.html) with the 100% score indicating they bind with high affinity to DNA (Figs. 22.4 and 22.5). Many residues previously shown to be critical for transposase activity were conserved in *Ppmar*1 and *Ppmar*2 (e.g., Met-216, Asp-237, Glu-238, Glu-256, Asp-360, Asp-370, Asp-395, and Asp-400 in *Ppmar*1 and Met-167, Asp-189, Glu-190, Glu-208, Asp-313, Asp-323, Asp-348, and Asp-353 in *Ppmar*2) (Izsvak et al. 2002; Watkins et al. 2004; Richardson et al. 2006; Yang et al. 2006, 2009).

In order to investigate the distribution of *Ppmar*1 and *Ppmar*2 in the *P. pubescens* genome, Southern blots of *P. pubescens* genomic DNA were probed with the *Ppmar*1 and *Ppmar*2 clones, revealing only several bands (only one and two copies identified for *Ppmar*1 with the BamHI/EcoRV-digested genomic DNA, respectively, and two and three copies identified for *Ppmar*2 with the same genomic DNA digestion pattern) for both transposons (Fig. 22.6). These data indicated that not more than three copies of *Ppmar*1 and *Ppmar*2 coexisted in the *P. pubescens* genome.

The vast majorities of identified MLEs have already undergone vertical inactivation and thus contain mutations such as internal stop codons, indels, and frameshifts (Hartl et al. 1997a, b; Hartl 2001). For example, genome-wide analysis identified 34 different MLEs in rice, 27 of which contained substitutions or small indels that

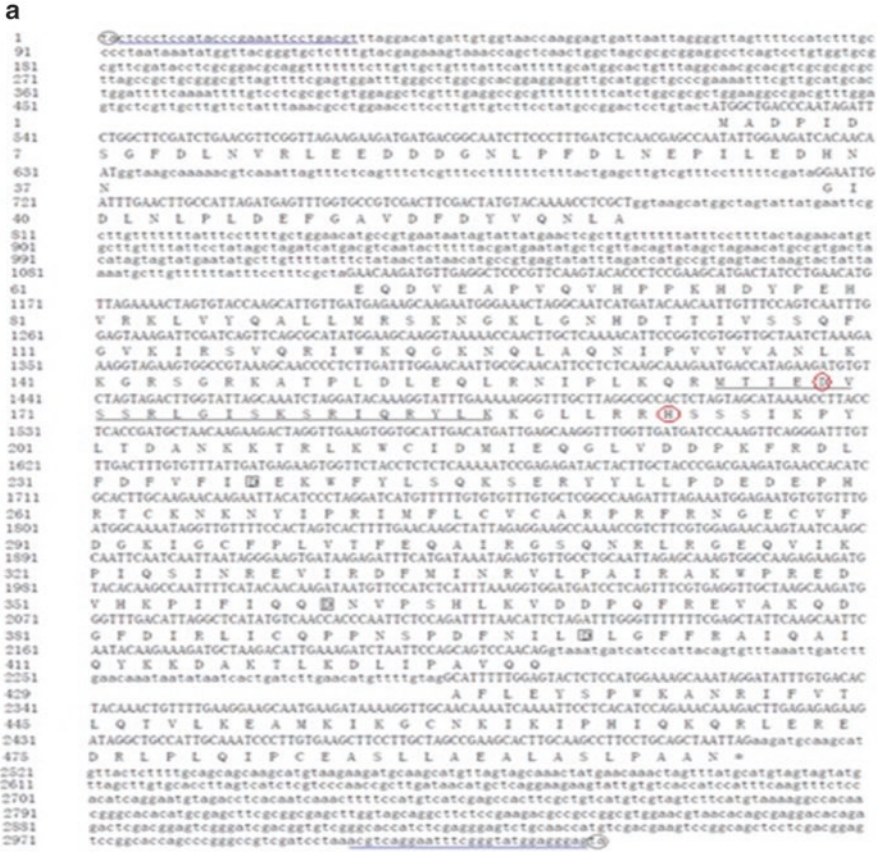

Fig. 22.4 The sequence and structure of (**a**) *Ppmar*1 and (**b**) *Ppmar*2. The ITRs are underlined in *blue* with *blue ovals* to indicate the TSDs. The aspartic acid residues of the catalytic triad are boxed, and helix-turn-helix motifs are underlined. The amino acid residues (Asp 169 and His 193 in *Ppmar*1, Asp 120 and His 144 in *Ppmar*2), which characterized as binding site of divalent metal ions by PAR-3D server, are marked with *red ovals*

Fig. 22.4 (continued)

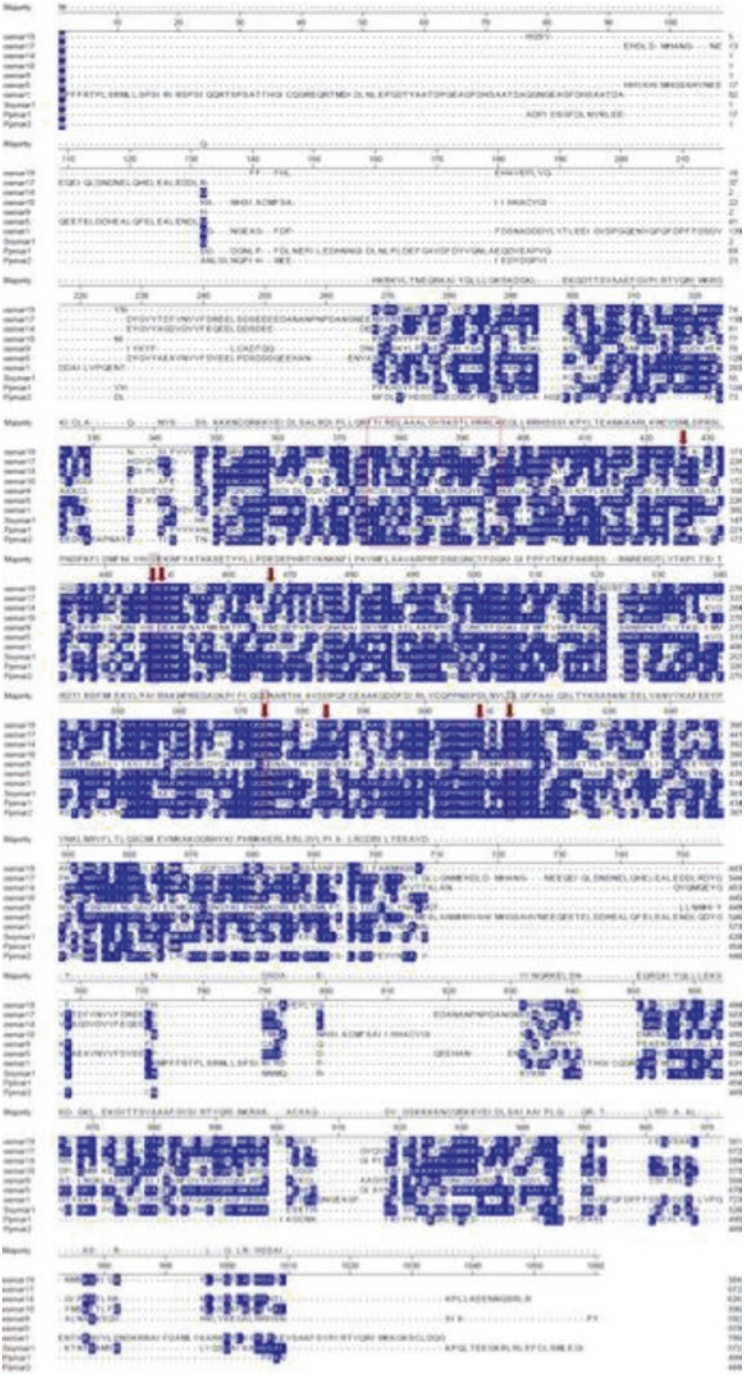

Fig. 22.5 Structural features of conserved amino acid domains in *P. pubescens* MLE transposases. Amino acids that match the consensus are shown with *blue* shading. The helix-turn-helix motif is outlined in *pink smooth box* and the acidic triads of residues representing the three Asps of the DD39D domain are outlined in *brown boxes*. The conserved residues which are critical for transposase activity are marked by *brown open arrows*. Sequences named according to the conventions introduced by Yang et al. (2009)

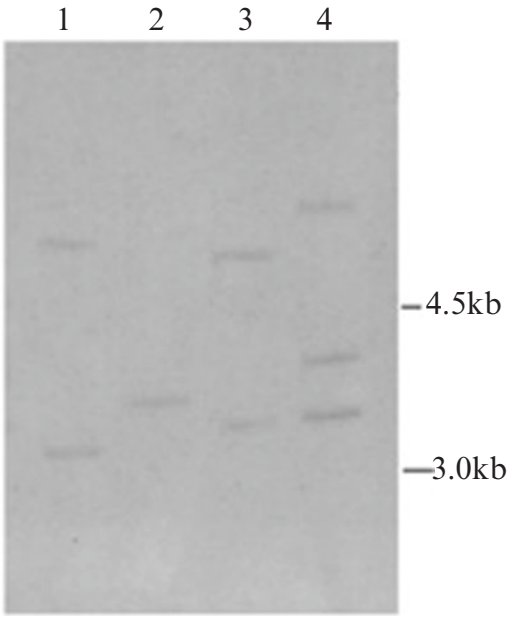

Fig. 22.6 Southern blot of *P. pubescens* genomic DNA probed with *Ppmar*1 or *Ppmar*2. Lane 1, 2: DNA digested with BamH III and EcoRV, probed with *Ppmar*1. Lane 3, 4: DNA digested with BamHIII and EcoRV, probed with *Ppmar*2

introduced premature stop codons in the transposase ORF (Feschotte et al. 2003). In contrast, there are no obvious mutations and indels in *Ppmar*1 or *Ppmar2* during the evolution of both transposons. The *Ppmar*1 and *Ppmar*2 transposases each contain intact catalytic and DNA-binding motifs. Analysis using the HELIX-TURN-HELIX MOTIF PREDICTION software indicates that the HTH motifs of both elements potentially bind with high affinity to DNA. The *Ppmar*1 and *Ppmar*2 transposases are most closely related to *Osmar*19 and *Osmar*10, respectively, which are synthetically active MLE transposases identified in rice (Yang et al. 2009). The 3D structures (Chen et al. 2006; http://ps2.life.nctu.edu.tw/) of the *Ppmar*1 and *Ppmar*2 transposases were very similar to the structure of *Mos*1 transposase, which is first isolated from *Drosophila mauritiana*, and intact and active (Richardson et al. 2006; Yang and Tung 2006; Tung et al. 2007). All these results indicate that the transposases from *Ppmar*1 and *Ppmar*2 are potentially active.

22.4 The Distribution and Insertion Polymorphism of *PPMAR*1 and *PPMAR*2 in the *P. pubescens*

Recently, Peng et al. (2013) reported the draft genome sequence of *P. pubescens*. De novo repeat annotation showed that approximately 59% of the *P. pubescens* genome consists of transposable elements. One complete copy and one truncated of *Ppmar*1 and five complete copies of *Ppmar*2 were identified in the draft genome sequence of *P. pubescens* (Table 22.2). Their insertion sites in the genome of ten pubescens cultivars or forms (its phenotype of stem shape and color refer to Lin et al. 2009)

Table 22.2 The copy of *Ppmar*1 and *Ppmar*2 in *P. pubescens* genome

Query	Scaffold no.	Location	E-value
*Ppmar*1	PH01002213	30849-34289	0.0
*Ppmar*1	PH01173692	272-709	e-136
*Ppmar*2	PH01000131	1070088-1073981	0.0
*Ppmar*2	PH01000740	49673-54675	0.0
*Ppmar*2	PH01000152	732857-737167	e-123
*Ppmar*2	PH01000135	758007-763490	e-119
*Ppmar*2	PH01001893	121231-124729	3e-91

plus the flowering *P. pubescens* and the radiation mutation *P. pubescens* were investigated by sequence-specific amplification polymorphism (SSAP), and amplified products were displayed by denaturant polyacrylamide gel electrophoresis and silver staining test. Results showed that there were dozens of copies of *Ppmar*1 and *Ppmar*2 coexisting in the individual species. A total of 356 electrophoretic bands were detected, of which 160 bands were polymorphism in sizes, 44.9% of total amplification fragments. There were 45 polymorphism bands amplified by the specific *Ppmar*2 primer (P2) and E09 primer, 50.6% of all bands detected in the same lane, which was most among all primer combinations. In contrast, there were only 37 polymorphism bands amplified by the specific *Ppmar*1 primer (P1) and E09 primer, 38.9% of all bands tested in the same lane (Fig. 22.7).

The polymorphism of electrophoretic bands in the denaturant polyacrylamide gel revealed high-level insertional polymorphism for copies of *Ppmar*1 and *Ppmar*2 among *P. pubescens* and nine intraspecies cultivars plus the flowering and the radiation mutation *P. pubescens*, indicating these two *mariner*-like elements appeared to have played a role in the differentiation of *P. pubescens* into the distinct cultivar groups. *P. pubescens* characterizes clonal reproduction in general and flowering at an interval of more than 60 years. In plants that use vegetative reproduction, somatic transposon activities are especially important, as TE insertions can be inherited and subsequently expanded in the population. Recently, a somatic DNA transposon insertion in the grapevine cultivar Carignan has been shown to cause a delayed flowering and altered fruit-clustering phenotype (Fernandez et al. 2010). Sequence analysis demonstrated this somatic insertion was the sole polymorphism in the 4-kb proximal interval of *VvTFL*1A gene that segregated with the phenotype. Inserted in the promoter region, the transposable element behaves as a *cis*-acting enhancer and upregulates *VvTFL*1A gene expression. Whether somatic transposon activity is a universal phenomenon in plants and how these activities may be selected for as a mechanism for generating genetic diversity at a population level will be interesting questions to address in the future (Huang et al. 2012). The study has argued that TEs might have played a role in the mutations of *P. pubescens* and the differentiation of *P. pubescens* into distinct cultivar groups. All the results provided the deeper understanding for impact of TEs on bamboo genomes during bamboo evolution (Zhou et al. 2010, 2011).

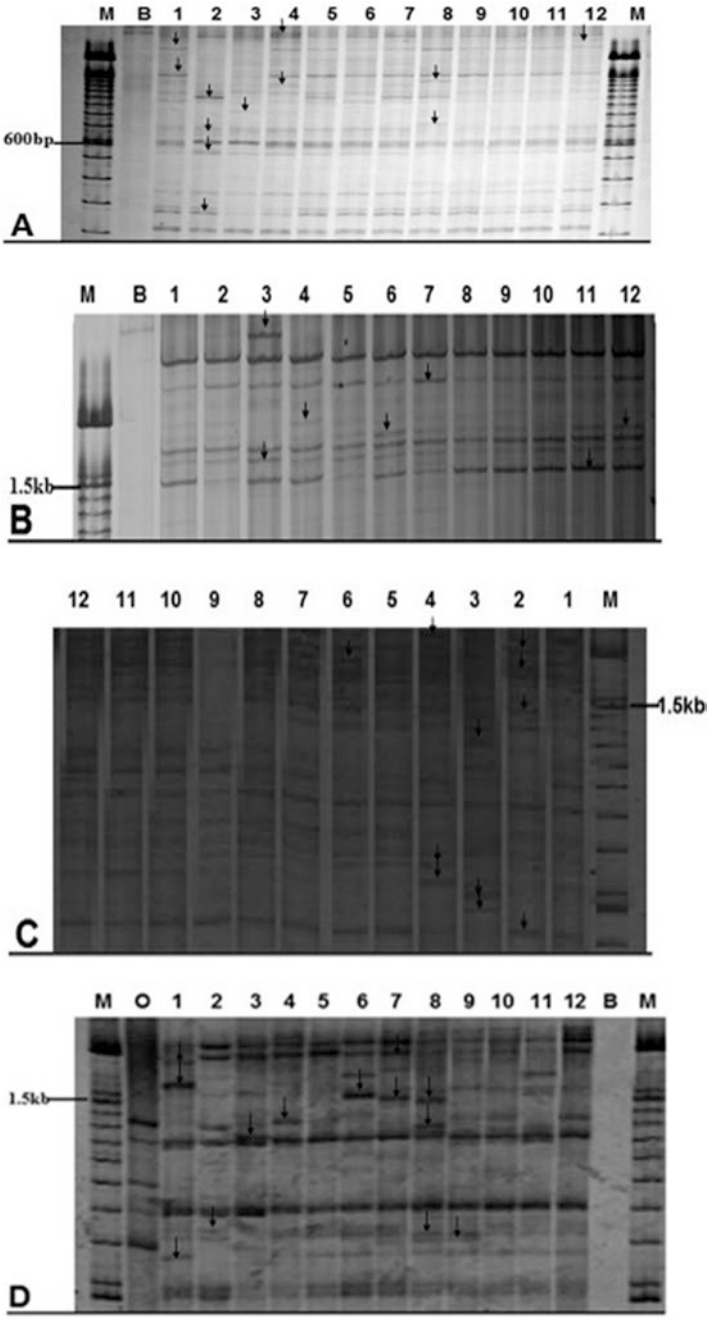

Fig. 22.7 Polymorphism detection of *Ppmar*1 insertion sites with different primers (**a**, **b**) and *Ppmar*2 insertion sites with different primers (**c**, **d**). The 12 genome DNA samples were extracted by the CTAB method and digested by Mse I/EcoR I enzymes. Then the digested DNA fragments were ligated to the adaptors. After two PCRs with the two different adapter primers (E07: 5′-GACTGC

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Fig. 22.7 (continued) GTA CCA ATT CAG C-3′; E09, 5′-GAC TGC GTA CCA ATT CAG A-3′) and specific primers (P1 for *Ppmar*1: ATT TCG AGC AAG CAA CTG GAC; P2 for *Ppmar*2, GAG GCC ACA ACG TCA GCT T) were designed from *Ppmar*1 and *Ppmar*2 sequences, the amplification fragments were displayed by denaturant polyacrylamide gel electrophoresis and silver staining test. *M* marker, *B* water, *O* primer control, *1 P. pubescens*, *2* flowering *P. pubescens*, *3* radiation mutation *P. pubescens*, *4 P. pubescens* cv. Tubaeformis, *5 P. pubescens* cv. Gracilis, *6 P. pubescens* cv. Viridisulcata, *7 P. pubescens* cv. Luteosulcata, *8 P. pubescens* cv. Anjiensis, *9 P. pubescens* cv. Ventricosa, *10 P. pubescens* cv. Tao Kiang, *11 P. pubescens* cv. Obliquinoda, *12 P. pubescens* cv. Heterocycla

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Novel Molecular Tools for Metabolic Engineering to Improve Microalgae-Based Biofuel Production

23

Yuan-Yeu Yau and Mona Easterling

Abstract

Biofuels are derived from biological material. They are an environmentally friendly, low-cost, and renewable energy source, making them potential fuels to replace nonrenewable fossil fuel. Over time, biofuel production technology/methodology has gone through several generations, from the simplest means of production (conversion of simple sugars into biofuel) to the current, more complicated means of production (using novel plant biotechnology). The next generation of biofuel production will involve genetic engineering to improve yields, especially metabolic pathway engineering. Both macroalgae and microalgae sources offer an alternative to fossil-based fuels. Single-celled microalgae use carbon dioxide and sunlight to produce energy. They provide an attractive alternative, due to the significance of their lipid yield, which can be further processed into biofuels and valuable coproducts. The use of microalgae to produce biofuels reduces overall carbon emission without taking away the lands needed for food crops. Despite vigorous research, deployment of large-scale algae-based biofuel production still faces challenges, including high demands of input (water, nutrients, CO_2, etc.) for algal growth. Genetic engineering of algal metabolic pathways holds potential for generating high lipid yields with minimal input. In this chapter, we discuss the use of modern molecular tools for metabolic engineering to improve microalgae-based biofuel production. These include multiple gene transformation, site-specific gene stacking, and precise gene/allele modification with currently developed genome-editing technology, such as CRISPR/Cas9 system.

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Biofuel • Genetic transformation • Genome editing • Metabolic engineering • Microalgae • Multiple gene transfer

23.1 Introduction

Due to depletion of nonrenewable fossil-based fuels, scientists are seeking other renewable energy alternatives. Biofuels are a promising alternative reserve. Biofuels are derived from biological materials. They are an environmentally friendly, low-cost, and renewable energy source. There are different types of biofuels, such as bioethanol (most common biofuel), biodiesel, biomethane, bio-oil, and others. Differing types of biofuels are produced from different materials or feedstock with different processes. For example, (1) bioethanol can be produced by conversion of starch or sugar-rich biomass food crops such as corn, other cereals, sugarcane, or from lignocellulosic materials. (2) Biodiesel can be produced by processing vegetable oils, used cooking oils, etc.

Technologies for biofuel production have rapidly developed and advanced, from the first-generation method into the fourth generation (Aro 2016). In first-generation method, a simple conventional way of converting accessible sugars from food crops, such as sugarcane and maize, into biofuel was used. Due to its reliance on food crops, first-generation methods caused serious concerns about competition of lands for food and fuel production. In a recent report, results indicated first-generation biofuels relied on approximately 2–3% of water and land used for agriculture globally, which could feed about 30% of the malnourished population (Rulli et al. 2016). In the second generation, improved technology allowed the utilization of more advanced processes with all forms of lignocellulosic biomass for biofuel production. Feedstocks used changed from food-based to non-food materials. The use of lignocellulosic biomass increased the varieties of available feedstock for the production of biofuels. In this case, cereal straw (e.g., wheat straw), corn stover, dedicated fast-growing biomass energy crops such as switchgrass (*Panicum virgatum* L.) and giant miscanthus (*Miscanthus x giganteus* Greef et Deu.), as well as agricultural and industrial residues/waste, could all be used for lignocellulosic bioethanol production (Hood 2016). Both switchgrass and miscanthus are C4 perennial grass species which grow well on marginal soils unsuitable for most conventional food crops. The third-generation biofuel refers to biofuel derived from microalgae (Behera et al. 2014). Microalgae have received increasing interest from scientists and industries to be used as an advanced biofuel production material, due to its many advantages over previous generations of biofuel feedstocks. Microalgae are photosynthetic microorganisms, which use sunlight to convert CO_2 into carbon-rich lipids (Fig. 23.1). Photosynthetically derived fuel is a renewable, potentially carbon-neutral, and scalable alternative. Microalgae are capable of producing a quantity and diversity of fuels no other feedstock can currently match. Additionally, microalgae can grow on nonarable lands and will not compete for agricultural lands. Also,

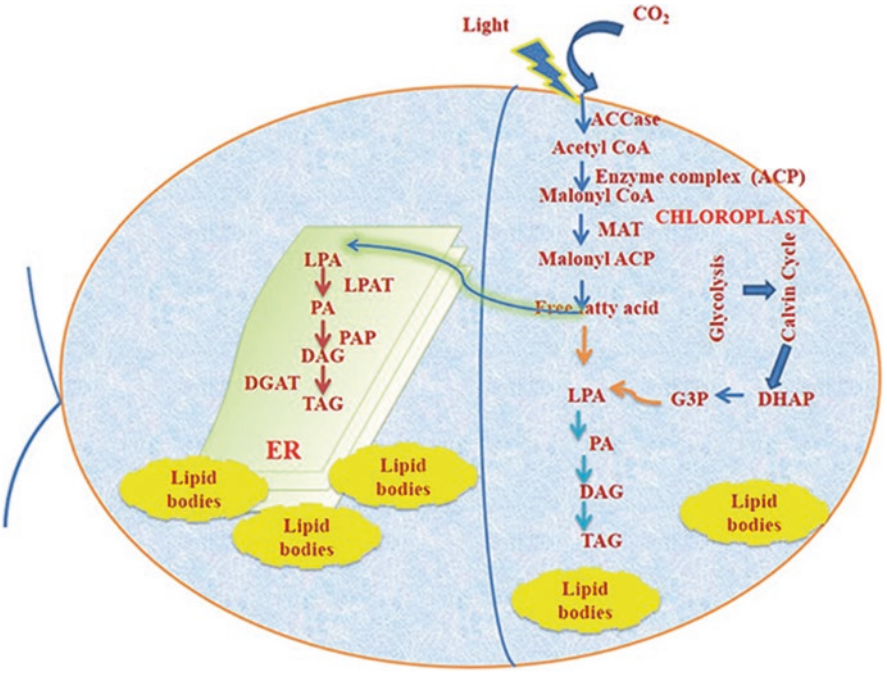

Fig. 23.1 Fundamental representation for TAG synthesis and accumulation pathway in *C. reinhardtii*. *DAG* diacylglycerol, *DGAT* diacylglycerol acyl transferase, *G-3-P* glycerol-3-phosphate, *ACCase* acetyl-CoA carboxylase, *ACP* acyl carrier protein, *FFA* free fatty acid, *DHAP* dihydroxyacetone phosphate, *MCAT* malonyl-CoA:ACP transacylase, *PAT* lysophosphatidic acid acyltransferase, *LPA* lysophosphatidic acid, *PA* phosphatidic acid, *PAP* phosphatidic acid phosphatase, *TAG* triacylglycerol (Figure reproduced from Banerjee et al.(2016), an Open Access article distributed under the terms and conditions of the Creative Commons by Attribution (CC-BY) license, https://creativecommons.org/licenses/by/4.0/)

the bio-oil produced is biodegradable and will not cause great impact on the environment if spilled. Current microalgae-based biofuels are bioethanol, biodiesel, biomethane, biohydrogen, and bio-oil.

Many eukaryotic microalgae have the ability to store energy-rich compounds such as triglycerides (TAG) and starch which can be extracted and converted to biofuels (Hu et al. 2008). Worldwide, scientists are carrying out different research programs needed to expand algal lipid (or TAG) production for biofuel. In early research, conventional methods using nutrient limitation, especially nitrogen stress, were the major approach to triggering TAG accumulation in microalgae (Benvenuti et al. 2016). However, one major disadvantage of this method was a reduced growth rate in these algae. New tools are needed to improve microalgae lipids production in the future. Biotechnology, especially the metabolic engineering, is striving to produce improvements within the fourth generation of biofuels. Metabolic engineering, an approach seeking to redesign natural pathways via manipulating up- or down-regulation of native genes or the introduction of transgenes, is one possible solution.

For example, scientists were able to modify the expression of critical genes involved in the TAG metabolic pathway and improve TAG production in microalgae (Banerjee et al. 2016). The development of genetic transformation systems for microalgae species is facilitating growth potential for a metabolic engineering approach. So far, successful nuclear transformation has been reported for ~25 microalgae species (Doron et al. 2016). In many cases, stable expression of transgenes in transformed microalgae was observed (Doron et al. 2016).

This chapter focuses on useful molecular tools for metabolic engineering, especially on multiple gene transformation and gene knockout that may be undertaken in order to improve microalgae-based biofuel production. The advantages of each technology and constraints of different approaches are also discussed.

23.2 Metabolic Engineering

Metabolic pathways producing complex molecules are usually complicated and involve many genes. One can manipulate the genes of metabolic pathways *in vivo* to increase the target metabolites through metabolic engineering. There are several different strategies to modulate metabolic compound levels in plants (Fig. 23.2;

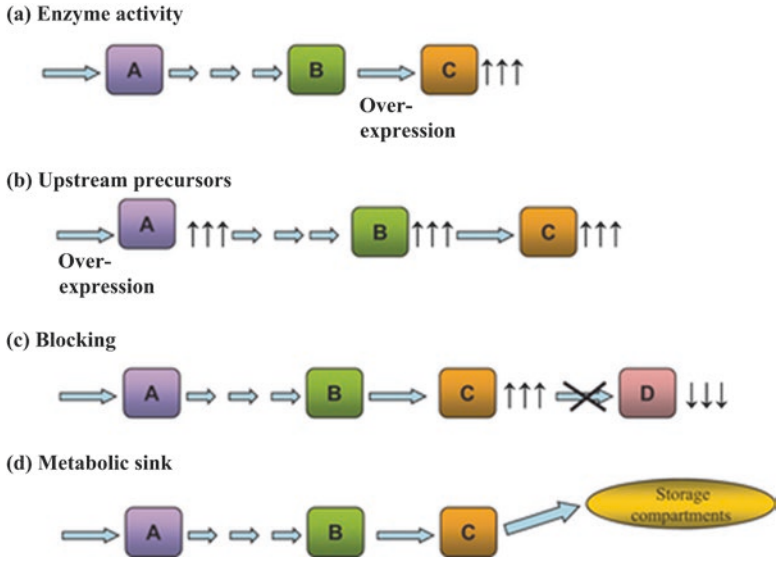

Fig. 23.2 Strategies to modulate organic compound levels in plants. These strategies comprise the modification of (**a**) activity of enzymes implicated in rate-limiting steps in target pathways by modulation of one or two key enzymes, or multiple enzymes, (**b**) upstream precursors to increase flux through the pathway by overexpressing the enzyme that catalyzes the first committed step of the target pathway, (**c**) pathway branch points by blocking and relieving feedback inhibition by RNA interference or antisense, and (**d**) enhancement of accumulation of target metabolites by increasing sink compartments to store target compounds (Figure reproduced from Zhu et al. (2013), an Open Access article distributed under the terms and conditions of the Creative Commons by Attribution (CC-BY) license, https://creativecommons.org/licenses/by/4.0/)

Zhu et al. 2013). Several examples of using metabolic engineering to increase the production of valuable metabolite compounds have been reported in plants. One example is *Saussurea involucrata*, a commonly used medicinal herb with great pharmacological value for human health. Qiu et al. have transferred two key phenylpropanoid pathway-inducing transcription factors (*PAP1* and *Lc*) into the rare wild species of snow lotus, *Saussurea involucrata*. By this process, the researchers activated most of the phenylpropanoid pathway genes, which increased accumulation of several phenylpropanoid compounds (Qiu et al. 2013). Another research group showed overexpression of the enzyme CrDGTT4 (type 2 diacylglycerol acyl-CoA acyltransferase) driven by the SQD2 (sulfoquinovosyldiacylglycerol synthase 2) promoter enhanced accumulation of TAGs in *Nannochloropsis* species (a unicellular photosynthetic microalgae) and increased yields of TAGs (Iwai et al. 2015).

Since metabolic pathways are usually complicated, involve many genes, and often feedback responses (such as feedback inhibition), significant interventions in metabolic flux toward desired compounds are likely to require the overexpression and/or silencing of multiple genes simultaneously (Zorrilla-Lópezet al. 2013). To accomplish this, the development of an efficient cloning method to assemble a group of available genes on a large-capacity vector and an efficient transformation system to deliver those vectors are needed. The development of other strategies to stack new gene(s) next to known gene(s) *in vivo* is also desirable. In the following paragraphs, different available molecular tools and strategies for multiple gene transfer and *in vivo* (or *in planta*) gene stacking are described.

23.3 Multiple Gene Transfer

Several established methods can be used to deliver multiple transgenes through genetic transformation. Some illustrations of successful methods are as follows:

1. Through consecutive transformation, multiple transgenes can stack into a plant line one by one through several runs of standard genetic transformation. For example, Singla-Pareek et al. (2003) stacked the *gly-II* gene into tobacco with *gly-I* transgenic background by retransformation, which led to enhanced salinity tolerance (Singla-Pareek et al. 2003). Consecutive transformation can help researchers study the role of each gene product in a metabolic pathway by comparing one-gene overexpression, two-gene overexpression, and three-gene overexpression results.

   There are some challenges for using the consecutive transformation strategy. One disadvantage is the need for unique selection agents and selectable marker genes (SMGs), which are limited. At this time, the most familiar and popular SMGs are kanamycin-resistance gene (*nptII*), hygromycin-resistance gene (*hyg*), and *bar*-resistant gene. One way to overcome this challenge is to recycle the SMG and reuse it in the next-run transformation. Application of site-specific recombination (SSR) systems is an option for recycling the SMG. SSR included in the transformation system can be used to delete SMG and produce SMG-free transgenic plants (Fig. 23.3). SMG-free transgenic lines will be used as the

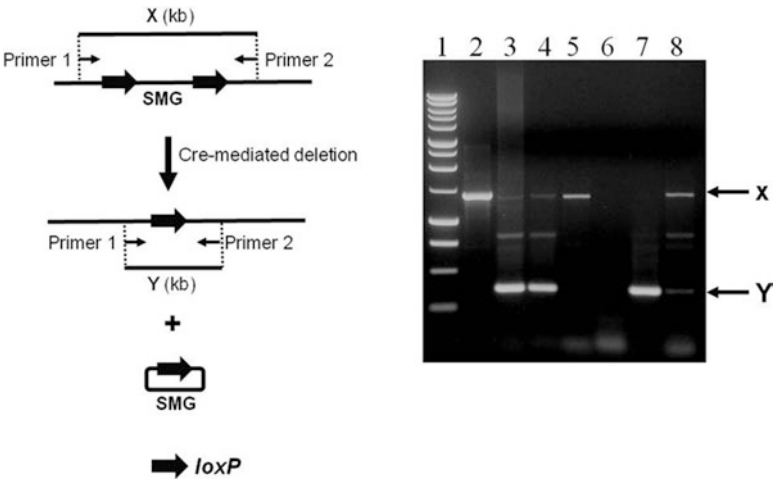

Fig. 23.3 PCR detection of Cre-mediated deletion in transgenic *Arabidopsis* plants. Primers flanking two *loxP* sites were used to detect Cre-mediated deletion. The amplification of PCR product "Y" indicates that excision events occurred. The selectable marker gene (SMG) was excised. 1% TAE gel. Lane 1, 1 kb DNA size markers; lane 2, positive control (without excision); lane 3, positive control (with excision). Lanes 4–8: transgenic plants

next-run transformation line, and the same SMG can be used for selection marker. Another benefit of removing SMG is to reduce food safety concerns of the public and regulatory agencies. Removal of SMG also reduces the metabolic burden of the transgenic plants. Several SSR systems have been proven functional *in planta*, including Cre-*lox*, phiC31-*att*, Bxb1-*att*, and others (for a review, see Wang et al. 2011).

One other disadvantage of this method is that transgenes are not linked. Since they are transformed one by one, they insert at different loci of the genome. It is difficult to obtain offspring with localized transgenes together in progeny following genetic segregation. Transgenes by nature unlink, so transgenic lines must be screened for position effects. This renders the consecutive transformation method less than optimal and practical. This challenge can be overcome by applying an *in planta* gene stacking method, where transgenes can be stacked at the same locus through transformation. *In planta* gene stacking will be discussed later in the gene stacking section.

2. Transgenes are built on different binary vectors for co-transformation. Individual transgenes are built into a single T-DNA on a binary vector (Fig. 23.4). For example, if four transgenes need to be transferred, then four binary vectors are to be constructed. The T-DNAs are then co-transformed into plant genome through *Agrobacterium*-mediated co-transformation. Co-transformation can be carried out through one species of *Agrobacterium* or mixture of *Agrobacterium* carrying different T-DNA (Fig. 23.4a, b). In general, the T-DNA cassettes insert at different loci of the plant genome. So, this method will have the same disadvantage as that described in method (1). Despite limits, there are examples of success with

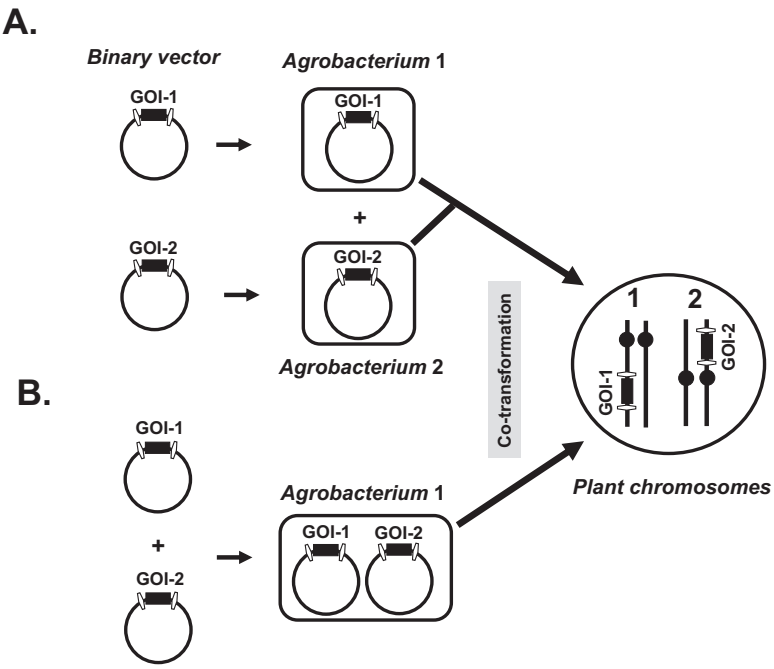

Fig. 23.4 *Agrobacterium*-mediated co-transformation. (**a**) Two individual binary vectors are carried by two different *Agrobacterium* for co-transformation. (**b**) Two individual binary vectors are carried by one *Agrobacterium* for co-transformation. *GOI* gene of interest, selectable marker gene is not shown on the plasmids

this method, including the engineering of starch biosynthesis in maize endosperm to increase total starch by using multiple transgenes (Jiang et al. 2013).

3. Multiple linked transgenes, each containing an appropriate promoter and a terminator, can be assembled within a single T-DNA on a standard binary vector for *Agrobacterium*-mediated delivery. The advantage of this strategy is that transgenes tend to insert at a single locus of a plant genome and co-segregate as a unit together into the subsequent generations. The limitation of this method is that only a small number of transgenes can be assembled onto a standard binary vector. When transgene numbers increase, the technique for cloning becomes cumbersome, due to a lack of unique restriction sites and the increased size of the vector, which can make it unstable for transformation. To overcome this challenge, two things need to be developed: (1) an efficient assembling tool to facilitate building of multiple transgenes onto a plasmid and (2) a high-capacity binary vector capable of holding a large DNA fragment for transformation and maintaining it stably during transformation.

   The binary bacterial chromosome (BIBAC) (Hamilton 1997) and transformation-competent artificial chromosome (TAC) vector systems serve these purposes (Lin et al. 2003). BIBAC vector is a high-capacity binary vector.

A size limit for a standard vector is reported around 50 kb, but BIBAC can take in a 200-kb fragment. Lin and colleagues have developed a system called "multigene assembly vector system" (Lin et al. 2003). The researchers used Cre-*lox* SSR system to facilitate the stacking of as many as ten genes and functional DNA fragments into the TAC-based vector and successfully transferred the genes into the rice genome using *Agrobacterium*-mediated transformation. In addition to the Cre-*lox* system (Lin et al. 2003), several other systems to increase the cloning efficiency and facilitate the cloning processes have been developed in recent years. These include Gateway™ technology-based gene assembly technology (Chen et al. 2006), recombination-assisted multifunctional DNA assembly (MISSA) system (Chen et al. 2010), modular cloning (MoClo) system (Werner et al. 2012), and GoldenBraid system (Sarrion-Perdigones et al. 2013). By taking advantage of the bacteriophage λ SSR system and circumventing traditional restriction enzyme-based cloning limitations, Gateway™ technology has been widely adopted for plasmid cloning (Hartley et al. 2000); GoldenBraid assembling system is based on type IIS restriction enzymes. According to Sarrion-Perdigones et al. (2011), the relative position of type IIS restriction sites inside destination plasmids (pDGB) introduces a double loop ("braid") topology in the cloning strategy allowing indefinite growth of composite parts through succession of iterative assembly steps (Sarrion-Perdigones et al. 2011).

4. Biolistic bombardment can also be used for multiple-gene delivery by directly shooting naked plasmids into plant genome. It has been reported that DNAs co-transformed by bombardment can integrate into the same locus (Makarevitch et al. 2003). However, the limitation of this method is that it tends to cause high-copy transgene insertion, and high-copy transgene insertion is prone to induce "gene silencing."

23.4 *In planta* Gene Stacking at the Same Genomic Site

As mentioned, the insertion of transgenes will be random using method (1) or (2) above, and different transgenes can integrate at different chromosomes or loci. Through genetic segregation, this set of transgenes can segregate in the next generation, with only a rare number of transgenic lines containing the whole set of transgenes. Researchers have to screen out the small number of successful transgenic lines from a huge number of putative transgenic population. It is time-consuming and labor intensive. To improve this situation, method (3) or (4) can be used. However, the use of method (3) or (4) requires all the transgenes be available while building the constructs. The other strategy is to stack transgenes at the same locus through consecutive transformation. This is particularly useful, if newly discovered genes are to be added to the same locus where other transgenes already exist. Several strategies for *in planta* gene stacking are discussed below. In this section, we focus on site-specific recombination system mediated methods for *in planta* gene stacking since SSR systems have been widely used in plants and other organisms for decades, although there are some other means.

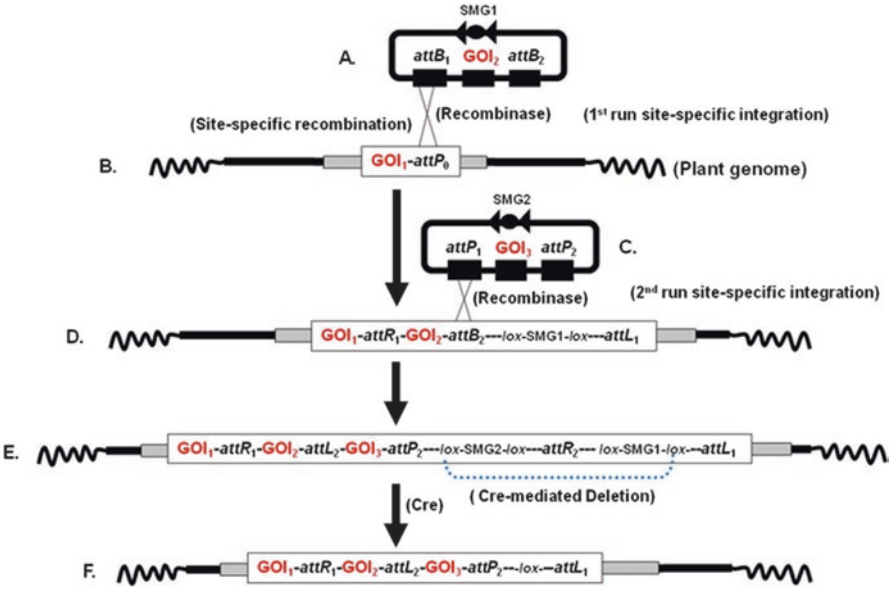

Fig. 23.5 *In planta* site-specific transgene stacking. The details of stacking steps have been described in the content. *GOI* gene of interest, *SMG* selectable maker gene, *attP* and *attB* recognition sites for Bxb1-*att* system, *attL* and *attR* hybrid sites generated after *attP* x *attB* site-specific recombination

23.4.1 Example 1

SSR systems can be used to repeat integrate transgenes at the same locus and stack them back to back in order (Fig. 23.5). Here in Fig. 23.5, we use Bxb1-*att* system as an example to study transgene stacking. Bxb1-*att* system is a unidirectional SSR system. Bxb1 recombinase performs site-specific recombination at two nonidentical specific recognition (or recombination) sites – *attP* and *attB* (Hou et al. 2014). For *in planta* gene stacking, "target" lines need to be generated and characterized first. Each target line contains a cassette with a gene of interest (GOI) and an *attP* site or GOI_1-$attP_0$ in Fig. 23.5b. DNA construct with GOI_1-$attP_0$ is first transformed into the plant genome through standard genetic transformation. Once the target lines containing GOI_1 are established, a construct (plasmid) carrying GOI_2 and two identical *attB* sites ($attB_1$ and $attB_2$) (Fig. 23.5a) is then transformed into them. This plasmid is then integrated into the same plant genomic site through site-specific recombination at genomic $attP_0$ and $attB_1$ of the plasmid ($attP_0 \times attB_1$). A recombinase-producing gene is also needed to carry out this reaction. Although $attP_0 \times attB_2$ can occur (at 50% chance), the recombination of $attP_0 \times attB_1$ is preferred. After $attP_0 \times attB_1$ recombination occurs, two hybrid sites, *attL* and *attR*, are generated. At the same time, a new trait gene GOI_2 is integrated next to GOI_1 (Fig. 23.5d). Plant lines with the desired recombination type ($attP_0 \times attB_1$) can be screened out through PCR on the integration junctions (containing *attL* and *attR* sequences) using two pairs of

specific primers. This integration will use up one *attB* site ($attB_1$) and bring in another *attB* site ($attB_2$). New *attB* site ($attB_2$) is generated for use with next-run integration (Fig. 23.5d). In second-run integration, another construct is built. It contains GOI_3 and two identical *attP* sites ($attP_1$ and $attP_2$) (Fig. 23.5c). A similar process to first-run integration will occur, but this time, the $attP_1$ of the plasmid will recombine with the genomic $attB_2$ site. After this integration, three trait genes (GOI_1, GOI_2, and GOI_3) will be stacked side by side in the same genomic locus (Fig. 23.5e). Finally, the selectable marker genes (SMGs) are removed through Cre-mediated site-specific deletion to obtain SMG-free product. Upon the expression of Cre recombinase, SMGs flanked with *loxP* sites of Cre-*lox* SSR system are excised (Fig. 23.5f).

Proof-of-concept studies using this strategy for *in planta* gene stacking have been done and published recently (Hou et al. 2014). In the paper, PEG-mediated protoplast transformation was carried out for transgene stacking. Integration plasmids and Bxb1-expressing plasmids were co-transformed into tobacco (*Nicotiana tabacum* L., Wisconsin 38) protoplasts of established target lines. The plasmid backbone and SMG were removed through Cre-mediated excision. Three transgenes (*gus, gfp, luc*) were successfully stacked and were able to co-transmit into progeny as a unit. No Bxb1 recombinase gene cassette was detected in the genomes of the final integration lines, indicating *bxb1* gene expression was transient.

Recently, using biolistic bombardment for transgene stacking with a similar strategy and Bxb1-*att* system was reported in rice (Li et al. 2016). For bombardment, biolistic particle delivery system (e.g., PDS-1000/He machine from *BioRad*) was used. The biolistic bombardment approach expands the use of this technology, because PEG-mediated protoplast transformation may not be applicable to many crops due to a lack of protoplast isolation and efficient transformation protocols. In contrast, the biolistic method has been widely used and has successfully produced transgenic plants for many crops in the past decades.

23.4.2 Example 2

Recently, Srivastava's lab also reported the use of Cre-*lox* system for gene stacking in plants (Nandy et al. 2015). In their approach, three biological systems were used to accomplish gene stacking: SSR (Cre-*lox*), homing endonuclease (meganuclease I-*Sce*I), and zinc finger nuclease (ZFN). The Cre-*lox* system was used to incorporate an integration construct into a pre-characterized locus. I-*Sce*I and ZFN systems were used to alternatively excise SMG and an unwanted *lox* site (wild *loxP* or mutated *lox*) later. The processes of integrating transgenes using Cre-*lox* is similar to using Bxb1-*att* system, as mentioned above. However, unlike unidirectional Bxb1-*att* system, Cre-*lox* is a bidirectional SSR system. The concern of using it for transgene integration is that the reaction can perform integration and excision simultaneously. The integrated fragments can be used as a substrate of Cre and excised out for as long as the recombinase presents. As a result, the integrated DNA fragment is unstable and the efficiency of site-specific integration is decreased. A few strategies can be used to prevent or limit the reversible nature of this reaction. They include (1) controlled Cre expression, e.g., transient expression of Cre, and (2) use

of mutant *lox* sites (one arm of *lox* was mutated). In the integration experiment, the authors use method (2) to trap the transgene in rice genome.

The other concern is the use of ZFN system for SMG deletion. When SMG is flanked by ZFN recognition sites, expression of ZFN can cause DNA double-stranded breaks (DSBs) at both ZFN recognition sites and release the SMG. The broken ends are then repaired and joined through the host cell's DSB repair system. However, the major DSB repair pathway in eukaryotic cells is nonhomologous end joining (NHEJ). The fixed join sites usually have an insertion or a deletion (indel). The deletion can be small (several bps) or large. In their report, out of a total of 68 lines analyzed, 38 of which (55.8%) were large deletions. Some large deletions are >1 kb. Large deletions create the possibility of generating truncated neighboring transgenes. So, quite a few transgenic lines need to be produced for screening. This is different from using SSR systems for SMG removal. Since the process of site-specific recombination through SSR systems is "conservative," there is no net gain or loss of sequence information. In other words, no extra bases are added or deleted following the recombination reaction. Another concern is that ZFN technology remains in its patent period and cannot be freely used by many researchers, while the Bxb1-*att* SSR system is an open source for use (Hou et al. 2014).

23.4.3 Example 3

Another method to stack transgenes is SSR-mediated "RMCE." RMCE stands for recombinase-mediated DNA cassette exchange (Turan et al. 2013). RMCE can be used to precisely replacing a genomic target cassette (which is embedded in the genome beforehand) with a compatible donor cassette (which is later induced into the genome) through SSR-mediated tag-and-exchange strategies. RMCE has been used in mammalian-cell and plant-cell researches. Li et al. used multiple runs of RMCE to stack seven transgenes in soybean (*Glycine max*) to improve its nutrition value, increase the contents of oil produced, and increase yield of the essential amino acids lysine and methionine (Li et al. 2010). It is important to note that their RMCE strategy had successfully stacked both "overexpression" and "gene-silencing" cassettes (of 7 genes) into soybean genome, and all were functional.

23.5 Metabolic Pathway Gene Knockout by Precise Genome-Editing Tools

As mentioned in Fig. 23.2c, the production of target metabolites can be increased by knocking out specific enzyme(s) in the pathway. For example, metabolite "C" in Fig. 23.2c can be built up by knocking out (or silencing) the key gene whose product catalyzes product "C" into "D." Efficient methods are needed to knock down or knock out unwanted genes in microalgae. RNAi technology was frequently used for gene silencing before new genome-editing tools were developed. It is important to note that RNAi usually does not completely eliminate the gene product but only

"knocks down" the gene. This means some functional RNA remains, and translation occurs at lower levels. In contrast, the new generation of gene-silencing technologies can be used to completely knock out a gene, by producing a null allele. These new tools have developed quickly in the past decade. They only require the sequence of a targeted gene to be available. Such novel genome-editing systems include ZFN, transcription activator-like effector nucleases (TALEN) (Bogdanove and Voytas 2011), and the most recent groundbreaking technology, *clustered regularly interspaced short palindromic repeat/CRISPR-associated proteins*, known as CRISPR/Cas system (Jinek et al. 2012). Among them, CRISPR/Cas9 system is the most popular, due to its simplicity of construction, efficiency (Cas9 endonuclease has demonstrated a better cleavage efficiency), precision, and low cost. CRISPR/Cas system has been used to successfully create DSBs at a specific gene locus in model plants and crop plants (Khatodia et al. 2016). The common feature among these three systems is an induction of DSB at a specific locus to generate stable and heritable mutations. As mentioned earlier, the major DSB repair pathway in eukaryotic cells is through NHEJ, which creates deletions or insertions (indels) at the fixed joint sites. If it occurs within an allele, the deletion or insertion can cause a frameshift mutation of the gene.

A recent report showed that CRISPR/Cas9 technology can be used to efficiently generate stable targeted gene mutations in microalgae, using marine diatom *Phaeodactylum tricornutum* (Nymark et al. 2016). By targeting the chloroplast signal recognition particle 54 (*CpSRP54* gene), a mutation frequency of 31% was given. Wang et al. also used CRISPR/Cas9-based system to successfully target nitrate reductase gene of the industrial oleaginous microalgae *Nannochloropsis oceanica* (Wang et al. 2016). Shin et al. also used CRISPR/Cas9 system to successfully induce knockout and knock-in mutations in *Chlamydomonas reinhardtii* (Shin et al. 2016). They found that the knock-in mutations were predominantly carried out through NHEJ-mediated repair pathway (Shin et al. 2016). Results from these pioneering studies indicate that the CRISPR/Cas9 technology can be used to efficiently generate stable mutations on targeted genes in microalgae.

23.6 Final Remarks

Biofuels are produced from biological materials and remain a promising alternative fuel reserve for the future. The use of biofuels reduces environmental pollution caused by fossil fuel. Microalgae have been a rising star as a feedstock for biofuel production, due to various advantages over competitors. In spite of intensive research worldwide, the use of microalgae for commercial-scale biofuel production is still not realistic. A major limiting factor is the lack of optimization for process methods and protocols needed to grow, maintain high lipid content, harvest, dry and extract oil. Biotechnology can play a vital role in enhancing overall microalgae biofuel production in the future, by manipulating critical genes in the oil (TAG)-producing pathway. Many novel molecular tools can be used for metabolic

engineering, and these novel tools have demonstrated success in plants. Although the research of these tools in microalgae lags behind that of other plant species, evidence suggests the tools can be used successfully in microalgae. With recent efforts in microalgae genetic transgenesis (> 25 species have been successfully transformed), genome sequencing (>10 species have been sequenced), genome-editing using CRISPR/Cas9, and other molecular tool applications, the advances in metabolic engineering show potential for increasing the production of energy-rich storage compounds, such as TAGs and starch for biofuel production (Radakovits et al. 2010).

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Synthetic and Semisynthetic Metabolic Pathways for Biofuel Production

24

Shikha Bhansali and Ashwani Kumar

Abstract

Recently, metabolic engineering is greatly benefited from systems and synthetic biology due to substantial advancements in those fields. The present review aims at importance of metabolic engineering and synthetic biology for production of compounds such as fatty acids, alcohols, and high-value chemicals. The C_3 plants, including important food crops like rice, wheat, barley, and soybean overcome RuBisCO's catalytic inefficiency by enriching some of the traits from algal system. Synthetic and semisynthetic energy conversion systems, based on photosynthetic processes, have recently been proposed. They envisioned that thylakoids with modified PSII can be used outside the living cell in potentially vast amounts and without the requirement of complicated isolation procedures. Another approach could be the use of a native and viable photosynthetic system adapted to serve as a direct source of either sustained electrical current or storable chemical energy and perhaps useful, conduit for electron transport. Balancing and optimization of metabolic engineering and systems biology to develop tailor-made microbial factories for the efficient production of chemicals and biofuels might replace products derived from natural sources in the near future.

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Electron transport • Photosynthetic process • PSII • Transmembrane complex

24.1 Introduction

The world population growth has outpaced improvements in agricultural productivity with conservative projections estimating a world population of 9.7 billion in 2050 and 11.2 billion in 2100. The volumes of bio-based products (derived from renewables, not necessarily fermentation based, and excluding biofuels) globally are estimated to be 50 billion kilos per year, and these volumes are anticipated to grow significantly in the near future (de Jong et al. 2012). Besides a growing demand for biofuels and biomass production will lead to increased competition for arable land (Kumar 2011). Plant biotechnology and synthetic biology can offer suitable solutions to meet the growing energy and food requirements.

Molecular biological approaches will be needed to improve agricultural and biofuel production (Kumar 2013, 2015a, b; Bhansali and Kumar 2014a; Bhansali and Kumar 2014b; Kumar et al. 2014). Jones et al. (2015) reviewed major improvements in yield, titer, and productivity that can be accomplished by balancing metabolic pathway gene expression (Pitera et al. 2007; Ajikumar et al. 2010; Xu et al. 2013). According to Lynch (2016), the production of numerous sustainable chemicals using engineered microbes has potential environmental impact with a significant reduction in greenhouse gas emissions (GGEs) while offering the potential of advanced products with improved properties. Wu et al. (2015) engineered the parallel repression of up to six essential central metabolic enzymes resulting in significant increases in the flux of the central metabolite malonyl-CoA and as a result an over sevenfold increase in the production of the flavonoid naringenin which has potential anti-cancer properties.

In plants, photosynthetic efficiency in terms of light energy converted to biomass is only ca. 1% and has been identified as one of the most promising targets for improving agricultural productivity (Long et al. 2015). An important limitation of photosynthetic carbon acquisition is the enzyme D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO).

Recent findings that plant metabolic pathways can be reconstituted in heterologous hosts and metabolism in crop plants can be engineered to improve the production of biofuels have given a new hope for molecular biological approaches for improving food and biofuel production. The major challenge of the modern era is the transition to a bio-based economy. Biofuels are a key part of this landscape, but challenges to efficiently and cost-effectively produce biofuels still remain a challenge to meet (Tyner 2012; Taheripour et al. 2012).

According to Sanford et al. (2016), the theme of "sugar is the next oil" connects chemical, biological, and thermochemical conversions of renewable feedstocks to products which are drop-in replacements for petroleum-derived chemicals and/or are new to market chemicals/materials.

24.2 Metabolic Engineering of Microbes

Metabolic engineering is a process of optimizing native metabolic pathways and regulatory networks or assembling heterologous metabolic pathways for production of targeted molecules using molecular, genetic, and combinatorial approaches (Zhu and Jackson 2015). Several excellent reviews on systems metabolic engineering and synthetic biology have highlighted the motivation and need for pathway balancing (Völler and Budisa 2017).

Jang et al. (2012) suggested systems metabolic engineering for successful development of microbes which are capable of producing several different biofuels including bioethanol, bio-butanol, alkane, biodiesel, and even hydrogen. Maximizing microbial biofuel production from plant biomass (i.e. lignocellulosic biomass or plant dry matter) requires reprogramming metabolism to ensure a seamless supply of carbon and energy (e.g. ATP) and reducing power (e.g. NAD(P).2H) toward engineered biofuel pathways (Martien and Amador-Noguez 2017). Nature exploits a very limited set of just 20 canonical alpha-L-amino acids (cAAs) for the ribosomal translation of peptides and proteins. According to Agostini et al. (2017), reprogramming this process enables incorporation of additional ncAAs capable of delivering a variety of novel chemical and biophysical properties into target proteins or protein-based complex structures. Significant progress has been achieved in understanding and engineering the *de novo* lipid biosynthesis in *Y. lipolytica* (Zhu and Jackson 2015).

Saccharomyces cerevisiae (Scer) and *Zymomonas mobilis* are native ethanol producers which can efficiently convert glucose to ethanol, but cannot use pentose sugars as carbon sources (Almeida et al. 2011). Thus, improving xylose utilization in industrially relevant yeasts is essential for producing economically viable biofuels from cellulosic material (Wohlbach et al. 2011).

24.2.1 Xylose Fermenting

Native *S. cerevisiae* does not consume xylose but can be engineered for xylose consumption with a minimal set of assimilation enzymes, including xylose reductase (*Xyl1*) and xylitol dehydrogenase (*Xyl2*) from the xylose-fermenting *Pichia stipitis* (*Psti*) (Jeffries 2006; Van Vleet and Jeffries 2009). Ha et al. (2011) engineered yeasts to coferment mixtures of xylose and cellobiose. Yeasts engineered to ferment xylose do so slowly and cannot utilize xylose until glucose is completely consumed.

In contrast to *S. cerevisiae* and *Z. mobilis*, *E. coli* can utilize most carbohydrate components present in lignocellulosics but produce only a small amount of ethanol during fermentation (Neidhardt et al. 1996). However, *E. coli* strains have been metabolically engineered for enhanced ethanol production through the introduction of foreign genes, elimination of competitive pathways, and disruption of by-product formation (Jarboe et al. 2007). The resulting strain, *E. coli* KO11, was constructed based on *E. coli* by introducing foreign genes encoding pyruvate decarboxylase and

alcohol dehydrogenase (PET operon) from *Z. mobilis* and disrupting fumarate reductase (Jarboe et al. 2007).

24.3 Methane to Biofuels

The availability of methane (from natural gas) and its oxidation product, methanol has been increasing, thus rendering them as attractive fermentation substrates. Methane and methanol as co-substrates, are more reduced than most carbohydrates. According to Whitaker et al. (2015) synthetic methylotrophy is the development of nonnative methylotrophs that can utilize methane and methanol as sole carbon and energy sources or as co-substrates with carbohydrates to produce metabolites as biofuels and chemicals. They discussed synthetic biology and metabolic engineering strategies based on the native biology of aerobic methylotrophs for developing synthetic strains grown on methanol, with *Escherichia coli* as the prototype.

24.4 Hybrid Processes

According to Beerthuis et al. (2015) hybrid processes, combining biochemical and chemical processes, will enhance competitiveness of bio-based products. For example, bio-based polymers and bioplastics will grow their market share by synergizing and collaborating with the chemical process industry (Babu et al. 2013). Creating the necessary process flow sheets, assessing cost sensitivities, and identifying bottlenecks upfront by the use of modeling, simulation, and techno-economic analysis will aid in a successful scale-up (Claypool et al. 2014; Harrison et al. 2015).

24.5 Synthetic Biology

The synthetic biology and metabolic engineering have made significant strides over the past 25 years. According to Lynch (2016), these fields have matured to the point, where currently, scientists skilled in these disciplines can readily engineer microbes allowing for the measurable ("proof of concept") production of numerous chemical products with a range of market applications. Tatsis and O'Connor (2016) highlighted a range of examples that demonstrate how the metabolic pathways of plants can be successfully harnessed with a variety of metabolic engineering approaches. One approach to harness plant metabolic pathways is to reconstitute the biosynthetic genes into a heterologous organism (O'Connor 2015).

Recent approaches of rebalancing or rewiring of the metabolic network by tuning the levels of essential enzymes and the use of dynamic metabolic control strategies to conditionally reduce essential competitive fluxes have yielded better results. The generalizable nature of newer gene silencing technologies, including CRISPR interference, makes transcriptional tuning an attractive platform for any desired microbe (Estrela and Cate 2016).

24.6 Terpenoids

Terpenoids represent one of the largest classes of secondary metabolites that includes pharmaceuticals, cosmetics, and potential biofuel candidates (Lindberg et al. 2009). They are important chemicals obtained from hydrocarbon-yielding plants like *Calotropis procera* and *Euphorbia* spp. which can be converted into biofuels using catalytic cracking system (Kumar 2013).Terpenoids are a class of isoprenoids with a large number of diverse structures exhibiting many biological activities, i.e. anticancer, anti-inflammatory, and anti-infectious properties. Terpenoids can be subclassified by their structure as monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40), and polyterpenes, where the chain length specificity dictates the final terpenoid structure generated by the terpene cyclase. Improving terpenoid biosynthesis could improve biofuel production (Niehaus et al. 2011). In nature, terpenoid biosynthesis is regulated at multiple metabolic branch points to create large structural and functional diversity (Tholl 2006; Keeling and Bohlmann 2006; Christianson 2008). The major metabolic branch point in terpenoid biosynthesis, the prenyltransferases, and terpenoid synthases catalyzes the formation of a wide range of structurally diverse acyclic and cyclic terpenoid molecules (Keeling and Bohlmann 2006).

Leonard et al. (2010) suggested that a common strategy of metabolic engineering is to increase the endogenous supply of precursor metabolites to improve pathway productivity. However, the ability to further enhance heterologous production of a desired compound may be limited by the inherent capacity of the imported pathway to accommodate high precursor supply. Several properties of a metabolic pathway, however, are not limited solely by the enzyme concentration, as is particularly true for the terpenoid pathway. Broadly, terpenoid pathways lead to compounds used in flavors, cosmetics, and potentially biofuels; our engineering approach is directly applicable for the high-level production of many commercially important compounds using microbial biotechnology (Leonard et al. 2010).

Efforts to increase terpenoid production in *E. coli* previously focused on (1) optimizing the expression of enzymes by codon bias (Dueber et al. 2009) and (2) overexpression of pathway enzymes. However, these approaches, which both aim to increase enzyme concentration to increase pathway flux, are still limited by the inherent low enzyme activity and specificity of the terpenoid pathways. Thus, in addition to metabolic engineering, the molecular reprogramming of key metabolic nodes such as prenyltransferase (GGPPS) and terpenoid synthase (LPS) through protein engineering is required to achieve substantial overproduction of a desired terpenoid product (Leonard et al. 2010).

24.7 Photosynthetic Systems

Photosynthesis is the major source of useful chemical energy in the biosphere (Larom et al. 2010). All photosynthetic processes require efficient electron transfer (ET) pathways that are utilized for proton gradient formation (to be used for the production of ATP) and/or accumulation of reducing equivalents.

24.7.1 Enriching C_3 and C_4 Plants

The C_3 plants, including important food crops like rice, wheat, barley, and soybean, overcome RuBisCO's catalytic inefficiency by accumulating large amounts of enzyme up to 50% of soluble protein (Feller et al. 2008). A number of strategies have been suggested for improving photosynthetic CO_2-fixation of agriculturally important plants. They include improving the catalytic properties of RuBisCO (Kreel and Tabita 2015), engineering accessory proteins like RuBisCO activase (Parry et al. 2013), and engineering other enzymatic components of the CBB cycle to improve carbon flux (Erb and Zarzycki 2016).

In C_4 plants, such as corn, sugarcane, and grasses as well as CAM (crassulacean acid metabolism), carbon-concentrating mechanisms (CCMs) have been developed to increase effective molarity of CO_2 near RuBisCO resulting in enhanced CO_2 fixation (Sage and Stata 2015). In contrast to this, algae have evolved CCMs consisting of active uptake systems for inorganic carbon, resulting in highly increased intracellular concentrations of bicarbonate.

Cyanobacteria have evolved a multicomponent CCM that consists of specific CO_2 and bicarbonate uptake systems. Giessen and Silver (2017) reviewed the current efforts aimed at transferring cyanobacterial carbon-concentrating mechanism CCMs to C_3 plants.

According to Meyer et al. (2016), many algae also contain pyrenoids, shell-less protein aggregates that co-localize RuBisCO and CA among other protein components. These approaches are aimed at engineering synthetic photorespiration bypasses (Shih et al. 2014) and at constructing synthetic CO_2 fixation pathways utilizing alternative carbon-fixing enzymes (Peter et al. 2015). This may ultimately improve the working conditions of RuBisCO by installing nonnative CCMs as promising strategies to improve plant photosynthetic efficiency (see review by Giessen and Silver 2017).

24.7.2 Energy Conversion Systems

Light-driven electron transfer occurs within a multistep pathway that is efficiently insulated from competing electron transfer pathways. Larom et al. (2010) reviewed synthetic and semisynthetic energy conversion systems, based on photosynthetic processes (Kruse et al. 2005; Nelson and Yocum 2006; Lomoth et al. 2006; Barber 2009). These include attempts to use dyes bound to solid-state materials, coupled

molecules that form novel ET pathways and the growth of photosynthetic organisms cyanobacteria green algae and plants (Dismukes et al. 2008; Li et al. 2008), which are subsequently converted to biofuels (Angermayr et al. 2009; Carroll and Somerville 2009; Manzanera et al. 2008).

The core of the electron transfer system, composed of six linearly coupled redox active cofactors that enable electron transfer from water to the secondary quinone acceptor QB, is mainly embedded within two proteins called **D1** and **D2** (Larom et al. 2010). PSII is an attractive choice for possible manipulations because it simultaneously produces three important products: electrons, protons, and oxygen. The resulting oxygen evolution could be the reason that PSII is highly sensitive to light, leading to the need for rapid turnover of the D1.

Larom et al. (2010) have successfully engineered a new electron transfer pathway and protein binding site into PSII that does not lead to loss of cell viability.

24.7.3 Bugs to Synthetic Biofuels

Lee et al. (2008) reviewed the ability to generate microorganisms that can produce biofuels similar to petroleum-based transportation fuels. The synthesis pathways of some potential biofuels were recently expressed in model organisms. Recombinant strains of *E. coli* have been successfully engineered into cell factories for the production of complex secondary metabolites of microbial, plant, fungal, and animal origin, as well as for specific biopolymers (Wang et al. 2015, 2017; Dai and Nielsen 2015). According to Becker and Wittmann (2016), *E. coli* has also entered the precious market of high-value molecules and is becoming a flexible, efficient production platform for various therapeutics, prebiotics, nutraceuticals, and pigments. This is enabled by systems metabolic engineering concepts that integrate systems biology and synthetic biology into the design and engineering of powerful *E. coli* cell factories.

The genes involved in the synthesis of isopropanol (Chen and Hiu 1986; Hanai et al. 2007; Scheffers et al. 2016) and butanol (Atsumi et al. 2008) from *Clostridium* were recently expressed in *E. coli*. Aside from producing ethanol during fermentation, *S. cerevisiae* is also known to produce higher alcohols and esters from amino acids (Schoondermark-Stolk et al. 2006). Recently, a similar pathway for higher alcohol production was expressed in *E. coli* to yield six different straight and branched-chain alcohols, and the same group has demonstrated production of 1.28 g/L of isopentanol by increasing the flux through the desired pathway (Connor and Liao 2008).

24.7.4 Solar-to-Chemical and Solar-to-Fuel Technology

Recent researches in solar-to-chemical and solar-to-fuel technology describe the use of solar energy to convert CO_2 to desired chemicals and fuels. Recent breakthroughs in the metabolic engineering of cyanobacteria, adoption of the light-harvesting

mechanisms from nature, and photovoltaic-derived water splitting technologies have been integrated with microbial biotechnology to produce desired chemicals. These studies on the integration of electrode material with next-generation microbes are showcased for alternative solar-to-chemical and solar-to-fuel platforms. The focus has been on chemical and fuel production by improving native pathways (e.g. CO_2 fixation) and by introducing heterologous pathways (Case and Atsumi 2016).

According to Liao et al. (2016), the direct conversion of carbon dioxide to chemicals and fuels presents a sustainable solution for reducing greenhouse gas emissions and sustaining our supply of energy. Woo (2017) reviewed carbon capture utilization (CCU) for reduction of greenhouse gas emission. Photoautotrophic cyanobacterial platforms have been extensively developed on this principle, producing a diverse range of alcohols, organic acids, and isoprenoids directly from CO_2.

According to Woo (2017), ultimately solar energy must be used for CO_2 reduction and conversions to provide a sustainable system, and this system is now available in the forms of solar-to-chemical (S2C) and solar-to-fuel (S2F) technologies. The S2C and S2F technology must be developed to capture and convert the essential feedstocks using only three inputs (CO_2, H_2O, and solar energy) to produce the desired value-added chemicals and fuels.

Photosynthetic organisms (including cyanobacteria) have been engineered to produce value-added chemicals, providing a number of promising S2C and S2F platforms. Thus, hybrid systems comprising an electrochemical *in situ* hydrogen evolution reaction at the electrode and the biological CO_2 fixation using autotrophic bacteria have been suggested as an alternative S2C and S2F platform.

24.8 Conclusion

The photosynthetic systems have three tunable components that together provide an optimal yet flexible system for energy conversion: (1) efficient light absorption, performed by pigments bound to light-harvesting complexes (LHC) (2) tuning of the LHC-RC cofactors to enable efficient transfer of the absorbed energy to enable charge separation and (3) the internal electron transfer chain, on both the acceptor and donor sides of the primary donor. This must be insulated against spurious electron transfer to alternative acceptors. It is enabled by precise positioning of the correct chemical functionalities along the pathway and stabilization of reduced states (Larom et al. 2010). By preserving photosynthesis, they envisioned that thylakoids with modified PSII can be used outside the living cell in potentially vast amounts and without the requirement of complicated isolation procedures. This phenomenon may be important in a possible initiative aiming the using of thylakoids in a future designed energy-producing biophotocell. Synthetic biologists are looking to either modify existing organisms or create from scratch microorganisms with minimal genomes and therefore with a minimal set of metabolic pathways (Gibson et al. 2008).

According to Jones et al. (2015), balancing and optimization merge the fields of metabolic engineering and systems biology to develop tailor-made microbial

factories for the efficient production of chemicals and biofuels at titers that are now approaching feasible levels to replace products derived from natural sources.

According to Lynch (2016), many challenges still remain; these recent efforts further support the potential of this discipline in making a significant impact in the production of high-volume industrial products, with the potential to displace petroleum with more sustainable alternatives. According to Woo (2017), synthetic biology inspired by metabolic engineering of next-generation microbes will be established to accommodate more efficient S2C and S2F platforms.

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