Microorganisms for Sustainability 42
Series Editor: Naveen Kumar Arora

Natarajan Amaresan
Dhanasekaran Dharumadurai
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Industrial Microbiology Based Entrepreneurship

Making Money from Microbes

Springer

Microorganisms for Sustainability

Volume 42

Series Editor

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Microorganisms perform diverse roles on our planet most of which are important to make earth a habitable and sustainable ecosystem. Many properties of microorganisms are being utilized as low input biotechnology to solve various problems related to the environment, food security, nutrition, biodegradation, bioremediation, sustainable agriculture, bioenergy and biofuel, bio-based industries including microbial enzymes/ extremozymes, probiotics etc. The book series covers all the wider aspects and unravels the role of microbes towards achieving a sustainable world. It focuses on various microbial technologies related to sustenance of ecosystems and achieving targets of Sustainable Development Goals. Series brings together content on microbe based technologies for replacing harmful chemicals in agriculture, green alternatives to fossil fuels, use of microorganisms for reclamation of wastelands/ stress affected regions, bioremediation of contaminated habitats, biodegradation purposes. Volumes in the series also focus on the use of microbes for various industrial purposes including enzymes, extremophilic microbes and enzymes, effluent treatment, food products.

The book series is a peer reviewed compendium focused on bringing up contemporary themes related to microbial technology from all parts of the world, at one place for its readers, thereby ascertaining the crucial role of microbes in sustaining the ecosystems.

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Dhanasekaran Dharumadurai • Diana R. Cundell
Editors

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Foreword

Industrial microbiology is an area of applied microbiology that uses microorganisms to produce high-value products such as chemicals, drugs, fuel, and electricity. This field constantly evolves with new techniques and new microbes being recruited to the area as microbiologists discover new possible applications.

The book *Industrial Microbiology-Based Entrepreneurship: Making Money from Microbes* is a carefully crafted text written by experts in the field that provides the reader with an overlook of the techniques used nowadays. Moreover, the book adds details and focuses also on the entrepreneurship angle of industrial microbiology, providing the reader with analysis of the economic impact of the techniques and products developed.

The first chapter is an introduction to the field and explains the scope and application of industrial microbiology, setting the information necessary for the reader to dive in the following chapters.

Following the introduction, the next chapters provide explanations and market analysis of mass production of enzymes, such as xylanase, protease, pectinase, and cellulase.

The book expands past enzymes production, providing insight to diverse topics in industrial microbiology spanning from vaccine productions to biogas.

Chapters 8, 9, 10, 11, 12, and 13 describe different topics and the employment of different microbes in the field, with chapters diving into the following topics:

- Production of chitinase using *Pseudomonas* species.
- Production of agar from *Gelidium.*
- Production of pro-vitamin A, exopolysaccharides, organic acids-citric acid, and organic acids-lactic acid.
- Production of agricultural effective microorganisms and bio-organic liquid fertilizers and plant nutrition enhancer.

Of current interest, Chap. 19 analyzes the costs and provides a business plan for large production of corona vaccines. Other interesting topics discussed in the book are the production of biogas, bio-stimulants, vinegar, and fertilizers.

In summary, *Industrial Microbiology-Based Entrepreneurship: Making Money from Microbes* is full of helpful information including about microbiology and from a marketing point of view. This makes this book a valuable publication for researchers, students, and other readers who are entering the world of industrial microbiology or who want more detailed information on this expanding field.

Best regards,

Manuela Tripepi

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Preface

When the first microbiologists discovered these tiny unicellular organisms in the 1800s, they were associated with disease and illness and it was not until the discovery of antibiotics in the 1920s that this notoriety began to abate. We now understand that microbes are very much "yin and yang," with both positive and negative members. They are both an integral part of our environment and manipulated for over 100 years for human gain to produce foods, remove waste products, and create advanced pharmaceutical products. These tiny entities are appealing to study as they have few needs and, when well chosen, can be grown easily and inexpensively thereby creating many new exciting products to improve lives around the globe. We are also at a point in history where harvesting raw materials from the Earth is beginning to fade and, unless we want our lives to change substantially, there has to be investment in future strategies that promise a very real payoff. So, instead of the classic "what can microbes make" approach, this book has looked at microorganisms from a new standpoint, namely how can they make money for those culturing them! Our three co-editors, Dr. Dharumadurai Dhanasekaran, Dr. Natarajan Amerasan, and myself, Dr. Diana R. Cundell, have together applied their expertise to compile a new type of book that seeks to encourage microbiologists to examine what might be needed rather than just creating a list series of what is already here. This collection of ideas reveals much about those who have edited this book as well as those contributing to it, but the reader should be able to find within something that will stimulate ideas of their own. Compiling these 19 chapters into *Industrial Microbiology-Based Entrepreneurship: Making Money from Microbes* and allowing them to flow easily into one another has allowed for the entrepreneurs included within to run the gamut from as individual enzyme production, which is anticipated to impact processing industries such as food production and biogas production, which could revolutionize fuels, to very general ideas such as biopolymers, food crop protection, and vaccine manufacture.

If we take *just* the latter three and look at them in more detail we can already see why this is a useful book for the microbiologist's library. Biopolymers are becoming big business in biomedical applications where they can enhance wound healing,

blood clotting, and prevent infections. Again, when natural products are used versus synthetic ones they are ultimately less expensive and patients show better recovery and less unwanted effects when exposed to them. Synthesis of biopolymers from microorganisms also has another advantage. When used as packaging material it allows for items that are used naturally by others in the environment and thus are biodegradable. The reader only needs to look at the piles of plastics with their hundreds of years of lifespan on garbage dumps around the globe and that pollute our waters to see the utility of these applications alone. Current estimates are that about up to eight million tons of plastic waste enters our global waters annually, with the majority being plastic packaging. Although many corporations have pledged to become biodegradable, an entrepreneur in this area would be able to significantly reduce both the micro- and macrodebris being produced in this fashion and accelerate the process by which our world is cleaned using natural strategies.

As a planet we cannot continue to maintain an omnivorous diet and must move towards a plant-based one. If this is the case, there will need to be considerable research towards examining methods to reduce the current one-third loss of cultivated crops to microbial disease. Botanists have long suggested that future seed usage should be designed to complement regional needs and insect/microbial infection issues rather than be optimized for productivity. Current countermeasures to crop and seed loss in developed countries have resulted in overuse and resistance to a whole series of insecticides and fungicides. In contrast, the pesticides available in developing countries are many times ineffective or toxic and represent those abandoned by the developed nations. The answer here lies in pitting microbe against microbe, which is in itself an organic solution. Utilizing a more natural approach also allows native ecosystems to persist. As can be seen in this book, those who would produce these new crop biocontrol agents would make money but, and this is more important, it would improve the foods being grown around the world to feed our increasing human populations. Studies have also shown that organic produce is healthier with much higher nutrient levels and antioxidants. Natural microbial products are also much less likely to be something that environmental pests will become resistant to, thus adding to their development appeal.

Using microbes to develop vaccines is another growth area. We have just lived through (and indeed continue to) an era of COVID-19, which was an RNA containing virus that jumped the species barrier into human beings. Why are we seeing this pandemic and will it be the last? The answer is sadly no. Many of the current zoonotic diseases are diluted in their effects on the human population due to carriage by a number of hosts. Studies suggest many of these are likely to be RNA-containing viruses and that rather than adapting to their hosts a series of "waves" or mini epidemics is likely to occur**.** In addition, global climate change is already selecting out the more virulent pathogens, including multi-resistant microbes as well as vectors of zoonotic diseases. This means that developing vaccine strategies that are novel and effective will occur under the extreme pressure we just witnessed with the COVID-19 pandemic and microbes that can be coopted for use in this category will be at a premium.

Realistically although these chapters stimulate the generation of revenue from their discoveries, it can be seen that they will also simultaneously benefit numerous communities around the world. Our society is facing some of the greatest challenges to its integrity due to climate change and population expansion. As this forces us to seek out new strategies in obtaining, packaging, and distributing food and medical care, it is clear that local sourcing of easily produced agents will be necessary. Much of the future can then be considered microbial, with Making Money from Microbes being the first of many explorations into this arena.

Surat, India — Natarajan Amaresan
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About the Series Editor

Naveen Kumar Arora, Fellow of International Society of Environmental Botanists (FISEB), PhD in Microbiology, is Professor and Head of the Department of Environmental Science at Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, Uttar Pradesh, India. He is a renowned researcher in the field of environmental microbiology and biotechnology. His specific area of research is plant-microbe interactions, particularly plant growth-promoting rhizobacteria. He has more than 75 research articles published in premium international journals and several articles published in magazines and dailies. He is an editor of 25 books, published by Springer. He is a member of several national and international societies, Secretary General of Society for Environmental Sustainability, serves on the editorial board of four journals, and a reviewer of several international journals. He is also the editor in chief of the journal *Environmental Sustainability* published by Springer Nature. He has delivered lectures at conferences and seminars around the globe. He has a long-standing interest in teaching at the PG level and is involved in taking courses in bacteriology, microbial physiology, environmental microbiology, agriculture microbiology, and industrial microbiology. He has been advisor to 134 postgraduate and 11 doctoral students. He has been awarded for excellence in research by several societies and national and international bodies/organizations. Although an academician and researcher by profession, he has a huge obsession for the wildlife and its conservation and has authored a book, *Splendid Wilds*. He is the President of Society for Conservation of Wildlife and has a dedicated website www.naveenarora.co.in for the cause of wildlife and environment conservation.

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Natarajan Amaresan is an Assistant Professor at C.G. Bhakta Institute of Biotechnology, Uka Tarsadia University, Gujarat. He has received his undergraduate, postgraduate, and doctorate degrees in Microbiology. Dr. N. Amaresan has over 16 years of experience in teaching and research in various allied fields of microbiology, mainly microbial ecology, plant-microbe interactions, and others. He has been awarded young scientist awards by Association of Microbiologists of India, National Academy of Biological Sciences, and recipient of visiting scientist fellowship from National Academy of India. He has published more than 60 research articles, 13 books, and 45 book chapters of national and international repute. He has handled various projects sponsored by DBT, DST, GEMI, etc., and guided students for their doctoral and master's degrees.

Dhanasekaran Dharumadurai is working as an Associate Professor, Department of Microbiology, Bharathidasan University, Tiruchirappalli, India. He was awarded UGC-Raman Post-Doctoral Fellowship and worked in the University of New Hampshire, USA. He was awarded Fellow of Linnean Society, London, UK, and INSA Visiting Scientist Fellowship by the National Science Academy. He has graduated 12 PhDs and 76 MScs with over 106 research publications, 49 book chapters, and an H-index of 30 and citations of 2850. He filed two Indian Patents and organized 8 national level seminar, conference, and workshop programs. He has edited 11 books and authored 4 laboratory manuals. His research focus is microbiome profiling of actinorhizal root nodules, lichen, poultry gut, and cattle's reproductive system.

Diana R. Cundell received her PhD in medicine from The Royal Postgraduate Medical Federation, London, U.K. in 1993 and is currently a Professor of Biology and the Pre-Medical Studies Program Director at Jefferson University. Her research spans over 30 years and during the past decade has focused on herbal medicines as antimicrobial and immunomodulatory agents. Dr. Cundell has published research on several phytomedicines including curcumin (turmeric) and their utility in safely managing chronic inflammatory disease. She is the recent author of the book *Allicin, the natural sulfur compound from garlic with many uses* as well as over 60 articles and holds 4 process patents associated with breathing wall systems. Dr. Cundell sits on the Editorial Board of *Plant Biology* and *Phytomedicine* and has acted as a guest editor for *Current Immunology Reviews*.

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Chapter 1
Microbiology-Based Entrepreneurship

Selvaraj Jayaraman, Durairaj Sekar, Ponnulakshmi Rajagopal, Vijayalakshmi Periyasamy, Mani Panangal, and Kumaran Subramanian

Abstract Microbiologists can use entrepreneurial microbiology, a multidisciplinary field that includes the investigation, discovery, and monetisation of living things, food, plants, and biological resources seeing how they could be beneficial to humans, to help address economic issues such as job insufficiency, fundamental, and national developments. According to Eniola (African entrepreneurship: challenges and opportunities for doing business. Springer, 2018), entrepreneurs are mentioned for technology innovation, economic growth, economic success, economic change, and application of possessed competencies. Science is continually being adapted for industrial, domestic, and ecological uses as a fast-evolving subject. With the rapid advancement of these novel aspects of microbiology, a new perspective is required to combine these innovative aspects into economic stability creation and growth. Commercialisation of microbiology and the integration of

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entrepreneurial microbiology in school and university educational plan will transform the way microbiology is viewed and make it among the economy's favourable influences.

Keywords Entrepreneurship · Microbiology · Self-employment · Diagnostics · Industry

1.1 Introduction

Science is an ever-changing, fast-paced subject that is always being changed for industrial, domestic, and ecological reasons. With how quickly these new ideas in microbiology are coming out, a new way of looking at things is needed to make sure that these new ideas can also be used to make money and grow the economy. The country's economy has been in a mess for a long time, with the inflation rate rising and the workforce still being underpaid, even though the government has tried to help. There has been no significant drop in the number of people looking for jobs, even though the government has tried to help. A lot of people are now saying that the entrepreneurial spirit in the country's economy is a great way to get out of the current financial downturn.

Microbiology as a course covers a wide range of topics, including healthcare, pharmaceuticals, and the food business, to mention a few. A microbiologist's job is to study microorganisms, but once the general public hears about controversial topics like the biologic-autistic disorder link, clone research, stem-cell research, and genetically altered foods, an anti-science attitude develops, despite the millions of dollars spent annually on microbial research and analysis, as well as the notable discoveries made daily. Now it is up to these microbiologists to defend themselves and their conclusions in front of the general public. A nation's population is compelled to launch entrepreneurship for economic growth, therefore it should come as no surprise that entrepreneurial microbiology, an intrinsic means of employment creation and invariably a means of predicting open market issues, would be pursued. With the advancements in advanced education and the rapid changes in technological breakthroughs, it is critical to explain to students and lecturers a biological system of biotechnological riches and entrepreneurial revolution (Bell 2010).

1.2 Microbiological Entrepreneurship

Entrepreneurs, according to Eniola (2018), are known for innovation development, economic growth, economic gain, economic transformation, and application of acquired competencies, taking into account their multidisciplinary features. While entrepreneurship microbiology is a multifaceted field that studies, discovers, and commercialises live organisms, foods, plants, and biological resources to see how

they could be helpful to humans (Akinkugbe and Onilude 2013), this is an open door for microbiologists to assist in addressing economic problems such as job scarcity, fundamental, and national developments. The lack of resources, amenities, and the necessary technical skills for recombinant DNA technology stymies innovative research and economic growth initiatives in the country, and has thus proven to be one of the barriers to pursuing entrepreneurial improvements and advancements (Bogoro 2015). This has demonstrated that, in order to counteract the society's ever-increasing rate of unemployment, our educational module must incorporate this entrepreneurial aspect to aid its economic process. Our country is bestowed and recognised with a plethora of scholastic intellectual natural resources and assets that have the potential to boost the economy and reduce the current level of cerebrum depletion. Entrepreneurship entails the ability to see new opportunities and take action to make a difference.

The commercial climate in India is far more favourable to sociologies and much less so to microbiology, which is why microbiology-based research and development struggles to thrive and survive. This can be shown in how microbiology is considered among bioscience courses in schools, universities, and potential workplaces. Those that persevere in the face of adversity and finally achieve do so because they are passionate about the subject or because microbiology is used as a basis for other, seemingly more relevant careers such as medicine and medical laboratory science. In India, the majority of microbiologists work in academia, with just a small number working in the healthcare and food industries, where specialities such as medical laboratory science, medical microbiology, pharmaceutical microbiology, and other related fields are widely desired after. Commoditisation of microbiology and the integration of entrepreneurial microbiology in school and university teaching modules will transform the way microbiology is viewed and make it one of the positive economic influences. By generating a niche of new job opportunities, entrepreneurial microbiology may be a driving force of economic transformation, transforming lives. Its long-term success will be determined firstly by public awareness, perception, knowledge, and acceptance; and, secondarily, an unbiased and cooperative relationship among industries, educational institutions, and investment groups. The United Kingdom's Biotechnology Young Entrepreneur Scheme (Biotechnology YES), for example, teaches students and scholars how to make money from microorganisms through a competition. Most importantly, the plan provides as a platform for students and scholars to gain a better understanding of the procedures involved in bioscience commercialisation (Eniola 2018). The fate of the Indian economy ultimately depends on a new generation of scientific business visionaries who emerge with goals and go out to achieve them, thereby creating economic prosperity and jobs. Diverse contenders in inventive biotechnology are in the scholastics with the assistance of the government or hierarchical financing, it is because stockholders and industries do not have the wherewithal for research, and thus rarely conduct forth new scientific innovations, while educational institutions are unable to underwrite the high value of product development, in as much as financial specialists and companies are more than willing to underwrite the high value of product development. As a result, it is critical

to transfer breakthrough scientific initiatives from educational institutions to businesses once enough scientific data is available to implement a build-out strategy. Nonetheless, the chance that the inventions would fail at the expense of the shareholders cannot be ruled out (Life Science Austria 2017). Protecting scientific entrepreneurial concepts is an important aspect of revolutionary technology that ensures it cannot be easily copied by competitors. Shareholders will want to ensure that a unique product concept does not become obsolete due to imitation (Shimasaki 2009). In other situations, it is also preferable to obtain legal protection through product licencing: particularly in the field of biosciences, timely licencing is frequently a great strategy. There are a variety of ways for biotech businesses and educational laboratories to collaborate in order to reach scientific consensus (Steven and Uma 2014). Because affluent biotech corporations produce biotech products, it is prudent to raise skilled individuals with research-based information that can be transformed into a result of resourceful objectives worthy of commercialisation. Regardless of how brilliant these individuals are at coming up with unique ideas, only a handful of them have the resources required to start a new firm. One solution could be to show their business plan to a financial speculator and acquire loans and subsidies. Investing in pharmaceutical and biotechnology start-ups might come with a lot of risks, but it also comes with the chance of bigger payouts and advantages. Creating scientific recreational grounds within university zones and regions is one way to enliven entrepreneurial activity within the academic community. The goal is to provide a large number of experienced advisers in intellectual property law, project finance, and commercial enterprise to newly founded biotech companies.

1.3 Microbiology's Scope and Importance

Viruses, bacteria, protozoa, and fungi, among other species and agents, are too small to be seen with the human eye in the world around us. A microbiologist is a scientist who studies bacteria that exist in a wide range of environments, including hot springs, the arctic, the human body, and many other living things, as well as the depths of the sea and ocean. Microbiology is thus an important science since bacteria have a harmful and beneficial impact on all aspects of life (Rama 2009). As a result, Prescott et al. (2005) defined microbiology as the study of organisms and agents too small to be seen clearly with the naked eye, that is, it is the study of microorganisms that requires sterilisation and the use of culture media to isolate and cultivate them. Microbiology is divided into two categories: fundamental and applied. Many microbiologists are primarily concerned with the biology of microbes. They are called virologists (viruses), bacteriologists (bacteria), phycologists or algologists (algae), mycologists (fungi), or protozoologists if they specialise in a certain category of microorganisms (protozoa). Others work on topics such as microbial physiology, microbial ecology, microbial cytology, microbial genetics, microbial taxonomy, and molecular biology, and are interested in microbial morphology or specific functional processes. Of course, a person can be viewed from both perspectives (e.g., as a

bacteriologist who works on taxonomic problems). Many microbiologists have a more applied focus and concentrate on real-world issues in sectors including medical microbiology, food and dairy microbiology, and public health microbiology (basic research is also conducted in these fields). Prescott et al. (2005) further highlighted the current career paths of professional microbiologists as follows:

Medical Microbiology This is one of the most significant and active branches of microbiology, and it deals with human and animal diseases. Medical microbiologists discover the infectious disease agent and devise strategies to eradicate or control it. They are frequently involved in the search for novel, unidentified viruses such as the hantavirus, SARS virus, and a variety of others. Microbiologists are also interested in how bacteria cause disease.

Public Health Microbiology Medical microbiology is intimately linked to this. Microbiologists in public health strive to keep contagious diseases from spreading. They frequently inspect neighbourhood food establishments and water sources to ensure that they are safe and clear of infectious disease pathogens.

Immunology Immunologists study how the immune system defends the body against viruses and how infectious agents react. It is also one of the fastest-growing fields in research; for example, monoclonal antibody production and use techniques have advanced at a breakneck pace. Immunology also deals with practical health issues including allergies and autoimmune illnesses like rheumatoid arthritis, as well as their causes and treatments.

Agricultural Microbiology The impact of microorganisms on agriculture is the subject of this study. Agricultural microbiologists research plant diseases that affect vital food crops, develop strategies to improve soil fertility and agricultural yields, and investigate the role of bacteria in the digestive systems of ruminants like cattle. There is currently a lot of interest in employing bacterial and viral insect diseases as pesticide alternatives. Microbiology of Food and Dairy Products: These microbiologists work to prevent food spoiling and the spread of foodborne diseases including botulism and salmonellosis. Microorganisms are also used to manufacture cheese, yoghurts, pickles, and beer. Microorganisms may become a more important food source for cattle and people in the future.

Industrial Microbiology Microorganisms are used by industrial microbiologists to create antibiotics, vaccines, steroids, alcohols and other solvents, vitamins, amino acids, and enzymes. Even low-grade ores can be leached of valuable minerals by microorganisms.

Microbial Ecology This relates to the interactions between microorganisms and their living and non-living environments. Microbial ecologists research how microorganisms contribute to the carbon, nitrogen, and sulphur cycles in soil and freshwater. The study of pollution's impacts on microorganisms is especially essential due to the environmental impact these organisms have. Microorganisms are also used in bioremediation by microbial ecologists to lessen pollution effects.

Microbial Genetics and Molecular Biology The nature of genetic information and how it governs the behaviour of cells and organisms are the focus of these studies. Understanding gene function has been greatly aided by the use of microbes. Microbial geneticists contribute to applied microbiology by developing new microbial strains that are more effective at creating useful products. Substances are tested for their ability to induce cancer using genetic approaches.

Genetic Engineering More recently, work in microbial genetics and molecular biology has spawned the discipline of genetic engineering, which will have a significant impact on microbiology, biology as a whole, and medicine. Engineered microbes are utilised to create valuable items more quickly and efficiently. New genes can now be put into plants and animals; for example, it may be feasible to give corn and wheat a nitrogen-fixing gene, allowing them to grow without nitrogen fertiliser.

1.4 Diagnostics

Clinical laboratory professionals in hospitals, public health laboratories, commercial medical or veterinary diagnostic laboratories, and private companies are diagnostic microbiologists. Patients or animal samples sent in by doctors or veterinarians are tested in hospitals and laboratories. These tests identify the bacterium that is making a patient/animal sick and can aid a doctor's or veterinarian's treatment options by evaluating whether the microbe is sensitive to antimicrobial drugs like antibiotics or resistant to them. Clinical microbiologists track and determine the cause of illness outbreaks in public health laboratories. Clinical microbiologists work in private companies to create new diagnostic tests and treatments. These specialists may supervise a complete clinical laboratory and its employees at higher levels of their careers.

1.5 Biosafety

Biosafety experts ensure that work in clinical and research laboratories is carried out safely, with the proper equipment and procedures, and that all federal, state, and local legislation and recommendations are followed. Their goal is to keep staff safe from injury or infection, as well as to keep germs and other biological agents out of the lab. They accomplish this through teaching researchers and clinical laboratory workers, establishing safety policies and procedures, and providing laboratory design advice. Professionals in the field of biosafety operate in a variety of settings, including colleges and universities, private enterprises, hospitals, and government agencies.

1.6 Hybrid Career Paths

Some microbiologists combine their scientific knowledge with other skills and interests. A bachelor's degree in microbiology is usually required, as well as a degree or additional training in a second subject. Business analysts assist companies and investment organisations in assessing a certain scientific or medical market in order to inform their strategy and judgments. A business analyst with a microbiological background, for example, could assist an investment firm in deciding whether or not to fund a biotechnology venture. Some business analysts work for a corporation directly, while others work for consulting firms or as independent consultants. They often hold a master's degree in business administration (M.B.A.).

Infectious disease physicians and veterinarians receive their training as doctors (M.D. or D.O.) or veterinarians (D.V.M.) before specialising in patient care for people and animals with infectious diseases such as HIV/AIDS, TB, or Q-fever. Infectious disease specialists may treat patients as well as conduct microbiology research.

Patent lawyers work at legal firms or private enterprises. Patents for innovative scientific devices, techniques, or goods are written and filed to safeguard intellectual property. Patent infringement lawsuits are also pursued or defended by them. Patent lawyers have both a law degree (J.D.) and scientific knowledge.

Professionals in public policy and regulatory affairs work for government agencies, non-profit groups, and private businesses. These individuals work in government to create rules, legislation, and regulations pertaining to biomedical products, healthcare, and laboratory research. These experts assist non-profits and private corporations in understanding and advocating for certain rules and regulations.

Science education or outreach specialists work at universities and colleges, non-profit organisations, museums and government agencies. Some also work for private firms' corporate social responsibility departments. These professionals plan and coordinate science-related programmes and events for the general public and K-12 students.

Science writers work for a variety of organisations, including newspapers, periodicals, and other forms of media, as well as government agencies. They also operate as freelancers on a regular basis. They must stay up with current events and new research published in order to study stories and create articles on technical issues.

1.7 Microbiology for Self-Employment and Self-Productivity

The following can be considered in order to re-engineer microbiological courses taught in universities in order to generate graduates capable of self-employment and self-productivity for the country:

1. All students should be required to take an entrepreneurial course as part of their university degree, according to national policy.
2. The entrepreneurship course should never be provided as a general studies education (GSE) subject that all students at a given level must take. Students will regard the course as a significant course in their studies, career development, and preparation for self-employment after graduation as a result of its inclusion as a departmental course.
3. The course tutor in microbiology, for example, should be a microbiologist with entrepreneurship training who can educate students on the fundamentals of entrepreneurship as well as familiarise them with the numerous self-employment prospects accessible in their field of study. The course instructor should also be able to assist students in creating a business of their choice inside their field of study's self-employment options. A professional entrepreneur teacher and a professional in the field of study that relates to the business in question should validate the designed business in order to assess the workability of the business as a small-scale business, the economic cost of the business, the market outlet of the business, the economic gain of the business, and its sustainability. As a result, you may decide whether it is worth putting up or not.
4. When creating a business, students should be required to work in groups of at least five people to avoid repeating business designs, lower the financial cost of starting individual enterprises, and enhance the business's workforce capability.
5. All students in all fields should be required to participate in the student industrial work experience scheme (SIWES). The plan can now be used to achieve two goals. The original goal was to provide students with actual job experience related to their field of study, but now it is being carried out under firms that are running the type of design business of their choice on a huge scale. As a result, students will become familiar with the industry and will be able to learn it practically from professionals in the sector. As a result, the government will save money on the costs of setting up student entrepreneurship centres for hands-on learning, which may not be enough to accommodate the growing number of students, as well as the costs of paying wages to staff at the centres, as is the case presently.
6. Entrepreneurial success stories should be featured in the curriculum, particularly in the student's field of study, to encourage students to develop an interest in self-employment and self-productivity.
7. Students with the greatest business design from each faculty must be recognised at graduation and given a financial reward from the federal government to start their own firm right after graduation. For other graduates, the federal government should establish the Enterprise Bank, which will be tasked with providing loans to students who want to start their own businesses. The bank will also serve as the graduates bank for the business in question, and the loan will be paid with low interest across the board for all businesses based on the capital provided. The Enterprise Bank should also hire a consulting firm to oversee the success and long-term viability of the enterprises it supports. The Enterprise Bank should identify the best business enterprises in each state and grant national recognition

and, if possible, a monetary award by the state government where the businesses are located.

1.8 Conclusion

Medicine, agriculture and food sciences, ecology, genetics, biochemistry, and molecular biology are just a few of the domains where microbiology has a significant impact. As a result, the importance of microbiologists in national development cannot be overstated. This highlights the importance of universities producing microbiologists. However, given the issue of mass student enrolment in universities as well as mass student graduation from universities, as well as the global economic recession, which has resulted in mass graduate unemployment in the country, it is necessary to re-engineer the microbiology taught in our universities in order to achieve the desired motive of education for self-employment.

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Chapter 2
Mass Multiplication, Production Cost Analysis and Marketing of Protease

Kartik Patel and Natarajan Amaresan

Abstract Proteases are widely distributed enzymes that play an important role in both synthesis and breakdown. The catalytic characteristics of proteases have allowed their use in a variety of industrial processes, including detergents, leather, textiles, medicine, feed, and trash. Researchers are exploring numerous techniques to discover, redesign, or artificially manufacture enzymes with improved applicability in industrial processes in response to the expanding demands and applications. Proteases have been successfully used as chemical substitutes and environmentally benign indications for nature and the environment. The most common protease producers are *Bacillus* sp. and *Aspergillus* sp., which are produced via submerged and solid-state fermentation, respectively. Thermostable and solvent-tolerant proteases are important for biotechnological and industrial applications because of their resistance to denaturing agents and chemicals. The current chapter highlights the microbial sources, mass production, existing and future uses of microbial proteases in various sectors, and the estimated costs to assist new entrepreneurs.

Keywords Proteases · Detergent industry · Leather industry · Textiles industry · Entrepreneur

2.1 Introduction

Enzymes are proteins produced by living organisms that catalyse chemical reactions in highly efficient and environmentally favourable ways. All enzymes, which are classified into six types, are essential for survival, and their malfunction causes disease (Homaei 2015). Proteases or proteolytic enzymes can break down peptide bonds in proteins. Proteases are classified as hydrolases in class 3 and peptide hydrolases or peptidases in subclass 3.4, according to the International Union of

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Biochemistry and Molecular Biology Nomenclature Committee. Proteases, often known as proteolytic enzymes, are the most basic and versatile enzymes involved in the functions of living organisms (Beg et al. 2003). Proteases are required for optimal cellular metabolism, particularly the mitochondrial processes, in biological contexts (Quiros et al. 2015). It also regulates the size, structure, and composition of essential proteins through numerous biochemical processes (Troncoso et al. 2022).

In terms of economics, new protease research is expanding because it accounts for 60% of all commercialized enzymes in the world. Proteases are now used in a variety of industries, including the leather and detergent industries, food technology, silver recovery, feed, chemical and waste treatment, and pharmaceutical manufacturing (Homaei et al. 2016). They are important in a variety of physiologic processes, including development, apoptosis, regulatory mechanisms, infection, fecundation, allergic responses, blood clotting, tumour growth, and bone remodelling, as well as in therapeutic targets (anti-inflammation, digestion, and wound healing) (Barzkar et al. 2018).

The classification of proteases remains difficult owing to the diversity of their methods of action and architecture. Protease enzymes are classified into two types based on the peptide bond cleavage site and their functions: exopeptidases and endopeptidases. The International Union of Biochemistry recognizes four mechanistic classes, each with six families of proteases: serine carboxy proteases (EC 3.4.16), serine proteases (EC 3.4.21), cysteine proteases (EC 3.4.22), aspartic proteases (EC 3.4.23), metallocarboxy proteases (EC 3.4.17), and metalloprotease I (EC 3.4.24), depending on the specific catalytic amino acid (or metal) involved in proteolysis (Jornitz et al. 2011). However, three novel systems have recently been discovered: the threonine-based proteasome system, glutamate-glutamine system of equolisin, and sedolisin serine-glutamate-aspartate system (Mamo and Assefa 2018). Aspartic proteases (EC 3.4.23) are peptidases with diverse properties. It contains two aspartic acid residues (Asp32 and Asp215) within its active site, which are important for catalysis. Most aspartic proteases have isoelectric points in the pH range of 3–4.5, with optimal enzyme activity at low pH (pH 3–4). The inability of plant and animal proteases to meet the current global enzyme demands has increased interest in microbial proteases. Microbial proteases were chosen over plant proteases because they possess the majority of the properties required for biotechnological applications.

Microbial enzymes play a wide range of biochemical, physiological, and regulatory roles. Microbial proteases have played an important role in the manufacturing of traditional fermented foods for generations, and the industrial enzyme industry, which is dominated by microbial protease products, delivers biocatalysts to a wide range of industries (dos Santos Aguilar and Sato 2018). Peptide bond cleavage in industry can be performed enzymatically or chemically. Chemical, alkaline, or acidic hydrolysis is more difficult to manage and produces hydrolysates containing altered amino acids (Tavano 2013). Proteases can catalyse specific and selective protein changes. Proteins with restricted proteolytic activity may have a wide range of applications. For example, using particular hydrolytic conditions, hydrolysed products with diverse functional qualities can be created and used in a variety of sectors.

These functional features are critical because they determine the main aspects of a finished product and define its use.

2.2 Mass Microbial Protease Production Technology

2.2.1 Microorganisms and Source

The inability of plant and animal proteases to meet the current global demands for the enzyme has prompted interest in microbial proteases. Isolation and selection of potential microbes is the first step in the synthesis of microbial proteases. Microbial protease enzymes are favoured over plant and animal proteases because of the desirable features for biotechnological applications. Microorganisms are isolated from various sources and ecosystems and are then selected for appropriate characteristics (Patel and Dudhagara 2020). For example, hot springs, dumping sites, soda lakes, soil samples from milk processing plants, meat waste contaminated soil, detergent industry, leather industry, poultry waste sites, wood factory, and tannery waste have specific or adverse features that affect the characteristics of the enzyme to be produced (Solanki et al. 2021). Protease producers from bacteria, fungi, and actinomycetes have been isolated and identified. Some bacterial species that have been reported to produce proteases include *Bacillus subtilis*, *B. amyloliquefaciens*, *B. halodurans*, *B. licheniformis*, *B. lentus*, *B. circulans*, *B. safensis*, *B. pumilus*, *B. pseudofirmus, B. clausili*, *B. alkaloophilus*, *B. lehensis*, *B. stearothermophilus*, *E. coli*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Pyrococcus furiosus*, *Thermus aquaticus*, *T. thermophilus*, *T. stature*, *Geobacillus* SBS-4S, *Streptomyces nogalator*, and *S. avermectus* (Mamo and Assefa 2018; Razzaq et al. 2019; Solanki et al. 2021). Among them, *Bacillus* sp. has been extensively studied for protease production on a large scale and is used in a variety of industries, including leather, detergents, pharmaceuticals, and textiles. The fungi reported as protease producers are *Aspergillus niger*, *A. sterreus*, *A. favus*, *A. oryzae*, *A. fumigates*, *A. nidulans* HA-10, *A. clavatus* ES1, *A. saitoi*, *Botrytis cinerea*, *Fusarium* sp. *Penicillium chrysogenium*, *P. italicum*, *Conidiobolus coronatus*, *Cryphonectria* (Endothia) *parasitica*, *Mucor* sp., *Cephalosporium* sp., *Rhizopus* sp., and *Trichoderma* (Naveed et al. 2021). *Aspergillus* and *Trichoderma* are two major strains used for industrial protease production. However, thermophilic and halophilic proteases are gaining popularity in biotechnological applications because of their thermal stability and ability to preserve their activity under high stress from organic solvents.

2.2.2 Bioprocesses for Protease Production

A variety of approaches are being used for enzyme production from a dominant microbial source for economic improvement, but the search for high-quality

enzymes from bacteria for industrial usage continues. Low protease titres have always been a major concern, so several researchers are attempting to improve production titres through a variety of approaches, such as using better bioprocess technologies, using cheaper or crude raw materials as substrates for enzyme production, and bioengineering the microorganisms (Razzaq et al. 2019). Microbial growth substrates account for nearly two-fourths of total manufacturing costs (Singh et al. 2015). Researchers worldwide have been working on bioprocess optimization strategies to boost protease production and specific activities. For cost-effective generation of microbial proteases, both solid substrate and submerged fermentation (SmF) are used. Solid-state fermentation (SSF) has regained popularity in the last few decades owing to high titres of enzyme synthesis using fungal cultures. Protease secretion was most strongly influenced by lignocellulosic substrates. In SSF, the readily available substrate wheat bran is shown to be more promising for protease synthesis, whereas other inexpensive substrate sources such as cow dung, agro-industrial waste, groundnuts, and wheat bran can be effective in the manufacture of proteases (Hamza 2017). Furthermore, easily available substrate sources such as molasses from sugar industry waste, dairy sludge, and effluents have the potential for value-added product enzyme synthesis, while also helping to reduce environmental pollution (Corral et al. 2018; Razzaq et al. 2019). Some substrates considerably increased proteolytic enzyme synthesis without the addition of specific inducers to the growth medium. Nonetheless, the benefits of improved monitoring and management are still connected to concealed culture. Protease production in cultures is growth dependent and is regulated by a variety of factors such as substrate type, medium pH, and nutrient availability. Large-scale production of proteases necessitates an understanding and effective control of the producer's growth and enzyme production capabilities (dos Santos Aguilar and Sato 2018). Microbial proteases include induction and repression mechanisms (Zhang et al. 2020), which must be considered in the process design and media formulation for protease synthesis. Genetic modification, however, provides researchers with a new way to manipulate the microbial genome using various biotechnological tools to improve the yield of proteases with desired properties. Cloning and overexpression, strain screening, fed-batch, and chemostat fermentation are all methods that scientists have used to boost protease yield for industrial use. Genetic engineering with the goals of enzyme hyperproduction, cost-effectiveness, and quality aids scientists in capturing the global biotechnology market.

2.2.3 Solid-State Fermentation (SSF)

Solid-state fermentation refers to fermentation that occurs in the absence or near the absence of free water. SSF for the synthesis of industrial enzymes is rapidly gaining popularity as a cost-effective method because microorganisms, particularly fungal cultures, produce comparatively high metabolite titres under fermentation conditions that are comparable to those found in nature (Singhania et al. 2009; Singhania 2011).

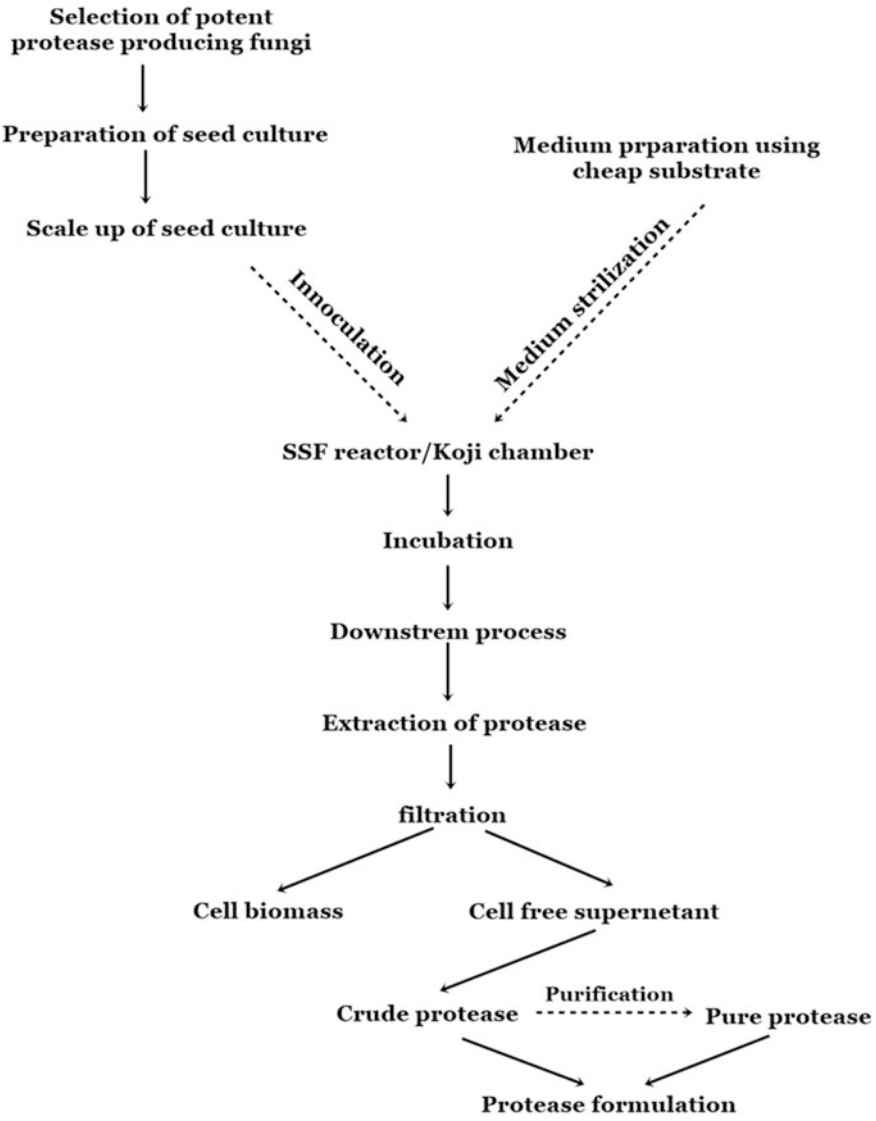

Fig. 2.1 Flowchart of protease production by SSF

Fungi such as *A. oryzae*, *M. miehei*, *C. parasitica,* etc. have been used to produce proteases utilizing SSF, in which a basal mineral salt medium was used to wet the substrate. Figure 2.1 shows the general steps of the SSF protease production process. For economic reasons, the Koji chamber can be utilized for large-scale production (Singhania 2011), but maintaining sterility is problematic. Agro-industrial waste can be used as a substrate. Inocula for protease synthesis can be created in a stirred tank reactor and sprayed onto a sterile medium in a shallow tray. Spores or mycelia can be

used as an inoculum. Temperature and humidity are controlled inside the koji chamber, and incubation is allowed for 7 days or as prescribed. A suitable buffer or distilled water with an adequate tTeen percentage is used as the extraction liquid. To remove biomass and cell detritus, the medium is homogenized with extraction liquid and centrifuged. The extracellular cellulase in the supernatant can be concentrated by acetone precipitation or salting out, or it can be used directly as a crude enzyme.

Substrate composition, pH, temperature, moisture content, and aeration are all critical parameters in protease production via SSF. The organism and substrate used may affect the working conditions. Fungi, for example, prefer an acidic pH and low moisture content (35–70%) compared to bacteria (70–90%) and grow best at 25–30 °C, whereas bacteria prefer a neutral pH and high moisture content and grow best at 35–37 °C. Owing to the low cost of input and the possibility of using naturally available sources, solid substrate fermentation may be a better technique for commercial protease production. Another key component is pH, which influences microbe development and, as a result, protease production. The pH of a solid substrate is difficult to monitor, but the pH of the basal medium, which usually contains nitrogen sources with buffering capabilities, can be altered. Heat transport is always limited because it is poor in the solid layer and overheating occurs in the substrate particle. This makes it difficult for spores to germinate, mycelia to proliferate, and enzymes to accumulate and secrete. Temperature management in a solid-state fermenter environment is very simple, but the temperature adjustment within the solid substrate layer is more complicated. Controlling the moisture content of the medium is also important for protease formation, which is a necessary component for microorganism development. The void space and gas-phase volume within the solid substrate are reduced when the water content is high, which increases the mass transfer resistance of oxygen and carbon dioxide, as well as the risk of contamination, whereas a low water content is unfavourable for spore germination and substrate swelling.

2.2.4 Submerged Fermentation

Fermentation in the presence of excess water is known as submerged fermentation. Owing to superior monitoring and handling, almost all large-scale enzyme production facilities use the proven technology of SmF. *B. subtilis*, *B. subtilis* var. *natto*, and *B. amyloliquefaciens* have been reported to produce proteases. As previously stated, the nature of the substrate, medium pH, nutrient availability, inducer supplementation, fermentation temperature, and other factors have a significant impact on protease synthesis in cultures (SmF). Increased production in the fermenter can be achieved through a gradient feed of a suitable substrate and maintaining ideal process conditions. Large-scale protease production requires an understanding and proper control of the producer's growth and enzyme production capabilities.

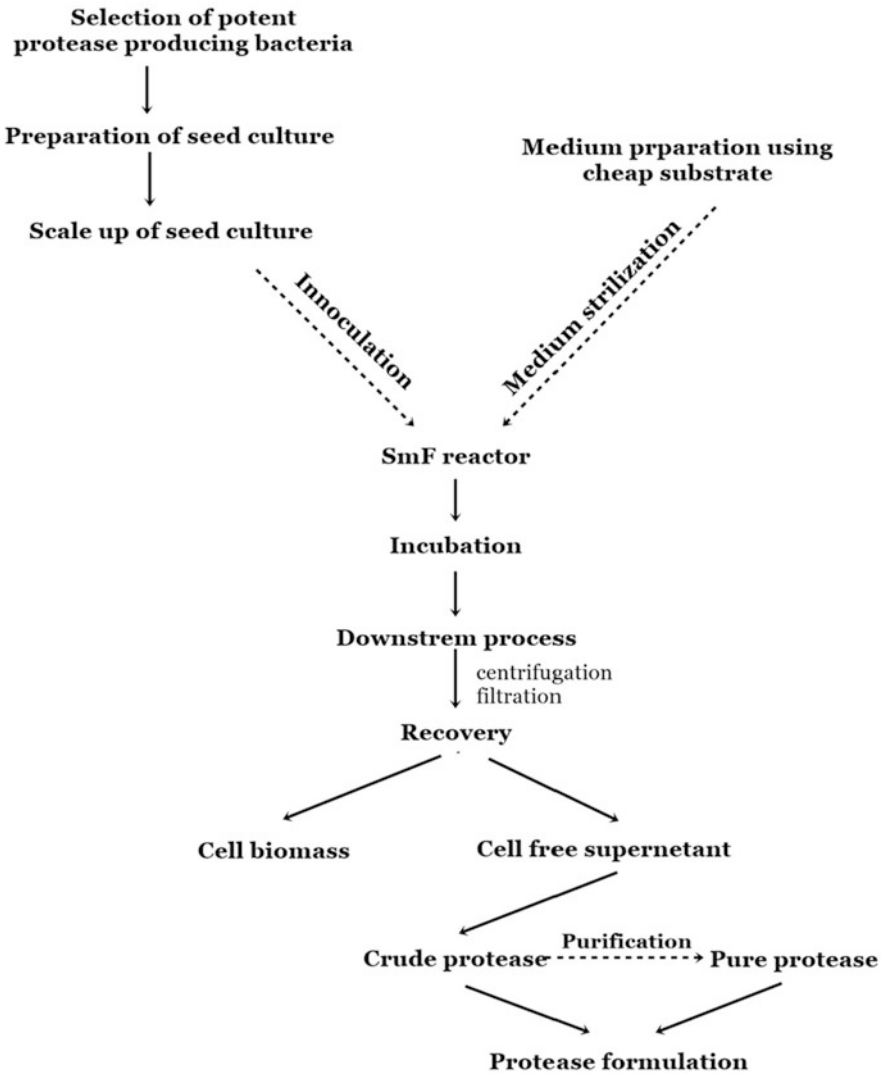

Fig. 2.2 Flowchart of protease production by SmF

Large bioreactors are available for submerged fermentation, and they also allow for easy control of many operating conditions such as pH, temperature, aeration, etc. Figure 2.2 depicts the general procedures involved in the manufacture of protease by submerged fermentation. SmF is the most widely used technology for the industrial production of primary and secondary metabolites. Because all the parameters required for modelling can be monitored in submerged fermentation, SmF has been used in the majority of modelling studies for metabolite synthesis (Singhania 2011).

2.3 Analysis of the Protease Production Cost

Here, we estimate the cost of generating proteases from bacteria and fungi, which will be useful for new entrepreneurs (Table 2.1).

2.4 Application of Protease

Proteases are the most important hydrolytic enzymes, where alkaline proteases are the most important enzymes in the enzyme market (Mahajan et al. 2015). Microbial acid proteases are primarily used in the detergent, food, leather, and pharmaceutical industries, whereas only a few alkaline protease products are marketed effectively (Fig. 2.3). Microbial proteases have a wide range of uses in the following industries.

2.5 Detergent Industry

Proteases are commonly used in the detergent industry on a commercial basis. Various detergent formulations containing proteases as essential components or ingredients have been used to clean domestic laundry, dentures, or contact lenses.

Table 2.1 Budget proposal for microbial protease production in small-scale operation

Cost item	Requirement	Amount (Rs)
Fixed capital cost estimation		
Total plant cost	Building construction	
	Electrical	
Equipment purchase	Laminar air flow	40,000
	Autoclave	20,000
	Fermenter (200 L)	5,60,000
	Incubator cum orbital shaker	45,000
	Centrifuge	20,000
Total cost		
Recurring cost estimation		
Raw materials	Wheat bran	7/kg; 105/month
Consumables	Glass wares and plastic wares	10,000[a]
Utilities	Media and chemicals	10,000[a]
Microbial strain	Fungal or bacterial strain from MTCC	2440/strain
Manpower	Microbiologist	25,000/month
	Labour	10,000/month
Transportation and waste treatment		
Total cost		

[a]Based on rate of utilization

Fig. 2.3 Application of microbial protease in various fields

Proteinaceous stains are particularly difficult to remove with regular detergents; nevertheless, such stains can be removed by utilizing microbial proteases. Furthermore, the addition of protease to detergent formulations increases the cleaning of proteinaceous stains and provides unique benefits that are not available with traditional detergent technologies. Enzymes are increasingly used in detergent formulations in industrialized countries, with enzymes found in more than 50% of all detergents (Hamza 2017). Most of these enzymes are produced by various bacterial species. *Bacillus* sp. have a wide range of applications in the textile and detergent industries. *B. cereus* BM1-produced protease has been reported as a good detergent ingredient, with stable action in a solution of 10% (w/v) commercial detergent, implying commercial use (Barberis et al. 2008).

2.6 Food Industry

Proteases are used in the food industry to modify proteins and increase the palatability and storage stability of protein sources. Proteases are added to milk cheese to hydrolyse kappa casein and prevent coagulation by stabilizing micelle formation. Because of its high specificity for casein, particularly the Phe105-Met106 link of k-casein, which is the first step in cheese manufacturing chymosin is the favoured protease in the cheese-making industry. Higher photolytic rates and shorter ripening times in cheddar-type cheeses were aided by microbial rennet from *B. amyloliquefaciens*. Proteases from *Mucor miehei* and *B. subtilis* have gradually replaced chymosin in cheese production. Alkaline proteases have been used to prepare protein hydrolysates with high nutritional value and well-defined peptide profiles. It also aids in tenderization of meat, particularly beef (Gupta et al. 2002). In the baking industry, endo- and exoproteases from *A. oryzae* have been used to change wheat gluten by restricted proteolysis, which reduces mixing time, improves dough texture, and boosts loaf volume. Wheat gluten solubility is substantially enhanced by the breakdown of wheat gluten by an acid protease from *A. usamii* under optimal conditions. The emulsifying activity index (EAI), water, and oil-holding capacity of wheat gluten increase dramatically after enzyme hydrolysis (Deng et al. 2016). Furthermore, after hydrolysis, the functional characteristics of the wheat gluten were enhanced.

2.7 Leather Industry

Proteases are enzymes utilized to degrade non-collagenous skin constituents and eliminate non-fibrillary proteins. The use of enzymes in leather processing improves the quality of leather, making it stronger and softer with fewer spots (Solanki et al. 2021). The elastolytic and keratinolytic actions of alkaline proteases have increased their use in the growing leather industry. Alkaline proteases are extremely useful in the leather processing industry. Proteases have been found to be useful in the soaking, bating, and dehairing stages of skin and hide preparation. Protease eliminates undesirable colours and aids in the creation of clean hides while lowering environmental pollution (Brandelli et al. 2010).

2.8 Medical Field

In medicine, various formulas including alkaline proteases produced by *B. subtilis*, such as gauze, non-woven tissues, and ointment compositions, exhibit promising therapeutic characteristics (Awad et al. 2013). The diagnosis of certain lytic enzyme-deficient diseases is aided by the oral introduction of alkaline proteases (Joshi and

Satyanarayana 2013). Fibrin breakdown has been found to be accomplished by alkaline fibrinolytic proteases. The utilization of fibrinolytic enzymes suggests that they could be used as an anticancer medicine or in thrombolytic therapy in the future (Jaouadi et al. 2012). The preparation of elastoterase immobilized on a bandage is used to treat various diseases such as burns, carbuncles, furuncles, and wounds (Palanivel et al. 2013).

2.9 Chemical Industry

Many alkaline proteases-producing microbes such as *B. pseudofirus* SVB1, *P. aeruginosa* PseA, and *A. flavus* have demonstrated significant success in peptide synthesis due to their durability in organic solvents (Razzaq et al. 2019). Several *Bacillus* and *Streptomyces* species that produce alkaline proteases in the water systems are active candidates for peptide and chemical synthesis (Yadav et al. 2015).

2.10 Miscellaneous Applications

Protease is also used in waste management, photographic industry, silk degumming, and beer and wine industries (Mamo and Assefa 2018; Razzaq et al. 2019). Poultry feathers with a very hard keratin structure account for 5% of body weight and are a great source of proteins for feed and food. Keratinolytic processes can convert poultry manure into feed and food. Alkaline proteases produced by *B. subtilis*, *Conidiobolus coronatus*, and *S. avermectnus* have been reported to successfully recover silver from X-ray films, ensuring that the process is more environmentally friendly than the use of chemicals. In bench-scale fermentation conducted at 200 °C, the addition of acid proteinase from *Saccharomycopsis fibuligera* 1570 and *Torulopsis magnoliae* 1536, as well as brewer's yeast, to brewer's wort demonstrated that the final bottled beer was resistant to haze formation. Acid protease enzymes can degrade the turbidity complexes formed by proteins in fruit juices and alcoholic beverages.

2.11 Market Trend of Protease

Proteases are one of the three major categories of industrial enzymes, accounting for more than 60% of the global enzyme sales. Microbes account for two-thirds of all commercial proteases sold worldwide. During this forecast period, the worldwide protease market is expected to develop at a CAGR of 5.8% (2022–2027). Since the late 1990s, proteases have been widely used in detergents. Protease use in the detergent industry accounts for 20% of the total enzyme sales. Proteases with the

names alcalase (*B. licheniformis*), Savinase (*Bacillus* sp.), Esperase (*B. lentus*), and Durazym (Protein designed, version of Savinase®) were introduced by Novoenzyme, Denmark, and used in detergents, degumming, and the textile industry. Denmark's Novo Industry is one of the world's leading protease producers, accounting for 40% of the global protease market. It produces three different types of proteases, Aquaderm, NUE, and Pyrase, which are used for soaking, dehairing, and bating, respectively (Sandhya et al. 2005). Fungal aspartic proteases (Aps) have been used as milk-clotting enzymes in the dairy sector for approximately 30 years because of the global scarcity of calf chymosin. Aspartic protease enzymes derived from *M. miehei*, *M. pusillus*, and *C. parasitica*, and sold under the brand names Rennilase®, Fromase®, Novoren®, Marzyme®, Hannilase®, Marzyme®, and Suparen® are commonly used in the manufacture of cheese (Mamo and Assefa 2018). The use of proline-specific endoproteinases (e.g., Brewers Clarex®, DSM, France) that target the degradation of haze-active proteins (e.g., hordeins) minimizes the production of storage haze in the finished beer. Similarly, proteases with the tread names proleather (*Bacillus* sp.), protease Savinase (*B. licheniformis*), and Biofeed protease (*B. licheniformis*) were utilized in the food and feed industry.

2.12 Conclusion

Microbial proteases are key hydrolytic enzymes that have been widely used since the beginning of enzymology. It has a wide range of uses in the detergent industry, bioremediation, food processing, and leather processing, and has been widely commercialized by a variety of companies. Owing to the use of low-cost basic materials and genetic manipulation, their use and manufacturing are rapidly increasing. New technology adoption is required to enhance cleaner production in all industries, particularly the leather and treatment industries, by replacing most existing chemical techniques with less expensive and more environment-friendly alternatives, particularly proteases.

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Chapter 3
Mass Multiplication, Production Cost Analysis and Marketing of Xylanase

Kartik Patel and Natarajan Amaresan

Abstract Microbial xylanases have received increasing attention because of their biotechnological potential in a variety of industrial processes. Lignocellulosic biomass is a renewable raw material that can be used to produce xylanase, thereby reducing the requirement for costly substrates and addressing the main issue of solid waste management. Submerged and solid-state fermentation have been used to produce xylanases. The majority of xylanases work best at temperatures ranging from 30 to 70 °C and pH level ranging from 4.0 to 8.0. However, thermostable and pH-stable xylanases are in high demand for use in a wide range of industrial processes that operate under harsh conditions. Xylanases modified for industrial applications can be created through genetic engineering. The xylanase market is in high demand owing to the use of microbial xylanases in industries such as pulp and paper, biofuel production, and food and feed. This chapter discusses the sources of xylanase, inexpensive substrates, mass production techniques, and various applications of xylanase. Additionally, it provides an approximate cost estimation for xylanase production, which will be helpful to new researchers and entrepreneurs.

Keywords Xylanase · Lignocellulosic biomass · Pulp and paper industry · Food and feed industry

3.1 Introduction

Since the 1980s, industrial enzyme-based processes have been steadily increasing owing to their availability and eco-friendliness (Patel and Dudhagara 2020a). Several important enzymes have been used in industrial and commercial processes. Enzyme-based products and processes are in high demand, and microbes are one of the largest sources of most enzymes (Adrio and Demain 2014). In the industrial

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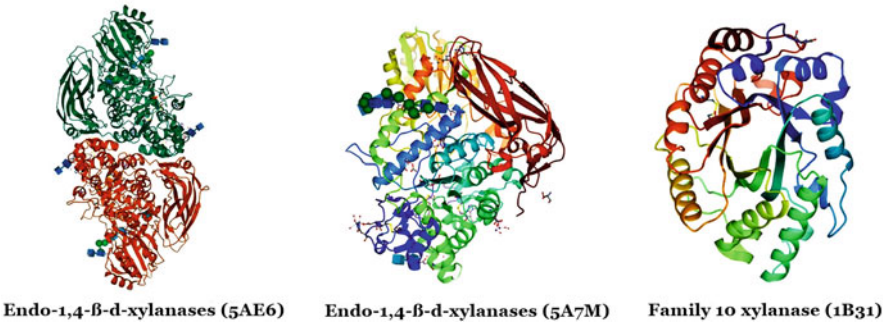

Fig. 3.1 Structures of selected microbial xylanases

market, more than 500 commercially available products are made from enzymes (Johannes and Zhao 2006; Kumar and Singh 2013). Additionally, enzyme-mediated processes are cost-effective, require low energy input, and are nontoxic, making them desirable for industries and the environment (Singh et al. 2016).

Xylanase is a group of enzymes comprising endo-1,4-β-d-xylanase (EC 3.2.1.8), β-d-xylosidases (EC 3.2.1.37), α-1-arabinofuranosidases (EC 3.2.1.55), acetyxylan esterase (EC 3.1.1.72), α-glucuronidase (EC 3.2.1.139), *p*-coumaric esterase (EC 3.1.1.B10), and ferulic acid esterase (EC 3.1.1.73) (Bhardwaj et al. 2019). Among them, endo-1,4-β-d-xylanase is considered the most important enzyme, which cleaves the glycosidic bonds in the xylan backbone and produces simple monosaccharides and xylo-oligosaccharides (Kumar et al. 2014). Classification of xylanases is based on their primary structure and comparison of their catalytic domains (Collins et al. 2005). According to the CAZy database (http://www.cazy.org), xylanases comprise 14 glycoside hydrolase (GH) families: GH 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, and 62. In particular, GH 10 and GH 11 families have been extensively studied (Fig. 3.1). The GH 10 family contains endo-1,4-β-d-xylanases and endo-1,3-β-d-xylanases (EC 3.2.1.32), which are capable of hydrolysing the aryl β–glycosides of xylobiose and xylotriose. However, only xylanases (EC 3.2.1.8) in family 11 are considered as "true xylanases", as they are active on d-xylose-containing substrates (Liu and Kokare 2017).

The biotechnological applications of xylanases in the production of ethanol and xylitol (Polizeli et al. 2005), pulp and paper industry (Patel and Dudhagara 2020b), food industry (Sharma et al. 2019), textile industry (Battan et al. 2012), and animal feed industry (Bajaj and Mahajan 2019) have attracted significant interest. The xylanases used in industries are mainly produced by microorganisms such as from bacteria, fungi, and actinomycetes. For the large-scale production of xylanase, inexpensive substrates that are readily and continually available are needed. Substrate selection is an important criterion for large-scale enzyme production (Patel and Dudhagara 2020a). Researchers are therefore becoming increasingly interested in low-cost substrates for xylanase production. The use of lignocellulosic biomass to produce bioethanol, xylitol, and other value-added compounds has increased greatly

over the past few years. In addition to these requirements, it is essential to determine the specificity of microbial xylanase, such as producing higher amounts of xylanase, and enzyme stability against various pH, temperatures, solvents, and metal ions are considered essential before mass multiplication of microbial xylanase and subsequent applications.

3.2 Source of Xylanase Production

Xylanase is ubiquitous in nature, and its presence is observed in living organisms, such as bacteria, fungi, yeast, protozoans, algae, plants, crustaceans, molluscs, and insects. Bacteria are a promising source for xylanase production. Bacterial genera including *Bacillus*, *Staphylococcus*, *Cellulomonas*, *Micrococcus*, *Microbacterium*, *Rhodothermus*, *Arthrobacter*, and *Pseudoxanthomonas* have been reported to produce xylanase (Singh 2019). Among these, several *Bacillus* species such as *B. subtilis*, *B. halodurans*, *B. pumilus*, *B. amyloliquefaciens*, *B. stearothermophilus*, *B. circulans*, and *B. tequilensis* have been reported as potent xylanase producers (Bhardwaj et al. 2019; Patel and Dudhagara 2020a). Several bacterial species such as *B. halodurans* TSEV1, *Streptomyces* sp., *Thermotoga thermrum*, *Clostridium thermocellum*, *Stenotrophomonas maltophila*, and *Rhodothermus marinus* have been reported to produce thermostable and alkali-stable xylanases (Gupta et al. 2015). Whereas, *Flavobacterium frigidarium*, *Pseudoalteromonas haloplanktis* TAH3A, and *Clostridium* sp. PXLY1 bacterial strains have been reported to produce psychrophilic xylanase (Singh 2019).

The fungal genera *Aspergillus* and *Trichoderma* are the most common xylanase producers and are widely used for commercial production. Whereas, *A. oryzae* LC1, *Talaromyces thermophiles*, *Malbranchea* sp., *Chaetomim termophilum*, *Rasamsonia emersonii*, *Thielayia terrestris*, *Malanocrpus* sp., *M. albomyces*, *Paecilomyces thermophile*, *Rhizomucor pusillus*, *T. leycettaanus*, *Humicola insolens*, *Thermoascus auranticus*, and *Achaetomim* sp. X2–8 are producer of alkaline and thermophilic active xylanases (Chadha et al. 2019). However, bacterial xylanase offers a wide range of pH and temperatures which makes it more applicable for many industrial applications.

3.3 Influencing Factors on Xylanase Production

Several factors need to be considered to produce xylanase on a large scale, such as strain selection, fermentation methods, substrate/energy sources, and external parameters. A strain to be chosen for the fermentation process should meet the following criteria: it should be able to produce the desired amount of product on a large scale, be able to grow faster, be safe to handle, and be grown in low-cost growth media, be genetically stable, easy to manipulate at the genetic level, and easy

to recover from culture (Parekh et al. 2000). In the natural environment, microbes often produce low levels of enzyme activity, which is why an increase in enzyme levels can be achieved by mutation. As an alternative, production of the enzyme in a well-optimized recombinant organism of choice has gained popularity.

Submerged fermentation (SmF) and solid-state fermentation (SSF) are two processes used to produce enzymes from bacteria and fungi, respectively. The main advantage of SmF is it maintains homogenous conditions throughout the enzyme production process and is easy to scale-up (Singh 2019); however, it is highly cost-prohibitive. In contrast, SmF processes have lower capital operation costs and higher productivity per reactor volume (Walia et al. 2017).

In addition, physical and nutritional factors during fermentation can influence xylanase production. In commercial production, substrate selection is important for determining the cost of xylanase. Large amounts of substrate are required for xylanase production on a large scale, and commercial substrates are not economical. Thus, low-cost lignocellulosic material is a better choice for the large-scale production of xylanase. Various substrates/energy sources have been reported to be suitable for xylanase production. These included rice straw, rice bran, wheat straw, wheat bran, sorghum straw, barley husk, oat spelt xylan, corn stover, corn cob, corn husk, sugarcane bagasse, molasses, apple pomace, and pineapple peel (Table 3.1).

Similarly, nitrogen sources such as yeast extract, beef extract, peptone, and soymeal increased xylanase production. In some cases, more than one nitrogen source has been used for high xylanase production (Singh 2019). Other factors influencing xylanase production include pH, temperature, agitation, and incubation time. Therefore, prior to large-scale fermentation, it is necessary to optimize all media parameters at the initial stage. Traditional (one factor at a time) and statistical (response surface methodology) medium optimizations are important criteria for removing unnecessary medium components and, as a result, making the entire process more cost-effective.

3.4 Bacterial Xylanase Production Using SmF

Substrate: Different agro-waste products (sawdust, oat bran, barley bran, corn cob, rice bran, wheat bran, and rice straw) are collected and cleaned by washing with water to remove unwanted debris. Then cut into small pieces (particle size 300–500 μm). ***Production:*** *Bacillus, Pseudoxanthomonas, Cellulomonas*, and other potent xylanase-producing bacteria are used for xylanase production using SmF, where 10% of prepared substrate is moistened with mineral salt solution (composition g/l: KCl, 0.5; $(NH_4)2HPO_4$, 2.5; $MgSO_4$. $7H_2O$, 0.5; NaH_2PO_4, 0.5; $FeSO_4$. $7H_2O$, 0.01; $CaCl_2.2H_2O$, 0.01; and $ZnSO_4.7H_2O$, 0.002). Autoclave the medium for 15 min at 121 °C for 15 Psi. Inoculate freshly prepared bacterial cultures (5%) into flasks and then incubate at 30 °C on a rotary shaker (150 rpm) for 7 days. Huge bioreactors are available for SmF, which allow for easy control of many operating conditions such as pH, temperatures, aeration, etc. Figure 3.2 depicts the

Table 3.1 Xylanase production by various microbes using low-cost lignocellulosic materials

Microorganism	Substrate	Fermentation conditions	Xylanase production[a]	Reference
Bacteria				
B. pumilus	Spelt xylan and wheat bran	SSF and SmF,35 °C, 9.0 pH and 72 h incubation	35.156 U/g; 580 U/ml	Poorna and Prema (2006)
Geobacillus thermoleovorans	Wheat bran	SmF, 70 °C, 7.0 pH and 72 h incubation	1.678 U/ml	Sharma et al. (2007)
B. subtilis	Oat spelt xylan	SmF, 55 °C, 9.0 pH and 36 h incubation	128 U/ml	Annamalai et al. (2009)
Chromohalo-Bacter sp. TPSV 101	Sugarcane bagasse	SmF, 40 °C, 9.0 pH and 140 h incubation	250 U/ml	Prakash et al. (2009)
B. pumilus SV-85S	Wheat bran	SmF, 37 °C, 6.0 pH and 48 h incubation	2900 U/ml	Nagar et al. (2010)
B. mojavensis AG137	Oat bran	SmF, 37 °C, 8.0 pH and 48 h incubation	302.46 U/ml	Akhavan Sepahy et al. (2011)
B. cereus BSA-1	Xylan	SmF, 35 °C, 6.0 pH and 84 h incubation	6.02 U/ml	Mandal et al. (2012)
Sphingobacterium sp. ksn-11	Corn husk	SmF, 40 °C, 7.0 pH and 24 h incubation	79.2 U/ml	Neelkant et al. (2019)
B. Tequilensis strain UD-3	Wheat bran	SmF, 45 °C, 7.5 pH and 72 h incubation	19.46 U/ml	Patel and Dudhagara (2020a)
Fungi				
A. foetidus MTCC 4898	Corn cob	SSF, 30 °C, 5.0 pH and 96 h incubation	3065 U/g	Shah and Madamwar (2005)
Paecilomyces thermophile J18	Wheat straw	SSF, 50 °C, 7.0 pH and 192 h incubation	77,450 U/g	Yang et al. (2006)
Thermoascus aurantiacus CBMAI756	Corn cob	SmF, 50 °C and 144 h incubation	130 U/ml	Oliveira et al. (2010)
A.niger	Barley bran	SSF, 35, 5.5 pH and 48 h incubation	42.5 U/g	Soliman et al. (2012)
Amycolatopsis sp.	Wheat straw	SmF, 30, 6.5 pH and 144 h incubation	530.13 U/ml	Kshirsagar et al. (2016)
Cladosporium oxysporum GQ-3	Wheat bran	SmF, 30 and 7.0 pH	55.92 U/ml	Guan et al. (2016)
Schizophyllum commune ARC-11	Rice straw	SSF, 30, 7.0 pH and 192 h incubation	6721.9 U/ml	Gautam et al. (2018)

[a]Xylanase production in SSF as U/g and SmF as U/ml

general procedures involved in SmF-based xylanase synthesis. ***Recovery:*** After incubation, the medium is filtered through a filter paper and centrifuged for 10 min at 4 °C at 10,000 rpm. Cell-free supernatants serve as crude xylanase. ***Advantages:***

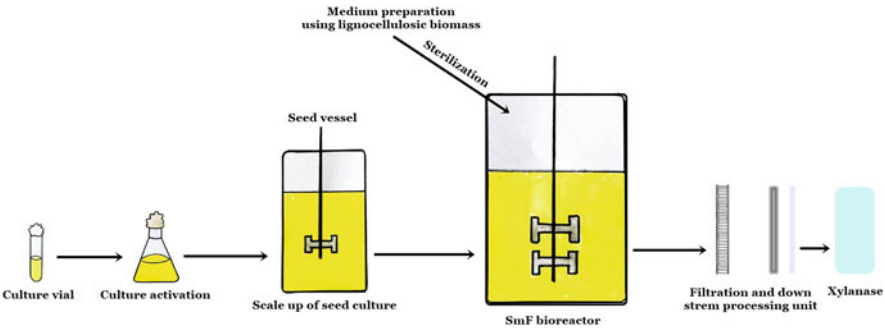

Fig. 3.2 General steps involved in xylanase production by SmF

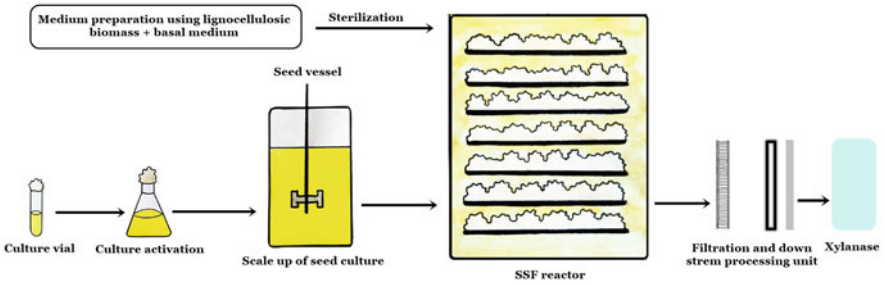

Fig. 3.3 General steps involved in xylanase production by SSF

Process control infrastructure is inexpensive and simple. ***Disadvantages:*** Low productivity and feedback inhibition.

3.5 Fungal Xylanase Production Using SSF

Substrate: Rice bran, wheat bran rice straw, etc. ***Production:*** *Aspergillus, Trichoderma,* and other potent xylanase-producing filamentous fungi can be used for xylanase production using SSF, where a basal mineral salt solution is used to moisten the substrate. Figure 3.3 depicts the general steps of SSF xylanase production. Lignocellulosic biomass can be used as the substrate. Inocula for xylanase production can be prepared in a stirred tank reactor and sprayed onto a sterile medium in a shallow tray. In filamentous fungi, spores or mycelia can be used as inocula. In this case, the temperature and humidity inside the chamber are controlled, and the incubation period is set to 7 days. Moisture content, temperature, pH, and aeration are all significant parameters in the production of xylanase via SSF. Therefore, all parameters must be kept constant to achieve maximum xylanase production via SSF. ***Recovery:*** The filtrate is harvested after incubation in a 50 mM sodium citrate buffer (pH 5.0). Filter the fermented slurry through muslin

cloth and centrifuge for 25 min at 4 °C at 10,000 rpm. The clear supernatants serve as crude xylanases. ***Advantages:*** Increased productivity due to increased biomass production, reduced protein breakdown, reduced proteolysis, and improved oxygen circulation. ***Disadvantages:*** High impurity and recovery costs; difficulty in managing process parameters (heat, pH, moisture, nutrients, etc.).

3.6 Analysis of the Xylanase Production Cost

According to Polizeli et al. (2005), hydrolytic enzymes such as xylanase, pectinase, and cellulase account for more than 20% of the total global enzyme production. However, the cost of each step of xylanase production in a biorefinery context is low and it is not available in the public domain. Klein-Marcuschamer et al. (2012) conducted a study on cellulase production using lignocellulosic biomass, and determined the baseline cellulase production cost to be $10.14/kg. They also proposed a percentage of cost for each component, such as raw materials (28%), facility-dependent (48%), consumables (4%), utilities (10%), labour (7%), and transportation and waste treatment (2%). Similarly, Ferreira et al. (2018) investigated the techno-economic parameters of *E. coli* β-glucosidase production at an industrial level. They discovered that the major costs during large-scale production are facility-dependent (45%), raw materials (25%), and consumables (23%). This clearly demonstrates that capital investment is the most expensive, followed by the cost of the substrate. Facility-dependent costs are incurred for infrastructure development, maintenance, insurance, and depreciation (Bhardwaj et al. 2019). Furthermore, upstream and downstream processes during enzyme production increase capital investment costs as well as consumables and utilities. However, under ideal conditions, the cost of enzyme can be drastically reduced (Ferreira et al. 2018). Based on these findings, we can conclude that the cost of substrate, consumable, upstream, and downstream processing influences xylanase production. As a result, optimization and the use of low-cost raw materials can help keep enzyme production costs as low as possible. However, we estimated the cost of xylanase production from bacteria (Table 3.2).

3.7 Application and Market Trend of Xylanase

In the global xylanase market, BioResource International, Inc., Elanco, Habio.Net, and Beijing Smile feed sci. & Tech. Co., Ltd., Enzyme Development Corporation, Royal DSM, Shenzhen LeveKing Bio-Engineering Co., Ltd., Shandong Longda Bio-Products Co., Ltd., etc., and other companies are identified as key players in the global xylanase market. The xylanase market is divided into three categories: application, grade, and end-user industry. The pulp and paper industry, biofuel production, food and feed industry, brewing industry, and waste paper deinking

Table 3.2 Budget proposal for bacterial xylanase production in small-scale operation

Cost item	Requirement	Amount (Rs)
Fixed capital cost estimation		
Total plant cost	Building construction	
	Electrical	
Equipment purchase	Laminar air flow	40,000
	Autoclave	20,000
	Fermenter (200 L)	5,60,000
	Incubator cum orbital shaker	45,000
	Centrifuge	20,000
Total cost		
Recurring cost estimation		
Raw materials	Wheat bran	7/kg; 105/month
Consumables	Glass wares and plastic wares	10,000[a]
Utilities	Media and chemicals	10,000[a]
Microbial strain	Bacterial strain from MTCC	2440/strain
Manpower	Microbiologist	25,000/month
	Labour	10,000/month
Transportation and waste treatment		
Total cost		

[a]Based on rate of utilization

are some of the most common xylanase applications (Basit et al. 2020). (a) Pulp and paper industry: xylanase is essential for removing lignin from wood pulp. Chemical bleaching (Cl_2, ClO_2, and hypochlorite) have been traditionally used to remove lignin and make paper bright and completely white. The most serious issue associated with the use of chemical bleaching agents is the generation of hazardous wastes. The use of xylanases during the pre-bleaching stage improves lignin extraction and increases pulp brightness. Xylanase reduces the amount of chlorine used from 10 to 50%, thereby lowering the overall cost of paper manufacturing (Kumar et al. 2018). According to Global Market Insights, Inc. (April 2020), xylanases account for approximately 17% of the pulp and paper enzyme market share. (b) Biofuel production: converting lignocellulosic biomass into fuel-grade ethanol has emerged as a global priority. Xylanase, in collaboration with ligninase, mannanase, gluconase, oxidase, and other enzymes, can be used to produce ethanol and xylitol from lignocellulosic biomasses. Xylanolytic enzymes are essential for hydrolysis. Hydrolysis and fermentation are critical steps for the conversion of biomass to bioethanol. *Geobacillus* sp. DUSELR13 is a thermostable xylanase producer that produces 3.53 and 3.72 g/l ethanol from prairie cord grass and corn stover, respectively (Bibra et al. 2018). According to a research published by Research and Markets (September 2021), the global biofuel enzyme market is expected to reach $2.0 billion by 2026, growing at a compound annual growth rate of 9.4%. (c) Food and feed industry: Xylanase plays an important role in the food and feed industries, such as dairy products, bread, juices, and animal feed (Kumar et al. 2018). Xylanase improves

bread quality by converting water-insoluble arabinoxylan into water-extractable arabinoxylan. In the juice and wine industries, xylanase was also used as a clarifier. The use of an enzyme mixture (xylanase and/or pectinase) reduces viscosity and improves juice clarity, aroma, and colour. Apple, peach, kiwi, apricot, grape, and pomegranate juices were treated with xylanase from *P. acidilactici* GC25, which successfully reduced the juice turbidity (Adiguzel et al. 2019). The majority of animal feed products are rich in lignocellulosic biomass, and using xylanase to break down arabinoxylan in the feed ingredients improves their nutritional value (Kumar et al. 2018). ECONASE XT is a well-known commercial endo-1,4-xylanase that is used as a feed additive for chicken fattening, weaned piglets, and fattening pigs (Rychen et al. 2018). (d) Waste paper deinking: Deinking is a green method of reprocessing waste paper by removing ink from paper. Chemical methods involving chlorine-based depravities such as sodium silicate, sodium carbonate, hypochlorite, and chelating agents have generally been used, resulting in the generation of hazardous waste. The combination of xylanase and laccase has been reported to improve the optical and physical properties of paper inks. Chutani and Sharma (2016) found that applying xylanase and cellulase from *T. longibrachiatum* MDU-6 to deinked newspapers increased brightness by 52%.

3.8 Conclusion

Microbial enzymes are referred to as a "Green" alternative and have been used as major catalysts by industrial product manufacturers to transform raw materials into end products. In conclusion, lignocellulosic biomass and enzyme-producing microbes are compelling subjects for research studies aimed at investigating the growing importance of xylanase in various industrial products.

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Chapter 4
Mass Multiplication, Production Cost Analysis, and Marketing of Cellulase

Kartik Patel and Natarajan Amaresan

Abstract Cellulases are a complex group of enzymes secreted by a variety of microorganisms such as fungi, bacteria, and actinomycetes, that depolymerize cellulose and convert it to glucose. The potential of cellulolytic enzymes in bioconversion processes has been extensively investigated, and they play an important role in a variety of industrial applications. Cellulase, a key enzyme for cellulose-rich waste feedstock-based biorefineries, is in high demand across a wide range of industries, including biofuel production, paper and pulp manufacturing, textile polishing and finishing, agriculture, and juice clarification. Furthermore, there has been continuous progress in developing new technologies to improve its production, such as the use of waste feedstock as the substrate, process parameter control, and genetic manipulation for enzyme production with increased yield, quality, and specificity. Furthermore, an understanding of mass production techniques for controlling the cost of enzymes on an industrial scale is presented. Although submerged fermentation (SmF) is the most commonly used method for commercial enzyme production, solid-state fermentation (SSF) is regarded as a promising method to produce higher enzyme titers than SmF. The purpose of this chapter is to provide new researchers and entrepreneurs with information on the classification, mechanisms, sources, mass production techniques, potential applications, market trends, and approximate cost estimation for cellulase production.

Keywords Cellulase · Biorefinery · Waste feedstock · Mass production · Market trends

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4.1 Introduction

Biomolecules obtained from natural resources play important roles in the production of everyday goods. Enzymes are one of those compounds that are widely recognized for their numerous industrial applications (Jayasekara and Ratnayake 2019; Patel and Dudhagara 2020a). Due to growing environmental safety concerns and the implementation of green processes to replace many of the traditional chemical processes, the demand for enzymes has expanded in many industrial applications over the past few decades. Their applications include brewing, dairy products, detergents, food and feed, pharmaceutical manufacturing, and paper and pulp industries (Patel and Dudhagara 2020b; Al-Ghanayem et al. 2022). Cellulase is one of the most extensively used enzymes. According to current worldwide cellulase market studies, the demand for this enzyme is expanding exponentially. The requirement for more stable and highly active cellulase enzymes is rapidly increasing, as is the predicted global market for industrial enzymes (Singhania 2011). Cellulases are essential enzymes in both the industrial and natural worlds because they contribute significantly to the global carbon cycle by converting insoluble cellulose into soluble sugars (Wilson 2009). Since the early 1980s, research on the biotechnology of cellulases and hemicellulases has been conducted first for the animal feed sector, and then for food applications. These enzymes have been used in the paper and pulp, textile, and detergent industries.

The most prevalent polysaccharide on Earth is cellulose, which serves as a substrate for cellulase. It is the primary component of plant materials. The first person to identify and isolate this substance from green plants was Anselme Payne (O'sullivan 1997). Cellulosic materials have historically been essential in everyday life. Currently, practical applications of cellulose in the industrial sector are widely acknowledged as cost-effective raw materials, and their complexity has significantly increased. This has created a significant foundation for scientists to conduct cellulose-based research by using interdisciplinary methods. The hydrolysis of cellulose is one such process. Cellulases typically perform this in the natural world.

Currently, India ranks second in the world in terms of agricultural production. Because of the differences in crop cultivation patterns across our country, the availability of lignocellulosic biomass varies from region to region. According to the Ministry of New and Renewable Energy, Report 2009, the Government of India, around 500 Mt. of leftovers are generated in India each year. Uttar Pradesh contributes the most residue (60 Mt./y), followed by Punjab (55 Mt./y) and Maharashtra (46 Mt./y) (Singh et al. 2019a, 2019b). Cereal crops provide the most residue (352 Mt./y), followed by fiber crops (66 Mt./y), oilseed crops (29 Mt./y), pulses (13 Mt./y), and sugarcane (12 Mt./y). Rice, wheat, maize, and millet account for up to 70% of the cereal crop production. Rice alone accounted for 34% of the total cereal crop residue, and wheat accounted for 22% of the total cereal crop residue. As shown above, fiber crops produce 13% of the total residues created by all crops. Cotton is the first fiber, creating 53 Mt./y (11% of crop leftovers), followed by

coconut at 12 Mt./y. Sugarcane residues (foliage and tops) generate 12 Mt./y, or 2% of crop residues.

Cellulases are currently the third-largest industrial enzymes in the world by dollar volume, and their popularity is growing owing to their wide range of uses, such as animal feed additives, cotton processing, paper recycling, juice extraction, and detergent enzymes. Utilizing cellulases has the potential to produce a variety of products with added value, including ethanol, by efficiently converting lignocellulosic biomass into glucose via enzymatic hydrolysis (Singhania 2011). Owing to the scarcity of fossil fuels and environmental concerns, there is a growing interest in the commercial exploitation of lignocellulosic biomass to produce ethanol for the transportation industry. According to Lynd et al. (2002), lignocellulosic biomass is the only source of energy that can be forecasted, and the future of humanity is expected to be built on a carbohydrate-based economy that relies only on the use of biomass. The main sources of fermentable sugars in lignocellulosic feedstock are cellulose and hemicelluloses; however, woody tissue is naturally created to be effectively resistant to microbial attack (Singh et al. 2019a, b). Therefore, crystalline cellulose is sometimes relatively impermeable to both larger molecules such as proteins and smaller molecules such as water. Very few reactive sites are available for enzyme attachment because of the compact and rigorous structure, as well as its complicated connection with other components, necessitating an effective pretreatment technique. In nature, cellulose is frequently incorporated into the lignin matrix and is associated with hemicelluloses. To make cellulose accessible to microorganisms, lignin must be removed using an appropriate pretreatment procedure. This is a critical stage in the commercial hydrolysis of lignocellulosic biomass. Currently, complex lignocellulosic biomass is pretreated using chemical and thermochemical methods, which makes cellulose more accessible to microbial attack (Rodríguez-Zúñiga et al. 2014).

4.2 Cellulase: Classification and Mode of Action

Cellulases are the most diverse class of enzymes that catalyze the hydrolysis of a single substrate, with 7 distinct protein shapes among the 10 real cellulase families (Wilson 2009). Cellulases are enzymes that break down the b-1, 4-D-glucan linkages in cellulose to create glucose, cellobiose, and cello-oligosaccharides as their main byproducts. Numerous microbes produce cellulases, that fall under a variety of enzyme classifications.

Endoglucanase or cellobiohydrolase (E.C. 3.2.1.4), exoglucanase (E.C. 3.2.1.91), and β-glucosidase (E.C. 3.2.1.21) are the three main forms of cellulase, and each has a unique structure and mode of action. Endoglucanase and exoglucanase combine their capabilities to hydrolyze cellulose into short cellooligosaccharides, which are then hydrolyzed by β-glucosidase into simple sugars (glucose) (Singh et al. 2019a, 2019b). There are several enzymes within these classifications; for example, the widely explored fungi for cellulase production, *Trichoderma reesei*, produce two

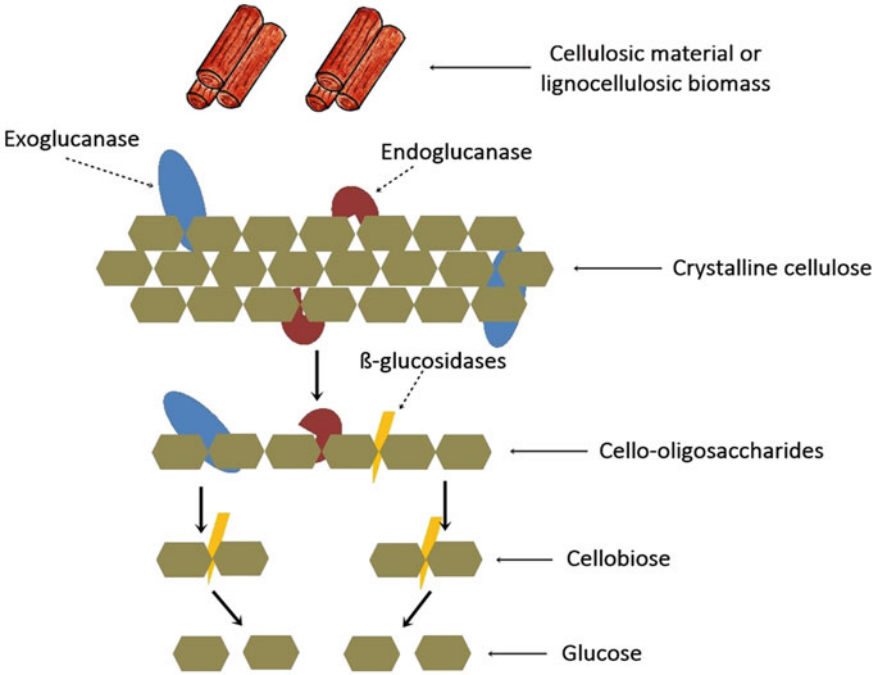

Fig. 4.1 Mechanism of various cellulase enzymes on cellulose

exoglucanase components, at least 8 endoglucanase components and 7 β-glucosidases (Aro et al. 2005). This is the most thoroughly investigated multiple enzyme complex, which includes endoglucanase, exoglucanase, and β-glucosidases (Singhania 2011). Figure 4.1 depicts the complete cellulase system, which includes endoglucanase, exoglucanase, and β-glucosidase components that work synergistically to convert crystalline cellulose to glucose.

4.3 Source of Cellulase Production

Cellulases are hydrolytic enzymes produced by a variety of microbes such as actinomycetes, bacteria, and fungi, when grown on cellulosic substrates. Because most bacterial cellulase systems are insufficient, they are unable to break down the crystalline cellulose. Therefore, the use of bacteria to produce cellulase is not common practice. Cellulolytic properties have been reported for some bacterial taxa, including *Cellulomonas*, *Cellvibrio*, *Micrococcus*, *Pseudomonas* sp., and *Bacillus* (Sethi et al. 2013). Filamentous fungi are the most common source of cellulases and hemicellulases. Most studies have focused on fungi such as *Phanerochaete chrysosporium*, *Ceriporiopsis subvermispora*, *Phlebia subseralis*,

Pleurotus ostreatus, and *Lentinus edodes* (Jayasekara and Ratnayake 2019). Cellulolytic enzymes generated by filamentous fungi include all three components of cellulase in varying amounts, and may thus completely degrade cellulose. The majority of cellulases used in industrial applications come from filamentous fungi such as *Trichoderma*, *Penicillium*, *Fusarium*, *Humicola*, *Phanerochaete*, and others, which contain a significant number of cellulases. *T. reesei* is one of the most potent cellulase producers used in the industry and is capable of producing two cellobiohydrolases (CBH I and CBH II) and two exoglucanases (EG1 and EG2). Owing to their thermostability, cellulases isolated from thermophilic fungi are of significant interest for industrial applications. *Chaetomium thermopile*, *Humicola insolens*, *Humicola agrisea*, *Myceliopthora thermophila*, *Talaromyces emersonii*, and *Thermoascus aurantiacus* are among the thermophilic fungi that produce cellulase (Singh et al. 2019a, b). In contrast to thermophiles, cellulose-producing alkaliphilic fungi are extremely rare. From mangrove leaves, alkaline-tolerant cellulases are produced by the marine fungi *Chaetomium* sp. (NIOCC36). Unexpectedly, no thermophilic archaea have been characterized by cellulolytic behavior.

4.4 Mass Production of Cellulase

Submerged fermentation (SmF) and solid-state fermentation (SSF) are the two most common methods for the mass production of enzymes.

4.4.1 Submerged Fermentation (SmF)

SmF refers to fermentation performed using freely flowing nutrient media. Enzymes are mostly produced industrially by SmF, owing to the much simpler processes involved in scale-up compared to those involved in SSF. This technology has developed to the extent that temperature, pH, aeration, agitation, and foam, among other parameters, can be accurately estimated depending on the type of reactor. Each of these factors is crucial for obtaining a desired product. When grown in a submerged culture, the majority of filamentous fungi tend to produce spherical pellets. This morphological distinction from SSF offers a potential explanation for the observed enzyme production. The filamentous fungi *T. reesei* and *A. niger* produce the majority of the commercial cellulases under SmF conditions. Generally, pure cellulose preparations such as Solka-Floc and Avicell have been used in liquid cultures of cellulolytic microbes to produce enzymes, but when using soluble substrates, the breakdown products may inhibit cellulase synthesis by promoting catabolite repression due to free sugar accumulation. Understanding and proper control of the producer's growth and enzyme production capabilities is necessary for large-scale production of cellulases. SmF is made more significant by a few other crucial factors such as the use of process parameter control, monitoring, and downstream

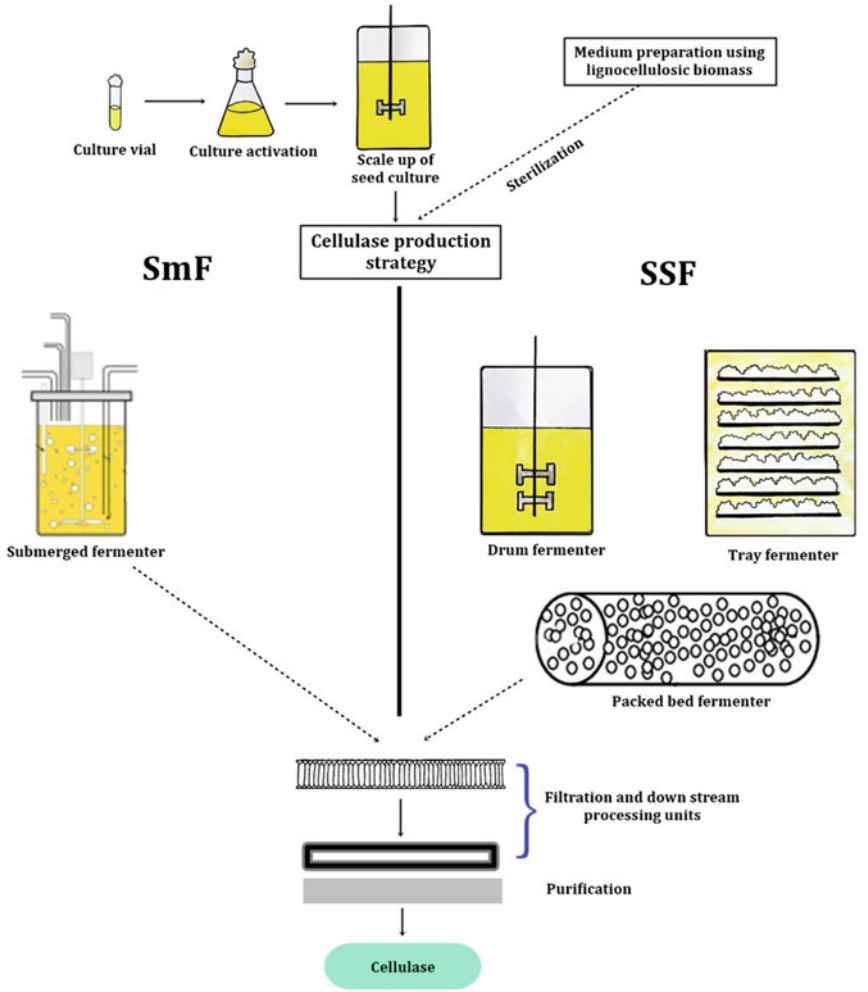

Fig. 4.2 The general steps involved in the production of cellulase formulation

processing. Few designs for SSF-based bioreactors are available in the literature. Stirred tanks (Fig. 4.2), which can be used to reduce labor intensity and where oxygen transfer is controllable, are the most popular bioreactors for SmF. However, if not used for continuous fermentation, stirred tanks are energy-intensive and can be labor-intensive.

4.4.2 Solid-State Fermentation (SSF)

The SSF process is described as fermentation, which contains almost no free water, but still has a moist substrate that is conducive to microbial growth. Therefore, when compared to submerged fermentation, SSF best mimics the natural habitat of most filamentous fungi (SmF). When their potential was compared, it was discovered that SSF offers more opportunities than SmF because it is more eco-friendly owing to lower energy demands, generates less wastewater, and is based on the use of waste solid biomass. Currently, filamentous fungi are grown using the SmF technology to meet the growing demand for cellulases. However, it was asserted that when comparing the same strains, the yield was higher for SSF than for SmF. As a result, attention has been focused on further developing the application of the SSF technology for commercial enzyme production. The type of bioreactor used for SSF is determined by the substrate, process variables, and the level of control required. Several types of bioreactors, including tray reactors, packed bed reactors, and drum reactors, have been developed for both small- and large-scale SSF applications (Fig. 4.2). The advantage of using tray reactors is that the labor intensity is very low if the system is run continuously, and it can be easily scaled to larger operations; however, the temperature control is complicated. The benefits of packed bed reactors include a larger substrate bed and relatively easy product recovery; however, heat transfer becomes challenging with a larger substrate bed. Drum bioreactors such as tray fermenters can operate with constant mixing and intermittent stirring, resulting in a stationary phase. The continuously stirred drum bioreactor has the disadvantages of forming agglomerates and increasing shear stress.

4.4.3 Fermentation Conditions

Fermentation conditions are critical for standardizing process parameters such as incubation time, pH, temperature, inoculum size, and carbon and nitrogen sources. The maximum cellulase production can range from 1 day to several weeks. Fungal cultures typically require longer incubation periods for cellulase production than bacterial cultures. The highest cellulase levels were obtained after 96 h of fermentation with *T. harzianam* and *P. chrysosporium* (Khan et al. 2007). pH was found to have a significant impact on bacterial and fungal cellulase production. *A. niger* showed the highest cellulase activity at pH 4.0, according to Milala et al. (2005). *A. niger* was tested against lignocellulosic biowastes such as sawdust and paper cellulose at various pH levels (4.0–7.0), with the highest activity recorded at pH 5 (Singh et al. 2015). The fermentation temperature has a significant impact on microbial cell growth and metabolic activity. The optimal temperature for cellulase production by *T. reesei* RUT C30 during solid-state fermentation is 33 °C (Mekala et al. 2008). Abd-Elzaher and Fadel (2010) investigated ethanol production from rice straw using cellulase produced by *T. reesei* F-418 cultivated in alkali-treated rice

straw under SSF, and found 162 U/g substrate cellulase activity when the fungus was incubated at 28 °C. Increasing the inoculum size up to maximum limits results in rapid growth and biomass production, resulting in increased cellulase production. Several studies have focused on the use of cellulase in the bioconversion of agro-industrial waste. Chandra et al. (2007) investigated the effects of various carbon sources on *A. niger* cellulase production, including wheat bran, rice bran, groundnut fodder, and sawdust. They discovered the highest cellulolytic enzyme titers in the solid-state fermentation of wheat bran. Similarly, peptone was found to be the best nitrogen source for *P. waksmanii* F10-2, *A. niger*, and *T. reesei* NRRL 11460 (Singh et al. 2019a, 2019b).

4.5 Analysis of the Cellulase Production Cost

The challenges in maintaining the cost of enzyme production are very important because of the application of cellulase in various industries. However, the cost of each step of cellulase production in biorefineries is low and not publicly available. Controlling the economics of cellulase and enzyme reusability is critical. Therefore, a focus on developing on-site cellulase production using low-cost lignocellulosic substrates is recommended. Barta et al. (2010) investigated the processing parameters and economics of softwood-based ethanol plants, focusing on on-site cellulase production using various carbon sources. According to the study, the capital cost (0.42–0.53 SEK/L ethanol) is the main contributor to industrial ethanol production, with enzyme production accounting for 60–78% of the cost. Furthermore, the results suggest that the addition of an enzyme production step may result in a 5–10 L/ton decline in the overall ethanol yield, which became 270 L/dry ton of raw materials when the commercial enzyme was used. Furthermore, pretreatment prior to using biomass for cellulase production is implied to increase cellulase production within a short incubation time. In a study on cellulase production using lignocellulosic biomass, Klein-Marcuschamer et al. (2012) calculated the baseline cellulase manufacturing cost as $10.14/kg. They also suggested a percentage of cost for each component, such as raw materials (28%), facility-dependent (48%), consumables (4%), utilities (10%), labor (7%), transportation and waste treatment (2%). Ferreira et al. (2018) investigated the techno-economic parameters of β-glucosidase production at the industrial level and found that facility-dependent (45%), raw materials (25%), and consumables (25%) were the major costs during large-scale production. Ferreira et al. (2021) investigated the cost of producing lignocellulose-degrading enzymes in *T. reesei* and filamentous fungi using SmF and SSF. The techno-economic parameter analyses suggested that the selection of substrates and capital-related expenditures are typically the major components of the cost of producing enzymes in a number of process designs. Therefore, optimization and the use of cost-efficient raw materials can help keep the cost of enzyme production as low as possible. Here, we provide a rough estimate of cellulase production in a reactor using cheap lignocellulosic biomass to assist new entrepreneurs (Table 4.1).

Table 4.1 Budget proposal for bacterial cellulase production in small-scale operation

Cost item	Requirement	Amount (Rs)
Fixed capital cost estimation		
Total plant cost	Building construction	
	Electrical	
Equipment purchase	Laminar air flow	40,000
	Autoclave	20,000
	Fermenter (200 L)	5,60,000
	Incubator cum orbital shaker	45,000
	Centrifuge	20,000
Total cost		
Recurring cost estimation		
Raw materials	Wheat bran	12/kg
Consumables	Glass wares and plastic wares	10,000[a]
Utilities	Media and chemicals	10,000[a]
Microbial strain	Fungal strain from MTCC	2440/strain
Manpower	Microbiologist	25,000/month
	Labor	10,000/month
Transportation and waste treatment		
Total cost		

[a]Based on rate of utilization

4.6 Applications

4.6.1 Paper and Pulp Industry

Cellulases and hemicellulases have been used in the pulp and paper industry for biomechanical pulping to modify coarse mechanical pulp and hand sheet strength properties, de-inking of recycled fibers, and improving the drainage and runnability of paper mills. Pulp dissolution containing more than 90% pure cellulose was observed in a special commercial pulp with numerous downstream applications, including microfilters, cellulose esters/ethers, viscose rayon, and lyocells (Li et al. 2016). Cellulase treatment was found to be an effective strategy with green and mild behavior that increased the reactivity of fock in the dissolving pulp. The cellulase-based pulping process not only saves energy and is eco-friendly but also improves the structural properties of the final paper product by improving inter-fiber bonding (Chen et al. 2012). The enzyme is used in the production of soft paper, such as paper towels and sanitary paper, and cellulase-containing preparations are used to remove the adhered paper. Moreover, cellulases improve the brightness and quality of recycled paper when combined with hemicellulases.

4.6.2 Textile Industry

The application of cellulase in textiles is critical for the growth of the textile industry. Stone washing is used in the textile industry to provide a worn-out look. However, fabric wear and tear, a large loss of water due to the lengthy washing process, and high labor costs are the main drawbacks of stone washing. Cellulases are used for bio-polishing cotton fabrics and enzyme-based stoning of jeans to give them a stone-washed appearance. By removing short fibers and surface fuzziness and improving color brightness, hydrophilicity, and moisture absorbance, cellulase treatment results in a smooth and shiny appearance (Singhania 2011). Although *H. insolens* cellulase is the most widely used cellulase in biostoning, acidic cellulase from *Trichoderma* combined with proteases has been found to be equally effective. In addition, actinomycetes from the genera *Streptomyces* and *Thermobifida*, as well as bacteria from *Pseudomonas* and *Sphingomonas,* are good sources of enzymes for the decolorization and degradation of textile dyes (Jayasekara and Ratnayake 2019).

4.6.3 Laundry and Detergents

The ability of enzymes to remove stains is the primary reason for their use in detergents. These detergent blends are primarily used for washing cotton and cotton blend fabrics. Because of their ability to modify the structure of cellulose fibrils, cellulases are mixed with detergents to improve brightness and hand feel, as well as to remove dirt from cotton and cotton-blended garments. There has been a lot of research done on cellulases that have been extracted from fungi like *T. reesei*, *T. viride*, *T. longibrachiatum*, *T. harzianum*, *A. niger*, *H. insolens*, and *Bacillus* sp. for use in detergents (Jayasekara and Ratnayake 2019). Washing powder frequently contains cellulase preparations, primarily from *Humicola* which are active at high temperatures and mild alkaline conditions. Celluzyme® and Carezyme® are two major microbial-based brands used in detergent blends.

4.6.4 Food and Feed Industry

Cellulases are now recognized as valuable resources in food biotechnology because of high applicability in a wide range of processes. Cellulases are used in a variety of processes in food biotechnology, including the extraction and clarification of fruit and vegetable juices, production of fruit nectars and purees, extraction of olive oil, and quality improvement of bakery products. “Rapidase pomaliq” is a commercially available enzyme preparation containing cellulase, hemicellulases, and pectinases derived from *T. reesei* and *A. niger* and used in the clarification of fruit juice. Cellulases are used in the extraction of carotenoids for the production of food

coloring agents. In the beer industry, glucanases are added to improve barley malting, whereas in the wine industry, exogenous hemicellulases and glucanases are used to improve maceration and color extraction. *Trichoderma* cellulase is used as a feed additive to improve the feed conversion ratio and digestibility of grain food.

4.6.5 Agriculture Sector

Cellulases are commonly used in agriculture as plant disease control agents and for promoting crop growth. Fungi such as *Trichoderma* sp., *Penicillium* sp., *Chaetomium* sp., and *Geocladium* sp. promote seed germination, root system improvement, rapid plant growth, flowering, and crop yield. The ability of some fungal cellulases to break down the cell wall of plant pathogens has been documented.

4.6.6 Medical Sector

Cellulolytic bacteria such as *Ruminococcus champanellensis* and *Bacteroides cellulosilyticus* can be used to cure phytobezoars disease, which causes the concretion of indigestible vegetable and fruit fibers in the digestive tract and may require surgical treatment. Biofilms are typically formed using pathogenic microorganisms. Cellulases have been discovered and have been used as effective antibiofilm agents against pathogenic biofilms.

4.6.7 Biofuel

The utilization of lignocellulosic biomass for biofuel production is perhaps the most important application currently being actively researched. Cellulase can be used to convert cellulosic materials to glucose and other fermentable sugars, which can then be used as microbial substrates for the production of single-cell proteins or a variety of fermentation products such as ethanol. Cellulase-producing microbes capable of directly converting biomass to alcohol have been reported in the literature. The current strategy for producing bioethanol from lignocellulosic residues requires pretreatment of the residue to remove lignin and hemicellulase fractions, cellulase treatment at 50 °C to hydrolyze the cellulosic residue to produce fermentable sugars, and the use of a fermentative microorganism to produce alcohol from the hydrolyzed cellulosic material (Singhania 2011). The cellulase preparation required for a bioethanol plant is made on-site using the same lignocellulosic biomass as the substrate, and the organism used is almost always *T. ressei*. However, effective strategies are yet to be developed and active research in this area is required.

4.7 Market Trend of Cellulase

In developed nations such as the United States, Japan, Canada, and Western Europe, the demand for industrial enzymes has remained relatively stable in recent years, while it has been rising steadily in developing nations like those in the Asia-Pacific, Africa, Eastern Europe, and the Middle East. Cellulases are the third largest industrial enzymes in the world by dollar volume, owing to their numerous applications in various sectors. Cellulase market was valued at USD 1677.7 million in 2019 and is projected to reach USD 2450.7 million by the end of 2026, growing at a CAGR of 5.5% during 2021–2026, according to Global Cellulase (CAS 9012-54-8) Market Research Report. Commercial cellulases are currently produced worldwide by companies such as Genencor (DuPont, USA), and Novozymes (Denmark). Quest International (USA), Kerry Group (Ireland), Chr. Hansen (Denmark), Advanced Enzymes (India), BASF (Germany), DSM (Netherlands), Zhongbei Bio-Chem Industry Co. Ltd., (China), and Chr. Hansen (Denmark). All of the aforementioned companies played a significant role in reducing cellulase production costs several fold through active research and are still working to reduce costs through the adoption of novel technologies. The National Renewable Energy Laboratory of the United States has established goals for lowering the cost of cellulases used in bioethanol production, with projects beginning in 2000 with contract partners Genencor Corporation and Novozymes. In 2004, Genencor announced that it had achieved an estimated cellulase cost of $0.10–$0.20 per gallon of ethanol using NREL's cost model (Singhania 2011). Genencor recently introduced AccelerateW1500, a cellulase complex designed specifically for lignocellulosic biomass processing industries. It is said to be more cost-effective and efficient than AccelerateW1000 for bioethanol industries. AccelerateW1500 was made from a genetically altered strain of *T. reesei*. CellusoftWAP and CellusoftWCR for bioblasting in textile mills, CarezymeW and Celluclean for laundry in detergent, DenimaxW 6011 for stonewash industry at low temperature, as well as many other cellulases tailored for particular applications, are just a few of the many cellulases offered by Novozyme based on application. Although the majority of enzyme manufacturing companies in the world produce and sell cellulases for a variety of applications, very few create cellulases for the conversion of biomass, with Genencor and Novozyme being the two most successful examples.

4.8 Conclusion

The current development in cellulase applications is remarkable and has gained attention worldwide. It already has an unbeatable hold in the global market. Because of their huge potential for producing cellulases, microbes are a fascinating subject of study for the production of these enzymes. The most abundant biomass on the planet and has the potential to produce biofuels and other useful chemicals. Microbial

cellulases derived from waste feedstock as substrates are an efficient method that can satisfy high industrial requirements. However, because the cost of enzyme production is critical to its intended industrial application, studies have suggested that on-site co-production of multiple enzymes with an emphasis on waste feedstock as a substrate and controlling process parameters are feasible methods for managing the industry's overall cost.

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Chapter 5
Mass Multiplication, Production Cost Analysis and Marketing of Pectinase

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Abstract An enzyme with numerous industrial and commercial uses is pectinase. The widespread use of pectinase may be highly restricted, nevertheless, by greater production costs. The use of microbial pectinase in several industries has increased the demand for it globally. Microorganisms, particularly bacteria, fungi, and yeast, are the principal suppliers of pectinase. In order to produce pectinase, it has been preferred to use inexpensive agro-industrial wastes as substrates. By adopting a distinct substrate and maximising the components of the fermentation medium and process conditions, researchers are attempting to lower the cost of pectinase production for eventual application in industrial processes. Temperature, pH, and production durations, which are the primary determinants in pectinase production, were among the parameters that could not be optimised for pectinase production. Due to its many benefits, the pectinase enzyme is receiving attention; however, more research is required to fully utilise this enzyme in a variety of industries. The structure of pectin, the substrate for pectinase production, factors that affect pectinase production, the industrial use of microbial pectinase, as well as obstacles and potential future applications of using microbial pectinase in industry, are all covered in this paper. This review discusses the ideas regarding its sources, production strategies, and alternative sources of substrate for pectinase production.

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5.1 Introduction

Pectinase is a heterogeneous group of enzymes that break down pectic materials. Pectinases were utilised by the home winemaker two centuries ago to produce wine. Pectinases were initially employed commercially in 1930 to clarify fruit juice, and they were afterwards used in the production of wine and fruit juice (Tapre and Jain 2014; Bhardwaj et al. 2017). Other enzymes were discovered after 1960, the chemical nature of plant tissues became clear, and researchers began to use more enzymes effectively. Pectinases are the most important enzymes in the commercial industry for maximising the yield of stable and clarified fruit juices (Tapre and Jain 2014). Pectinase applications are growing all the time, and pectinases account for 25% of the global enzyme market (Amin et al. 2017). Yeast, bacteria, fungi, and plants are some of the natural sources of pectinase. Enzymatic catalysts are replacing chemical catalysts because they are more specific, less aggressive, environmentally benign, and save energy (Garg et al. 2016; Amin et al. 2019). As the demand for pectin and pectinase grows, this review seeks to briefly explain on the production strategies, and alternate substrate sources used for pectinase production, so that researchers can design different strategies for optimum pectinase production and lower the cost.

5.2 Pectinases

Pectinases are complex enzymes that breakdown pectic compounds and are used as biocatalysts (Khan et al. 2013; Oumer 2017). Pectinases were previously solely identified as a virulence factor involved in the degradation of plant cell walls. On the other hand, they are now considered essential enzymes due to their numerous applications in a variety of industries, including food, textiles, and biofuels, and account for over 25% of total enzyme market sales (Oumer 2017; Shrestha et al. 2020). Pectinases are expected to become a major biocatalyst in the near future due to their versatility, broad substrate specificity, inducibility, stability, and ability to act on a wide range of pectic compounds (Martín et al. 2019).

5.3 Classification of Pectinases

Pectinases are a group of enzymes that catalyse the degradation of pectic substances, either by depolymerisation (hydrolases and lyases) or de-esterification (esterases) reactions. Based on the action of pectinase on a substrate, the pectinase enzymes can

be classified into three groups: protopectinase, pectinesterase, and depolymerase (hydrolase and transeliminase) (Garg et al. 2016; Oumer 2017; Patidar et al. 2018).

5.3.1 Protopectinase

Pectinase of this type is found in unripe fruits and transforms insoluble protopectin to a soluble form of pectin. As a result, protopectinase is thought to be the first enzyme involved in pectin breakdown. Protopectinase is another name for pectinosinase (Tapre and Jain 2014; Patidar et al. 2018).

5.3.2 Pectin Esterase (PE, De-esterase)

Pectin methyl hydrolase, pectin methylesterase, and pectase are all names for this pectinase. PE is a carboxylic acid esterase that catalyses the de-esterification of the galacturonan backbone of pectin's methyl ester bond, resulting in pectic acid and methanol (Garg et al. 2016). According to reports, the resultant pectin is subsequently acted on by PG and lyases after PE action. PEs can be found in the fruits, leaves, flowers, stems, and roots of higher plants. They are thought to change the pH balance from acidic to alkaline (Nighojkar et al. 2019).

5.3.3 Depolymerising Enzyme

This class constitutes hydrolysing enzymes and cleaving enzymes.

(a) *Hydrolysing enzymes* (hydrolases) include polygalacturonase (PG) and polymethylgalacturonase (PMG). Both PG and PMG hydrolyse the pectin chain by acting on α 1→4 glycosidic bond in the presence of water molecule and produce polygalacturonate and polymethylgalacturonate, respectively (Garg et al. 2016).
(b) *Pectin transaminase* (transeliminase) includes polygalacturonatelyase (PGL) and polymethylgalacturonatelyase (PMGL), which cleave α (1→4) glycosidic linkage by transelimination reaction producing unsaturated galacturonates. The breakage of the pectin chain is nonhydrolytic; the action is by eliminating H from C-5 and producing D4:5 unsaturated products (Tapre and Jain 2014).

Based on the position of pectinase action on the pectin chain, depolymerising enzymes, including hydrolases and transeliminases, can be exo or endo. Pectinase is known as endopectinase when it acts randomly in a long chain of pectin, and exopectinase when it functions at the reducing end of the chain terminally. Depending on the pH range in which pectinases work, they are classed as alkaline

or acidic pectinases. Most fungi create acidic pectinase, which can function at an acidic pH, whereas most bacteria produce alkaline pectinase, which can function at an alkaline pH (Nighojkar et al. 2019). Pectinases are classed as psychrophilic, thermophilic, or mesophilic based on how they react to temperature. The majority of pectinases used in industry are mesophilic, meaning they work in a temperature range of 20–50 °C. Pectinases that demonstrate activity at 37 °C, on the other hand, are not suitable for companies that use low- or high-temperature processes. Furthermore, extremophiles may be the finest options (John et al. 2020). Because of the growing demand for pectinase in many industries, there is a need to develop hyperactive pectinase that can withstand a wide range of pH and is thermostable.

5.4 Pectin

Pectin is a naturally occurring biopolymer that is a major component of the cell walls of all higher plants. The only polysaccharide responsible for cell cohesion is found in the middle lamella. It is a non-toxic anionic heteropolysaccharide that makes up 0.5–4.0% of the plant's fresh weight (Picot-Allain et al. 2020). Pectin supplies the rigidity and structure of the cell in plants and fruits. During growth, ripening, and storage, the type of pectin used affects the texture of vegetables and fruits. The pectin in fruits is affected by enzymatic and chemical modifications. Pectinase is responsible for the solubilisation of pectin and softening of fruits during ripening. Pectin's structure is complicated, and its exact composition is unknown. The "smooth and hairy area" model and the "RG I backbone" model are the two most common pectic models. D-galacturonic acid is the primary component of pectin polysaccharides, which is connected by 1-4-glycosidic linkages. Other sugar units, such as ribose, galactose, arabinose, and sucrose, are also introduced into the polymers, and the cell wall contains around 70% galacturonic acid (Picot-Allain et al. 2020; Shrestha et al. 2020).

5.5 Sources of Pectinases

Pectinases can be found in a variety of places across nature. Plants, bacteria, fungi, yeasts, insects, nematodes, and protozoa are all sources of pectinases. The microbial source, on the other hand, is crucial because of its rapid growth, vast distribution, shorter fermentation time, and easier access to genetic alterations. Plant pathogenicity, symbiosis, and breakdown of plant deposits are all aided by microbial pectinases (Amin et al. 2019). In addition, unlike plant and animal sources, the synthesis of microbial pectinases has no political or societal implications. According to reports, 35% of pectinases come from bacteria, 55% from fungus and yeast, and just 5 per cent come from plants or animals. Many investigations are being conducted on

pectinases of high quality, such as their excellent resilience to various physicochemical circumstances derived from microorganisms of various origins.

5.5.1 *Bacteria*

For the manufacture of pectinases, many microorganisms are excellent sources. Bacteria are easy to grow in a variety of laboratory conditions, such as temperature and pH, have a short lifespan, are genetically manipulable, and are ecologically friendly (Amin et al. 2019; John et al. 2020). Pectinases derived from bacteria are normally neutral or slightly alkaline. Pectinases produced by *Bacillus tequilensis* were stable at pH 10 and had higher pectinase activity, and pectin lyase from *Paenibacillus xylanolyticus* was stable at pH 9. *Bacillus* sp., *Pseudomonas* sp., and actinomycetes are the most common producers of alkaline pectinase (Kavuthodi and Sebastian 2018).

5.5.2 *Fungus*

Fungi are the most prevalent source of acidic pectinases with *Aspergillus* sp. being the most important fungal strain for pectinase production. Because it produces non-toxic and commercially useful metabolites, this strain is extremely essential. Furthermore, *Aspergillus* sp. is classified as a microorganism that is usually considered to be safe (John et al. 2020). Fungus like *Aspergillus*, *Candida*, *Geotricum*, and *Rhizopus* have a longer optimal time for producing pectinases than bacteria.

5.6 Insect

Pectinases for commercial purpose are usually made from microbial sources. Insects, on the other hand, can be a source of pectinases. *Sphenophorus levis*, the sugarcane weevil, has pectin methylesterase and endo-polygalacturonase. Sugarcane weevil pectinase has been expressed in *Pichia pastoris*, which could be exploited as a source of commercial pectinases in the future (Habrylo et al. 2018). *Bacillus* sp. that produces polygalacturonase was isolated from the intestines of an alkaline and slightly thermostable *Apis mellifera* (honey bee) (Paudel et al. 2015).

5.7 Strategies for Mass Cultivation to Enhance the Production

Submerged and solid-state fermentation can both yield pectinases (SSF). The growing of microorganisms in liquid broth is known as submerged fermentation. It uses a lot of water, requires constant agitation, and produces a lot of waste. Solid-state fermentation involves microbial growth and product creation on or inside particles of a solid substrate under aerobic circumstances, in the absence or near absence of free water, and does not usually necessitate aseptic conditions for enzyme production.

5.8 Fermentation Strategies

Pectinase is traditionally produced using two fermentation techniques: solid-state fermentation (SSF) and submerged-state fermentation (SmF). Different factors such as growth medium, cultivation conditions, pH, temperature, aeration, moisture, salts, carbon supply, nitrogen source, inoculum volume, inoculum age, type of strain, and inducers are taken into account when using microorganisms to improve pectinase synthesis (Amin et al. 2017). The procedures, on the other hand, are arduous and time-consuming. The majority of enzyme manufacturing firms employ SmF to generate enzymes since it is a simple method that can be scaled up quickly (Oumer and Abate 2018). There is homogeneous nutrition mixing, greater heat and mass transmission, and better microbe diffusion during SmF. As a result, SmF is commonly employed in the commercial manufacturing of pectinase.

SSF is replacing SmF due to the high cost of medium components, low productivity, high energy consumption, necessity for an antifoaming agent to decrease foam occurrence, and high effluent output (Patidar et al. 2018). Different agricultural wastes, such as wheat bran, orange bagasse, sugarcane bagasse, and banana peel, are evaluated using SSF to find the most cost-effective and acceptable source of carbon for pectinase production. In SSF, only a small amount of water is supplied to a solid substrate so that microbes, primarily fungi, can develop as they would in their natural environment and produce the most pectinase. Microbes also obtain enough nutrients from the added substrate, thus there is no need for additional nutrients in SSF (John et al. 2020).

5.9 Immobilisation Strategies

Another useful strategy for increasing enzyme production and improving enzyme characteristics is immobilisation of enzyme or microorganisms onto a solid carrier. Adsorption, covalent bonding, and entrapment are all strategies used to immobilise enzymes and entire cells. For enzyme immobilisation, a variety of inorganic supports

(bentonite, silica, ceramics, polyacrylamide, glass beads, etc.), organic supports (chitosan, proteins, alginate, agar–agar, etc.), and organic–inorganic hybrids (magnetic nanoparticles, silica–dialdehyde starch, etc.) have been used (Sánchez et al. 2015). Different types of immobilisation carriers must have specific features such as biocompatibility, low toxicity, biodegradability, and customised surface chemistry. The various immobilisation-supporting ingredients must be stable, inexpensive, nontoxic, and nonreactive in the reaction solution. The choice of immobilisation method and supporting material is critical. When an enzyme is in free form and has short time stability, immobilisation makes it easier to recover it. The advantage of using immobilised enzymes or cells includes proper attachment or confined in a specific region for their catalytic activities, can be exploited repeatedly or continuously, can be easily separated from the media, enhanced stability or tolerance of enzymes to temperature and pH, minimise enzyme loss, enhance enzymatic properties, and decrease product contamination (Martín et al. 2019).

Dextran polyaldehyde was used as a cross-linking agent to immobilise the pectinase enzyme onto the surface of magnetic nanoparticles, which was then used to clarify apple juice. The immobilised pectinase outperformed the free enzyme in terms of thermal stability, residual activity (87%) after seven cycles of recyclability, and fast turbidity reduction (up to 74%) (Sojitra et al. 2017). Pullulan made of polyaldehyde could be a viable alternative for immobilising pectinase on glass beads (Hosseini et al. 2020). Pectinase encapsulated in magnetic chitosan particles was used to clarify juices in a similar way (Magro et al. 2019). Polygalacturonase was immobilised in calcium alginate, agar–agar, and polyacrylamide gels. Low-cost agrowaste, such as corn cob, was also used as an economical matrix support for yeast (*Geotrichum candidum*) immobilisation, resulting in higher pectinase output as compared to free yeast cells (Ejaz et al. 2018).

5.10 Genetic Modification Strategies

Another technique/method for upgrading bacterial strains used in the commercial development of fermentation processes to promote bioproduct production is microbial strain development or improvement. Classical methodologies, biochemical engineering, and molecular genetics are all used in this procedure. Different procedures, such as mutagenesis and molecular processes, are used to produce or improve bacteria strains. These processes primarily change the microbial DNA (Heerd et al. 2014). To boost pectinase synthesis, microbial recombinant DNA technology tools such as gene cloning and metabolic engineering have been successfully employed in cloning and expressing the gene of interest for relevant enzyme production efficiently and effectively (Amin et al. 2019). Using *Penicillium griseoroseum* under SMF, recombinant technology resulted in a significant increase in polygalacturonase and pectin lyase production of 14 and 400 folds, respectively (Gonçalves et al. 2012). Gene shufing can also help bacteria boost their pectinase activity; for example, in *Bacillus subtilis*, two rounds of genomic shufing increased

pectinase activity by 1.6-fold (Yu et al. 2019). Strain development has economic benefits as well, as it lowers production costs while not increasing capital investment. Recombinant technology has allowed scientists to successfully transfer genes of interest to different organisms and express the desired enzyme or protein.

5.11 Microbial Pectinase for Large-Scale Production

Pectinases are found naturally in plant fruits and play a part in the ripening process; however, microbial sources are used for large-scale manufacturing because they are easy to multiply and maintain under controlled circumstances. Pectinases are produced using a variety of fungal, bacterial, and yeast strains. Negatively charged or methyl-esterified galacturonic acid is abundant in pectic compounds. The degree of esterification and placement of esterified residues along the pectin molecule varies depending on the plant life cycle and between species. As a result, the ability of some phytopathogenic bacteria to produce a variety of pectinolytic enzymes with different features, mostly in substrate specificity, might provide them with greater efficacy in cell wall pectin breakdown and, as a result, greater plant infection success. Several polygalacturonases can be produced from a single gene or many genes. Caprari et al. (1993) discovered that the pathogenic fungus *Fusarium moniliforme* produces four endo-polygalacturonases that varied in the level of glycosylation of the same polypeptide derived from a single gene. The polygalacturonases of *Aspergillus niger*, on the other hand, are encoded by a set of divergent genes. The substrate specificity, kinetic characteristics, and optimal pH of the PG isozymes differ significantly. Different fermentation conditions were used to separate the polygalacturonases isozymes generated by the phytopathogenic fungus *Botrytis cinerea* (Cruickshank and Wade 1980).

Submerged fermentation is a well-developed technique for producing a wide range of microbial metabolites on an industrial scale. Technically, SmF is easier than SSF, and it has been heavily developed since the 1940s due to the need to generate antibiotics on a big scale (Sunnotel and Nigam 2002). SSF, on the other hand, is more productive due to increased enzyme yields (Hölker and Lenz 2005). SSF processes mimic the life circumstances of many filamentous fungus at a higher level. Ascomycetes, basidiomycetes, and deuteromycetes evolved on wet substrates in terrestrial settings. Higher fungus, as well as their enzymes, spores, and metabolites, are adapted to growing on solid wet substrates. Fungal spores produced by SSF, for example, have higher stability, are more resistant to drying, and have higher germination rates after freeze-drying for longer periods of time than spores produced by SmF. Despite its benefits, SSF's industrial use is difficult to envision, at least for the time being. The inability of the process to be regulated, the highly heterogeneous fermentation conditions and the resulting frequently unsatisfactory reproducibility of the results, difficult scale-up, often impractical biomass determination, and complicated product purification by downstream processes due to the use of heterogeneous organic growth substrates are the main obstacles (Sunnotel and Nigam 2002).

The pectinase-producing genera Aspergillus and Penicillium are the most common fungal strains. *Aspergillus fumigatus*, *Aspergillus kawachii*, *Aspergillus niger,* and *Aspergillus giganteus* have all been identified as pectinase-producing Aspergilli. *Penicillium occitanis* and *Penicillium notatum* are potent pectinases that produce Penicilli. *Aureobasidium pullulans*, *Thermoascusindicaeseudaticae*, and *T. aurantiacus* are other fungi that potentially yield significant pectinases.

Bacterial strains that produce pectinase include *Chryseobacterium indologenes*, *B. subtilis*, *Pectobacterium carotovorum*, and *Sclerotium rolfsii*. Microbially generated pectinases have limited activity and are less resistant to acidic, alkaline, and high-temperature environments. Because of their greater activity and capacity to tolerate a wide range of pH and temperature, recombinant pectinases are created from a variety of microbiological sources. Among the few potential recombinant pectinase-producing organisms are *Bacillus clausii*, *A. aculeatus*, *Paenibacillius* sp., *Bacillus halodurans*, *Bacillus licheniformis*, and *Bispora* sp.

5.12 Cost Analysis Factors in Pectinase Production

Pectinases market value has risen in recent years, and it now ranks first among commercially utilised enzymes in the sectors, with a projected market value of $41.4 billion in the future (Garg et al. 2016). Because enzymes are widely employed in a variety of fields, lowering their production costs has become a major issue that must be addressed. The majority of fruit dump yard wastes cause dangerous pollution, and their disposal in less developed countries need an environmentally appropriate strategy. Citrus waste residues collect as undesirable trash that is regarded as worthless, but after decomposition by pectinolytic actinomycetes, by-products like cellulose, fibres, and pectin are produced, which can be used in a variety of industrial processes. Many companies discharge agro-industrial residues as municipal solid wastes, which can be used as a substrate for pectinase synthesis (Marzo et al. 2019). These wastes contain high levels of carbon, nitrogen, and other minerals, and they can be used as a substrate for pectinase production (Mehmood et al. 2019). Approximately 34 million tonnes of citrus peels and seeds were thrown in food processing businesses, causing major environmental problems. The cost of the medium, according to the industrial point of view, is primarily responsible for the cost of enzyme synthesis, and it must be minimised.

By doing trials on flask experiments, the Taguchi technique has been used to forecast the important contribution of variables that are designed as well as the optimum settings of each variable (John et al. 2019). Valorisation of industrial wastes and statistical analysis of their use could be a positive way to lowering enzyme production costs. Response surface methodology is primarily used for optimising the components of media required for microbial-derived pectinase, but it has also been applied to biochemical and chemical reactions such as enzyme yield (Barbosa et al. 2010), media optimisation (Kunamneni and Singh 2005), and enzyme hydrolysis constraints (Ozer et al. 2004).

5.12.1 Usage of Alternative Substrates

Pectin derived from various plant sources has a wide range of uses. To gain economically and reduce agricultural waste disposal, it is prudent to employ agricultural wastes as alternative sources for pectinase manufacturing. Agro-waste is a renewable, low-cost, and natural resource that can be used to produce pectinases at a low cost and with minimal environmental impact. Natural resource usage will aid in the resolution of energy shortages, pollution concerns, and waste disposal challenges while posing no threat to the food supply chain (Govindaraji and Vuppu 2020; Shrestha et al. 2020). Agricultural wastes are extremely nutritious, allowing and promoting the growth of a variety of bacteria. Pectinase has been made from a variety of carbon sources, including apple pomace, sunflower heads, orange peel, barley grain, and grape pomace (John et al. 2020). However, there are lots of agro-waste that have the potential to be used as the source of pectin for pectinase production.

5.12.2 Agro-industrial Waste

Wheat bran is a common agro-industrial waste that accounts for 15–20% of total agro-industrial waste and is discarded during the wheat flour production process. Wheat bran could be utilised to make industrially essential enzymes. Under SSF at 50 °C and pH 4, wheat bran and tea extract together create a 15.28-fold rise in polygalacturonase with a specific activity of 33.47 U/ml (Anand et al. 2017). In another work, polygalacturonase of 535.4 U/g substrates was generated when *Aspergillusojae* was grown on wheat bran media at 37 °C for 4 days under SSF (Demir et al. 2014). Similarly, *Bacillus subtilis* produced the most pectinase (1272.4 25.5 U/gds) when grown in SSF with an initial pH of 6.5 and at 37 °C. For pectinase and pectin lyase production, Kaur and Gupta screened 25 different agro wastes, including orange peel, coconut fibre, paddy straw, mustard straw, mustard oil cake, rice bran, and lemon peel. At pH 7 and 35 °C, the greatest pectinase (450.50 12.8 U/gds) was found in an orange peel-containing medium. However, after 4 days of incubation, a 4:1 mixture of orange peel and coconut fibre established itself as the dominant substrate, yielding 3315 U/gds of pectinase with a moisture content of 60% at 35 °C and pH 4 (Kaur and Gupta 2017).

Tobacco stalks, which are also considered waste, contain a lot of pectin, and *Bacillus tequilensis* showed pectinase activity of 1370 U/ml in ideal fermentation conditions of 40 h, pH 7, and 3% inoculum (Zhang et al. 2019). *Aspergillus awamori* was used to produce pectinase from *Ficus religiosa* leaves in SSF at 30 °C, 60% v/w moisture content, and pH 5, the maximal pectinase was detected in 72 h (Dasari 2020).

5.13 Fruit and Vegetable Waste

When pectinase activity was measured in peels from fruits such as orange, mango, pomegranate, and mosambi, the orange peel had the highest pectinase activity of 98.65 U/ml at 35 °C, pH 6, and 48 h of incubation (Govindaraji and Vuppu 2020). Under SSF, the use of mango peel for pectinase synthesis by *Aspergillus foetidus* resulted in the greatest polygalacturonase and pectin lyase levels. At 40 °C and 150 min of incubation, the pectinase produced in this technique gave the highest mango juice clarification (92.5%). Lignocellulosic enzymes, including pectinase, were produced using grape skin and olive pomace. *Aspergillus niger* and *Aspergillus fumigatus*, two local species of Aspergillus, were cultivated in solid media including grape skin and olive pomace, with wheat added in some cases (Sánchez et al. 2015). To manufacture pectinase from *Aspergillus niger*, orange peel was introduced to Czapeck media as a carbon source under SmF. They also demonstrated the highest pectinase output (117.13.4 M/mL/min) after 4 days of incubation at 30 °C and pH 5.5. Under SSF, orange peel and coconut fibre in a 4:1 ratio were also employed to make pectinase. Maximum pectinase (3315 U/gds) and pectin lyase (10.5 U/gds) were measured in this environment at pH 4, 60% moisture content, and 4 and 8 days of incubation, respectively (Kaur and Gupta 2017). Papaya is a popular tropical fruit with medicinal and nutritional properties, as well as pectin. As a result, papaya peel was employed to produce pectin methylesterase (246.83 U/gds) using *Aspergillus tubingensis*.

5.14 Algal Biomass

Algal biomass has also been used to make pectinases as a natural resource. Because the middle lamella of the algal cell wall contains a substantial amount of pectin, it is suitable for this use. *Penium margaritaceum*, a charophyte green algae, has been examined in the manufacture of pectinase (Domozych et al. 2014). Pectinase was also produced using brown or green algae such as *Dictyopteris polypodioides*, *Sargassum wightii*, *Dictyopeteris divaricate*, *Ulval actuca*, and *Codium tomentosum*. Furthermore, algal biomass can be employed as a substrate for the manufacture of additional hydrolytic enzymes. Under SmF (2457 3.31 U/mg) compared to SSF (1432 1.46 U/mg), green algal biomass from *U. lactuca* was shown to be the best suitable substrate for *Bacillus licheniformis* in generating pectinases over rice husk and sugarcane bagasse.

5.15 Application and Market Demand

Pectinases have been employed in a variety of applications, including plant fibre processing, tea and coffee fermentation, industrial wastewater treatment, and so on. Pectinases are employed in the food processing industry for the clarification of juices, as colour and yield enhancers, and in the treatment of fruit mash. Pectinases are also utilised in degumming/retting fibre crops, as enzyme cocktails for animal feed production, filtering plant viruses, oil extraction, and textile desizing agents. Because of its unique qualities, pectin is used in the food and cosmetic sectors as a gelling, stabilising, and emulsifying ingredient. Pectin also provides a number of health benefits, including decreasing blood cholesterol and serum glucose levels, preventing cancer cell proliferation, and activating the immune system (Lara-Espinoza et al. 2018). Pectin is also utilised to make a variety of unique goods, including as edible and biodegradable films, adhesives, paper alternatives, foams and plasticisers, surface modifiers for medical devices, and biomedical implantation materials. Pectin is also employed as a carrier material in colon-specific medication delivery systems (Lara-Espinoza et al. 2018), which successfully removes harmful substances such as lead, cadmium, arsenic, and mercury from the gastrointestinal tract and respiratory organs (Lara-Espinoza et al. 2018). Pectin has also been employed as a pectin hydrogel in the manufacture of controlled-release matrix tablets for water purification (Thakur et al. 2019).

5.16 Conclusion

Microbial pectinases are rapidly expanding enzymes with numerous uses in a variety of fields, highlighting the need for a green and cost-effective technique for optimum pectinase production. The stability of the enzyme in a wide range of industrial environmental conditions, as well as the cost-effectiveness of the procedure, are the most important considerations. The most important aspect that gives a microbial strain an additional advantage is the stability of enzymes over a wide range of temperatures and pH. Hence new microorganisms with high pectinase activity that are stable over a wider temperature and pH range for a longer period of time, as well as their cost-effective production, must be highlighted to enhance the quality of the product.

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Chapter 6
Production, Cost Analysis, and Marketing of Citric Acid

Bharathi S and Radhakrishnan M

Abstract Citric acid is the most widely used organic acid in the world, with applications in the food, pharmaceutical, and beverage sectors. This chapter discusses the microbial synthesis of citric acid, methods of fermentation, commercial strain, fermentation media including carbon source, nitrogen source, phosphorus source, trace elements, and fermentation conditions including pH, temperature, aeration, recovery, extraction, cost analysis, and marketing of citric acid.

Keywords Citric acid · Fermentation · Commercial strain · Cost analysis and marketing of citric acid

6.1 Introduction

Citric acid (2-hydroxypropane-1,2,3-tricarboxylic acid) gets its name from the Latin word citrus, which refers to a tree bearing lemon-like fruit. Citric acid is composed of three carboxylic functional groups (Gargul et al. 2019) with pKa values of 3.1, 4.7, and 6.4, respectively. The molecular weight of this tricarboxylic acid is 210.14 g/mol (Max et al. 2010). In plants and animals, citric acid is found to be present in trace quantities since it is the primary metabolite obtained from the tricarboxylic cycle (or Krebs cycle) (Tong et al. 2019) (Fig. 6.1).

Karls Scheel from England was the first person to isolate citric acid from Italian lemon juice in 1874. In 1893, Wehmer first demonstrated the accumulation of citric acid from Citromyces (now Penicillium) in the presence of sugars and inorganic salts. Currie, an American, pioneered the industrial manufacture of citric acid from *Aspergillus niger* in 1917.

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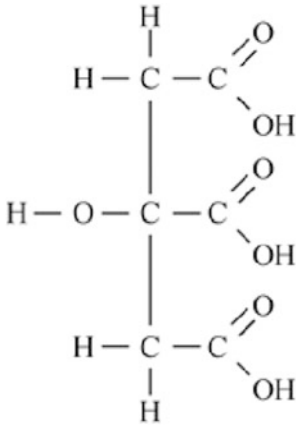


Fig. 6.1 Structure of citric acid

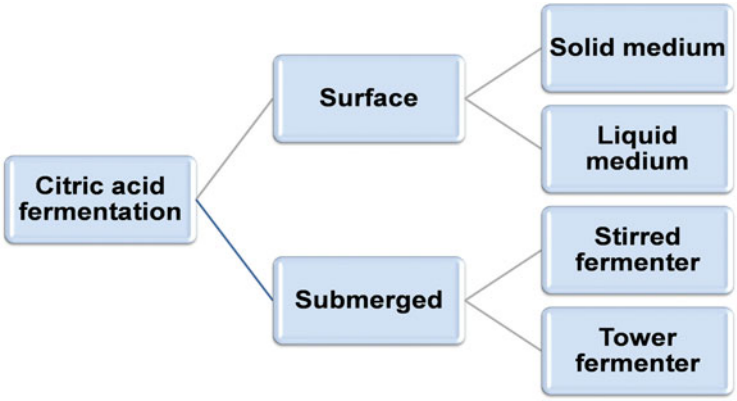
6.2 Uses

Citric acid has many industrial applications, as flavoring agent, acid for vegetable oils and fats, stabilizer and preservative in the food and pharmaceutical industries. It is also used as an antioxidant and emulsifier in cheese and cream, to prevent oxidation and generate a deeper color in sweets, jams, jellies, and dairy goods. In beverage industries, it is used to inhibit turbidity of wine, invert sucrose, eliminate haze due to trace metals, and prevent color and flavor deterioration. In cosmetic products, it is used as an antioxidant. In addition, it is used for, metal plating, detergents, tanning, and textile processes (Swain et al. 2012).

6.3 Commercial Strain

Citric acid is produced by a wide range of microorganisms, including bacteria, fungi, and yeasts. Some of the citric acid-producing bacteria include *Klebsiella* sp., *Brevibacterium* sp., *Bacillus* sp., *Corynebacterium* sp., and *Pseudomonas* sp. (Anastassiadis et al. 2008). The molds reported to produce citric acid include *Penicillium*, *Eupenicillium*, *Absidia* sp., *Botrytis*, *Acremonium*, and some *Aspergillus* sp., such as *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus luchensis*, *Aspergillus nidulans*, and *Aspergillus awamori* (Papagianni 2007). The yeast that produces citric acid is *Saccharomycopsis* sp., *Candida* sp., and *Yarrowia* sp. (Mattey 1999). Among these, only *A. niger* and *Saccharomycopsis* sp. are utilized for large-scale production of citric acid. The fungus *A. niger* has remained the organism of choice for commercial production. The following are the key benefits of utilizing *A. niger*: (a) convenience in handling, (b) ability to utilize a variety of low-cost basic materials, and (c) increased product (Wayman and Mattey 2003).

6.4 Fermentation



6.5 Submerged Fermentation

Submerged fermentation is found to be responsible for around 80% of global citric acid production. Higher yields and cheaper labor costs are two advantages of submerged fermentation for citric acid manufacturing (Roukas 1991). The kind of material utilized to construct the fermenter, as well as the aeration, are two elements that are very important to be considered for citric acid synthesis using submerged cultures. Fermenters have to be acid-resistant and made of stainless steel because ordinary steel dissolves at pH 1–2 and prevents fermentation (Show et al. 2015). Although *A. niger* has a low oxygen requirement, it is quite sensitive to its complete absence. Aeration rates of 0.2–1 vvm are commonly utilized during the manufacturing phase (Max et al. 2010). Due to the low viscosity of cultures, no mechanical agitation is necessary, and in some cases, airlift reactors are employed (Papagianni and Mattey 2004). The major issue with submerged fermentation is the development of foam that can be overcome with the use of animal and vegetable fats or by mechanical methods (Max et al. 2010) (Table 6.1).

6.6 Surface Fermentation

The liquid surface culture was launched in 1919 by Products Organiques in Belgium. In 1923, Chas Pfizer & Co. in the United States developed as the first individual technique for citric acid synthesis (Vandenberghe et al. 1999). The surface approach requires less time and effort to operate and install, as well as lower energy costs

Table 6.1 Raw materials used in submerged fermentation for citric acid production

S. No.	Raw materials	Strain	References
1.	Coconut oil	*C. lipolytica* N-5704	Ikeno et al. (1975)
2.	Cane molasses	*A. niger* T 55	Kundu et al. (1984)
3.	Wood hemicellulose	*A. niger* IMI- 41874	Maddox et al. (1985)
4.	Brewery wastes	*A. niger* ATTC 9142	Roukas and Kotzekidou (1986)
5.	Beet molasses	*Yarrow lipolytica* A101	Kautola et al. (1992)
6.	Corn starch	*A. niger* IM-155	Nguyen et al. (1992)
7.	Starch hydrolysate	*Y. lipolytica* A-101	Wojtatowicz et al. (1993)
8.	Soybean oil	*Y. lipolytica* A-101	Wojtatowicz et al. (1993)
9.	Rapeseed oil	*Y. lipolytica* A-101	Wojtatowicz et al. (1993)
10.	Date syrup	*A. niger* ATTC 9142	Roukas and Kotzekidou (1997)

(Grewal and Kalra 1995). Surface techniques are separated into solids and liquids depending on the substrate used, with the latter having a larger economic value.

6.7 Liquid Surface Fermentation

The early efforts at citric acid fermentation exploited surface liquid nutrients, which still account for 20% of global output (Kubicek 2001). Surface liquid cultures are commonly done in batches, with fermentation broths made from wheat bran, potato starch, glucose syrup, or molasses at 160 g/l concentrations (Max et al. 2010). The presence of trace metal ions and dissolved oxygen does not influence surface fermentation. Surface liquid fermentation has a myriad of benefits, including minimal initial investment, low cooling energy, and a very simple process. The disadvantage is their maintenance cost is higher due to the labor needed to clean reactor walls, pipes, and trays (Kubicek 2001). The sterilized fermentation media is added to aluminum trays with a depth of 3–200 cm, and is inoculated with a spore suspension of 2–5 $\times \cdot 10^7$ dry spores/m^2 and is then incubated at 28 °C. The pH of the medium dropped from 6.0–6.5 to 1.5–2.0 after 24 h of inoculation (Marzona 1996). Sterile air is passed through nutrient solution to maintain the temperature, avoid contamination, and remove CO_2. The excess iron in the medium enhances oxalic acid production and yellow pigment that affect the recovery of citric acid. The cycle lasts 7–15 days, with daily output ranging from 1.2 to 1.5 kg citric acid monohydrate/m^2, equivalent to a 70–80% yield (Marzona 1996).

6.8 Solid Surface Fermentation

Solid-state fermentation for citric acid production known to be the Koji process has been used to produce citric acid from agro-industrial substances (Pandey 1991). Citric acid production by SSF was first developed in Japan. Apple pomace, orange waste, grape pomace, sugar cane, pineapple waste, beet molasses, orange waste, and cassava bagasse are some of the raw materials that can be used in SSF. Depending on the substrate absorption capability, the substrate is soaked to around 70% moisture. The optimal pH is adjusted around 4.5–6.0 and the incubation temperature is in the range of 28–30 °C. *A. niger* is often used on the other hand yeast has been reported (Tisnadjaja et al. 1996). With liquid surface fermentation trace elements have no impact on production. SSF has employed a variety of fermenters for citric acid fermentation, including conical flasks, glass incubators, and trays, among others (Vandenberghe et al. 1999).

6.9 Fermentation Media

6.9.1 Carbon Source

The choice of polysaccharides depends on the hydrolytic enzyme possessed by the production strain. The enzyme must also be resistant and active at low pH (Hossain et al. 1984). Sucrose has been the primary choice over glucose for *A. niger* since it possesses invertase bound to mycelium that is effective at acidic pH (Angumeenal and Venkappayya 2013). Glucose from starch hydrolysis, beet molasses, and low-grade sugarcane byproducts are the most often utilized carbon sources in industrial fermentations. Citric acid accumulation decreases when starch, pentoses (xyloses and arabinoses), sorbitol, pyruvic acid, and galactose used as carbon source. Low production with reduced growth has been evidenced with other carbon sources such as ethanol, mannitol, cellulose, sorbitol, malic, lactic, and α-acetoglutaric acid (Yokoya 1992). The maximal output for citric acid is influenced by the starting sugar concentration in batch fermentation and the feeding rate of glucose in continuous fermentation (Xie and West 2009). The optimal sugar concentration was found to be 14–22%. The increased carbon source may reduce the activity of α-ketoglutarate dehydrogenase resulting in low yield (Hossain et al. 1984), while the low glucose level results in the reduction of the size of mycelium thereby the yield is affected (Papagianni and Mattey 2004).

6.9.2 Nitrogen Source

Molasses is a nitrogen-rich complex medium that seldom has to be supplemented with a nitrogen source. As a nitrogen source, urea, ammonium sulfate, ammonium chloride, peptone, and malt extract have all been successful. Consumption of nitrogen sources lowers pH, which is a critical factor in citric acid production (Rohr et al. 1983; Kubicek and Rohr 1986). Citric acid fermentation demands a nitrogen input in the concentration of 0.1–0.4 N/l. An increased nitrogen concentration stimulates fungal growth and sugar consumption, but it reduces citric acid production (Hang et al. 1977).

6.9.3 Phosphorus Source

The most acceptable phosphorous source has been identified as potassium dihydrogen phosphate. For an optimal citric acid generation, the fungus required phosphorus at a concentration of 0.5–5.0 g/L in a chemically defined medium (Shu and Johnson 1948). Citric acid production is favored when phosphate levels are low; conversely, when phosphate levels are high, citric acid synthesis is suppressed and fungal growth is accelerated (Vandenberghe et al. 1999).

6.10 Trace Elements

Citric acid formation by *A. niger* has been observed to be influenced by many divalent metals, including zinc, manganese, iron, copper, and magnesium. Citric acid synthesis necessitates the presence of additional trace components (Karaffa et al. 2021). The addition of iron reduced the buildup of citric acid, which influenced on mycelial development. Magnesium is essential both for the growth and the generation of citric acid. The optimal magnesium sulfate concentration was determined to be between 0.02 and 0.025% (Kapoor et al. 1983). The ideal Zn and Fe values are 0.3 and 1.3 ppm, respectively (Shu and Johnson 1948).

6.11 pH

All fermentations begin with spores, which require a pH of more than 5 to germinate. The intake of ammonia by developing spores leads to the release of protons, which lowers the pH and improves citric acid synthesis. The manufacturing phase's low pH (pH 2) decreases the danger of invasion by other microbes and prevents the

development of undesirable organic acids (gluconic and oxalic acids), making product recovery easier (Max et al. 2010).

6.12 Aeration

It is well established that citric acid formation mainly depends on the rate of aeration and the deficit in aeration will have a detrimental effect on production. The partial pressure of dissolved CO_2 in the fermentation tank would be too minimal if the aeration rate increased. The supply of oxaloacetate for citrate synthase is dependent on carbon dioxide. High levels of CO_2 in the gas, on the other hand, are harmful to the ultimate concentrations of citrate and biomass. Aeration is carried out continuously during the fermentation at a rate of 0.5 to 1.5 vvm at a uniform intensity (McIntyre and McNeil 1997).

6.13 Temperature

The incubation temperature for citric acid production using *A. niger* is in the range of 26–28 °C.

6.14 Recovery and Extraction

The recovery of citric acid from the fermentation broth is performed by the following steps:

1. The fermentation liquor is drained off to separate mycelium.
2. The slightly soluble tri-calcium citrate tetrahydrate is then precipitated by adding calcium oxide hydrate (milk of lime) to the fermentation liquid.
3. Filtration is used to extract the precipitated tri-calcium citrate, which is then rinsed multiple times with water.
4. It is then processed with sulfuric acid to produce calcium sulfate, which is subsequently filtered off, releasing citric acid.
5. Active carbon is used to treat citric acid-containing liquor before it is transported through cation and anion exchangers.
6. Finally, at 20-25°C, the liquid is concentrated in vacuum crystallizers, yielding citric acid monohydrate. Anhydrous citric acid is produced through crystallization at temperatures greater than this (Max et al. 2010).

6.15 Cost Analysis of Citric Acid Production

The cost of the finished product is largely determined by the substrate used. Citric acid is made from a variety of substrates, including carob pod extract, n-paraffin, black strap molasses, corn starch, hydrolysate starch, glycerol, yam bean starch, wood hemicellulose, olive oil, beet molasses, rapeseed oil, palm oil, cane molasses, and soya bean oil (Show et al. 2015). Pretreatment of substrate for citric acid fermentation is essential to remove the trace metals (Kristiansen et al. 1999). Hence the amount of citric acid yield is directionally proportional to the purity of the substrate (Lesniak 1999).

In search of substrate for cheap production of citric acid to reduce production cost, many substrates were exploited. The most commonly used low-value substrate molasses have higher sugar content (40–55%) (Dronawat et al. 1995). It was also found that adding phytate (an essential plant element) to beet molasses at the onset of the incubation process causes a 3-fold increase in citric acid buildup (Soccol et al. 2006). Ramesh and Kalaiselvam (2011) used seaweed *Gelidiella acerosa* as an alternative sugar substrate. It was reported that seaweed powder along with 10% sucrose produced 50g of citric acid with the cost of Rs. 35 as compared to control media (Sucrose) with the cost of Rs. 77 and 80 g of citric acid yield. Another cheap substrate palmyra jaggery from palmyra palm utilized for citric acid production (Ambati and Ayyanna 2001).

The higher yield of citric acid can also be achieved by strain improvement. Strain improvement had been practiced over years and it was well evidenced that penicillin production increased 500-fold upon strain improvement of *Penicillium chrysogenum* (Adrio and Demain 2006). Although there are numerous research conducted on strain improvement for citric acid production very few reports have been published. This is because production companies want to keep their successful methods. X-rays and UV rays were used by Gardner et al. (1956) for the increased production of citric acid and found the mutant strain of *A. niger* showed six-fold increased citric acid as compared to the wild strain.

In earlier days many researchers widely used protoplast fusion to produce intra-specific recombinants of *Aspergillus niger* for industrial production of citric acid (Kirimura et al. 1986, 1987, 1988a, 1988b; Martinkova et al. 1990). Chemical mutagenesis of *Aspergillus niger* was carried out using ethyl methane sulfonate (EMS) and ethidium bromide (EB), with ethidium bromide-treated *A. niger* EB-3 being the effective mutant for increased citric acid synthesis. The hyper-producing mutant EB-3 (treated with 1 mg/mL ethidium bromide for 120 min) yielded the maximum citric acid output (64.2 mg/mL) after 72 h (Javed et al. 2010). Xue et al. (2021) found that over-expression of high-affinity glucose transporter (HGT1) leads to increase production of citric acid by the industrial strain *A. niger* CGMCC 10142.

6.16 Marketing of Citric Acid

The increasing application of citric acid in the field of food and beverages, pharmaceuticals, detergents, and personal care products such as acidulants, preservatives, buffering agents, and antioxidants impose great demand. Currently, citric acid-enriched foods including diet beverages, snacks, low calories confectioneries, and ice cream grab attention in the market (Swain et al. 2012). In addition, there has been raising awareness of the use of natural and organic food additives that foster the market growth of citric acid. Due to the non-toxic nature of citric acid, it is generally recognized as safe (GRAS) by the Joint FAO/WHO Expert Committee on Food Additives (Kirimura et al. 2011). With a compounded annual growth rate (CAGR) of 4.5%, the global market for citric acid is expected to reach US$3.9 billion by 2024. Western Europe, which constitutes the largest regional market for citric acid, contributes around 34.6% of the total. The United States, China, the Middle East, Africa, Central/Eastern Europe, Brazil, and India follow Western Europe (Source: Research and Markets, 2019). In India Anil Bioplus Ltd., Bharat Starch Inds. Ltd., Citurgia Biochemicals Ltd., Diviya Chemicals Ltd., Parry & Co. Ltd., Parry Enterprises India Ltd., and Vantech Industry Ltd. are few industrial manufacturers of citric acid.

6.17 Conclusion

The rapidly growing applications of citric acid spur great demand for citric acid in the market. Even though citric acid production using *Aspergillus niger* supplies citric acid in satisfactory amounts, there is still a need for promising strain, and novel fermentation techniques using cheap raw materials for greater yield. Hence research has to focus on the exploration of microbes for efficient and eco-friendly production of citric acid.

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Conflict of Interest Authors have no conflict of interest.

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Chapter 7
Production, Cost Analysis and Marketing of Lactic Acid

Rajeswari Somasundaram, Vimala Guttha, and A. Sankaranarayanan

Abstract Lactic acid is an important organic acid having numerous applications in diversified areas like food, pharmaceutical, cosmetic and chemical industries, hence it is considered as an important industrial product. There are various processes involved in the production of lactic acid including chemical and biological processes. It has received the attention of industrialists, by playing a prominent role in the production of poly (lactic acid) (PLA), a bio-based, biodegradable product. The production cost of lactic acid depends on the sugar to lactic acid conversion rates, price of raw substrate, plant size and annual operation hours. This chapter provides an overview of the various microorganisms, diversified raw materials (substrates), challenges in downstream processing, cost analysis, safety and issues involved in the production of lactic acid and listed out the commercial uses of the product. This chapter may give an overall outline for the entrepreneurs involved in the production of this product.

Keywords Lactic acid · Substrates · Microbes · Downstream processing · Entrepreneurship · Commercial applications

7.1 Introduction

Microorganisms are exceptionally effective and adaptable complex assembling apparatuses, which can be utilized to synthesize usable products to boost the economy of a country. So, microorganisms can be exploited to make many helpful items/products on large scale. As of now, microorganisms are utilized commercially to flavour substances, nutrients, plastics and much more. Bio-transformations of economically important substances at an industrial scale are mainly dependent on the community requirement and usage of efficient microorganisms. The fundamental attributes for a developing community are population expansion, adequate food

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supply, renewable energy sources and steady employments. Moreover, consumption is the beginning and end of all economic activities. Microorganism-based industries could support with tending to these difficulties both ecologically and financially.

From ancient times, humanity has utilized microbial in fermenting bread dough, wine, cheese and pickles making. In recent years microbial science has turned into a formal logical discipline, through the characterization of microbes, understating the physiological properties, advancement in the facilities, industrial development, and systematization.

In this context lactic acid is one of the important products produced by many microorganisms. Lactic acid is a naturally occurring organic acid in living organisms as it is an intermediate in anaerobic metabolic (Bouguettoucha 2008). It is found in fermented products such as bread, beer, cheese, pickled vegetables and pickled meat. The IUPAC name of lactic acid is 2-hydroxypropanoic acid with the molecular formula $C_3H_6O_3$ ($CH_3COHCOOH$) (IUPAC 2021). Karl Wilhelm Scheele (1742–1786), a Swedish German pharmaceutical chemist, first isolated an impure lactic acid from sour milk in 1780 (Kompanje et al. 2007). In the year 1857, Pasteur noticed lactic acid as a fermentation metabolite produced by microbes. A French scientist Frémy successfully fermented lactic acid by a microbial process (Abdel-Rahman et al. 2013). It made a revolution in the industrial production of lactic acid by various microbes especially bacteria and fungi.

The worldwide demand for the polylactic acid market is having a projected estimated value of USD 1.756 billion to 2.1 billion by 2025, recording a compound annual growth rate (CAGR) of 12.8% and 17.4% (Lorenzo and Androsch 2018). Due to its wide usage and versatility, the market value of lactic acid may be reached US$ 9.5 billion in the year 2027 (Oliveira et al. 2022). The consumers' knowledge about the green environment and awareness for biodegradable plastic/biocompatible substances is the main reasons for the market. The well-established market for food and beverage industries in North America requires a large quantity of lactic acid as an additive. The developing market in Asia also requires biodegradable eco-friendly materials to wrap the food products.

Heat-resistant one-time use coffee cups, transparent cups for water and beverages, curd and yoghurt packaging cup, ice cream cup, bottle, sauce cup, takeaway food boxes, straw, cutlery items such as spoon, knife and fork are some of the examples for PLA products. The market for lactic acid is endless and high demanding (Oliveira et al. 2022).

The European Association for Bioplastics in partnership with the Nova Institute in Germany estimated that the worldwide bioplastics manufacturing capability will grow up to 2.62 million tons by 2023. The total requirement for plastic is 335 million tons in that bioplastics represent nearly 1% of the produced. Hence packing materials utilize the major share of biopolymers. So, PLA (polylactic acid) is expected to be one of the major reasons which drive the development of biological-based and biodegradable plastics manufacturing units. The significant reason for the establishment of the lactic acid-based industries and the estimated utilization hence the market is

- The rise in demand for advanced biopolymers
- End-user applications
- Innovative products

7.2 The Fermentation Processes

According to Endres, 2009 bacterial fermentation of carbohydrates is the major process of the industrial production of lactic acid. Which contributes not less than 70%. Even it reaches up to 90%. Lactic acid is a simple carboxy acid with asymmetric carbon. Hence quantitatively and qualitatively the purity of freshly synthesized lactic acid can be assessed by its optical properties such as optical rotation (α) and specific rotation ($[\alpha]_{\lambda}^{\theta}$). Unlike chemical synthesis, microbial fermentation yields optically pure L (+)-lactic acid. Hence it possesses better polymerization, high crystalline and high melting point. L (+) isomer of lactic acid is present in mammals. It is observed that few bacterial species could synthesize both D (–) and L (+) enantiomers. The homolactic acid can be synthesized from fermenting various carbohydrate-rich raw materials such as whey, corn or potato starch, wheat straw, wheat or bran flour, corn cob, alfalfa fifer, grape marc, pineapples syrup, date juice, molasses, corrugated grains, camel or cow milk, yucca, etc.

Lactic acid is synthesized by two major processes or pathways. They are homolactic fermentation and heterolactic fermentation. During homolactic fermentation processes, one molecule of glucose is getting converted into two molecules of lactic acid and in heterolactic fermentation gluconeogenesis, one molecule of glucose yields at least three different compounds that are carbon-di-oxide, ethanol, and lactic acid one molecule of each. During the anaerobic condition, lactate is formed from pyruvate by the enzyme lactate dehydrogenase. According to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database, lactic acid is produced by at least twelve pathways in biological systems (Fig. 7.1).

7.3 Microorganisms Used for the Production of Lactic Acid

Food and Drug Administration (FDA) or European Food Safety Authority (EFSA) must grade the organisms as safe to use in food processing industries. The strain of the microbes should be periodically reviewed on their genetic makeup, metabolic and fermentation capability. The organisms used to produce lactic acid belongs to the gram-positive group and the genus *Aerococcus*, *Alloiococcus*, *Atropobium*, *Bacillus*, *Bifidobacterium*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Kluyveromyces marxianus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vegococcus*, and *Weissella* (Table 7.1).

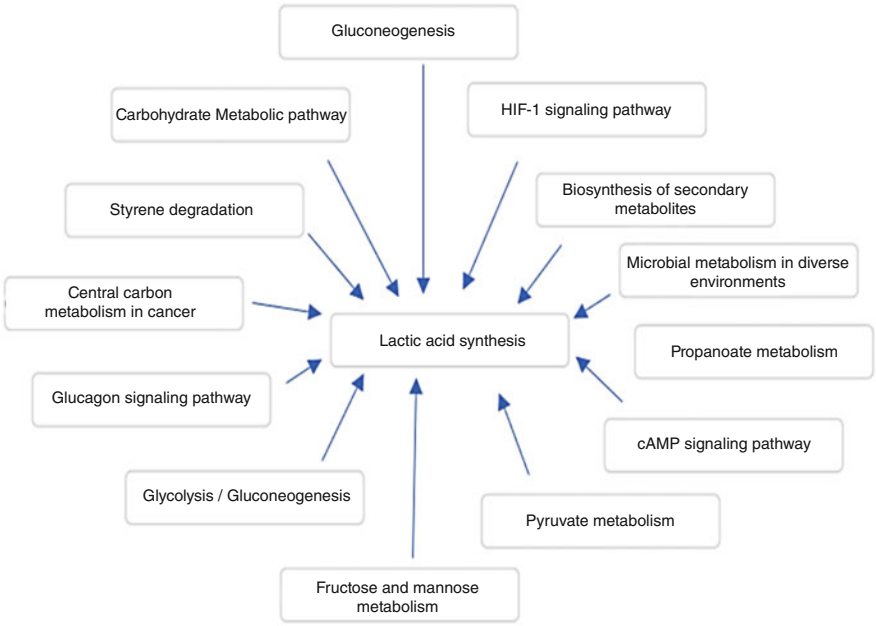

Fig. 7.1 The metabolic pathway for the synthesis of lactic acid in the biological system is based on the KEGG database

Among the bacterial genera *Lactococcus*, *Enterococcus*, and *Streptococcus* few *Lactobacillus* species are homolactic fermenters under non-limiting conditions on hexose metabolism followed by the Embden–Meyerhof pathway (EMP). At low glycolytic flux, this process may result in the production of other organic compounds such as ethanol, acetic acid, and formic acid (Abedi and Hashemi 2020).

Lactobacillus is the most common bacteria used for industrial fermentation as it produces purely one enantiomer. It is a non-spore-forming rod when fermented with a suitable substrate which is often very complex. The carbohydrates substratum should be fortified with minerals, vitamin B complex, amino acids, and nitrogen bases. Whereas the filamentous fungi can tolerate low pH and can be even active.

Apart from those microorganisms listed (Table 7.1), *Aerococcus*, *Enterococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Saccharomyces cerevisiae*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* are also considered to produce lactic acid (Liu et al. 2014).

Table 7.1 The common microorganisms and the raw materials used for the production of lactic acid and detail about its consolidated bioprocessing

Organism	Lactic acid	Raw material	Consolidated bioprocessing	References
Bacillus coagulans GKN316 *Lactobacillus plantarum* SKL-22	45.39 g/L	Corn stover hydrolysate	–	Jiang et al. (2016)
	34.26 g/L	Lignocellulosic by-products	–	Yadav et al. (2020)
Trichoderma reesei	34.7 g/L	Lignocellulosic biomass	Hexoses and pentoses aerobic process	Shahab et al. (2018)
Lactobacillus delbruckii NCIMB 8130	90 g/L	Molasses	–	Kotzamanidis et al. (2002)
Lactobacillus plantarum	27.3 g/L	Alkali pre-treated corn stover and sorghum stalks	Sequential saccharification and simultaneous saccharification and co-fermentation	Zhang et al. (2016)
	22 g/L		No feedback inhibition on higher lactic acid yield	
Lactobacillus pentosus, *Lactobacillus brevis* and *Lactococcus lactis*	60 g/kg	Xylose	Phosphoketolase pathway	Mathew et al. (2018)
Escherichia coli AC-521	49 g/kg	Glycerol	Aerobic condition	Huang et al. (2005)
Rhizopus oryzae NRRL 395	2.3 g/kg	Pineapple waste	–	Abedi and Hashemi (2020)
Rhizopus oryzae MTCC5384	27 g/L	Wastepaper sludge	Saccharification and fermentation	Dhandapani et al. (2021)
	75 g/L	50:50 blended cellulase cocktail and paper sludge		
Bacillus coagulans d-DSM1	48.65 g/L	Non-sterile media	Thermophile (grows at 52 °C)	Zhang et al. (2017)
Lactobacillus paracasei H4-11		Rice-acid ferment	–	Liu et al. (2021)
Pseudomonas putida KT2440 and *Bacillus coagulans* NL01	35.8 g/L	High-toxic lignocellulosic hydrolysate	–	Zou et al. (2021)
Rhizopus oryzae 36017 *Rhizopus arrhizus* 2062	85–92 g/kg	Potato starch wastewater	Saccharification and fermentation	Huang et al. (2005)

7.4 Raw Material Used

Lactic acid fermenting microbes require complex nutrition, this complicates the industrial processes and enhances the cost. Hence identification of the low-cost raw materials which give high production may help to decrease the overall cost. The use of paper industry sledge and recycling paper is an economic and ecological way to produce lactic acid. Lignocellulosic materials availability is limited availability due to seasonal harvest, dispersed distribution, and transportation costs.

The raw materials used in lactic acid production are immense. It includes the food industry waste and agro-industrial materials such as straw of crops (barley, rice, millets, etc.), fruit peels, fruit press filter waste, sugar mill waste, household wet waste, yard waste, oil cakes (palm, coconut, canola, sunflower, groundnut, etc.), wastepaper sledge, soy molasses, whey powder, and potato starch (Table 7.2).

Direct purchase of raw materials from the formers or industries is the best way to reduce production costs. But the availability of the materials depends on the growing season of the crop and the harvest. During the fall season harvest of crops is common. So, it is possible to get raw material in abundance. But the continuous supply of raw material is unplausible. Buying it from the vendors or market has the advantage of getting the raw materials to supply constantly. But the traders will fix the price based on the demand and supply. Now, it is possible to procure the raw materials used for lactic acid production through online purchase.

Localization of industry is mainly related to territorial, adequate, and availability of trained labour. It is also based on climatic condition, nearness to raw material and market and availability of finance. The production may be of small and large scale. From an economic point of view, the large-scale industry is advantageous as it may

Table 7.2 The common raw materials used for lactic acid production

Raw material	Cost (Rs/kg)
General agriculture waste	3
Wheat straw	1.14
Soybean agro-waste	2.80
Rice husk	4.50
Wheat sticks waste	2
Chickpea waste	6
Rice bran	2.80
Papermill waste	22
Orange peel	35–90
Banana peel powder	75–350
Corn cob	6
Groundnut shell	2–3
Tamarind husk	5
Cashew husk	20–25

- Use the machine efficiently to its maximum potential
- Decrease in cost management and low-cost production
- More production of the product

Small-scale industries also have their advantages. They are

- Need small capital to start
- Close supervision
- Easy management
- Direct relationship with the consumer and the producer may know the demand

7.5 Downstream Process

7.5.1 Clarification

During the fermentation process as time increases, the microorganism's population density increases inside the bioreactors. After the completion of the fermentation process, extraction of the product and decontamination of the fermenter is important for subsequent fermentation. In this initial step, the fermenter stock is clarified to eliminate the spent cells (biomass) and other suspended solids from the fermenter substance to improve the quality of the product.

Clarification employs mostly filtration techniques. Feed and bleed mode is mostly used in which ceramic membranes are chosen to withstand higher chemical concentration and temperature. The flow rate can be regulated by having a pump. But the cost of the product will increase as it is an energy-requiring process. The product cost also depends on cleaning of the membrane, water and detergent used for the cleaning procedures and the number of times the membrane to be replaced when damaged.

7.5.2 Purification and Concentration

The recovery of lactic acid should be worked on to minimize lactic acid degradation and purity (Din et al. 2021). Purification is a significant stage of lactic acid fermentation. Fermentation stock usually contains various contaminations like remaining sugars, salts, supplements, and other natural acids. These impurities must be removed from the broth altogether to make a commercial product (Komesu et al. 2017). Purification of lactic acid is often needed as it may be contaminated with other inorganic salts. The most common salts include Na^+, K^+, Ca^{2+}, Mg^{2+}, PO_4^{2-}, CO_3^{2-}, and Cl^-. Among them, Na^+ concentration will be much higher. Nanofiltration, ion exchange, and osmosis are some of the technologies that were explored to purify the lactic acid from the fermentation broth.

The concentration of fermented products is usually very low in the broth (the maximum concentration is up to 90 g/L). Lactic acid concentration is done by

reverse osmosis (RO) and evaporation. During evaporation of lactic acid concentration by polymerization and degradation. To preserve the structure and function intact the process must be done below atmospheric pressure, so that broth evaporates at a lower temperature than its boiling point at atmospheric pressure.

7.5.3 Packaging

Packaging is also a relatively expensive process in industrial organic acid production. Packaging is an impartment process to keep the quality unaltered during transport and storage. Packaging safeguards the industrial products which minimize the environmental impact during sales and delivery. Properly packaged products attract consumers to buy the product. There are several aseptic packaging processes available now. That includes vacuum packaging, thermoforming carton systems, and wrapping. Quality packaging prolongs the shelf life of lactic acid and facilitated its marketing to a distant area. Packaging material should be acid-resistant and non-corrosive. The sealing should be leakage-proof, oxygen-tight, aroma-tight and odour-tight. The shape stability of the material, resistance to moisture and shock are some of the mechanical properties of the packaging material to be considered along with the published research insisted on the efficiency in industrial packaging, minimize the environmental impact on packaging materials used for industrial products (Silva and Palsson 2022).

7.5.4 Downtime Management

Downtime in the production unit is defined as the time when an industry is not manufacturing the product. Any halts in manufacture affect the profit/income of the manufacturing unit. If the downtime is prolonged a company can go in loss. Downtime (nonproduction time such as cleaning, sterilization, long lag phase and preparation of another batch cultivation) in batch cultivation always yields low productivity during the subsequent cultivations (Srivastava and Gupta 2011). Industries require to be conscious of area hazards and the different types of outages that disturbs production which can be predicted to prevent any concerns. Proper training is vital for the work teams to get a maximum outcome. The employee and the labours should be allotted precisely for supervision, restoration, and handling different tasks. The downtime critical event index deals with which instrument/process dominates downtime in an industry over a point of time.

7.6 Cost Analysis

Any sort of industry the investment is divided into fixed cost and operating cost. For more details refer Table 7.3. Production in the fermentation industries addresses the essential constraint regarding the total expenditure to manufacture the product. Total yield and total expenditure are inversely proportional; as yield increases the cost comes down exponentially. In industrial fermentation, the product yield indicates the quantitative measure that is the output of the process per unit volume of the reactor. In general, the fluctuation in the profit percentage depends on variability on the raw material value in large-scale industry. But it is of less concern in small-scale industries and also low-yielding plant. The annual plant output of the fermentation industries depends on the fermentation method employed, batch operation cost, capacity of the plant and fixed costs, together with handling charges (utility, labour, maintenance, depreciation, and miscellaneous expenditure). Therefore, the expenses

Table 7.3 Investment, operating, and production costs for lactic acid production

Particular	Approximate cost (Rs/5 ton)
1. Fixed cost (a) Land cost (subjected to) (b) Construction charges (may vary based on size and area)	1,000,000–2,000,000 1,000,000–1,400,000
(c) Equipment Compressor Pre-heater Reactor Cooler Mixing tank Storage tank Filters Distillation column Gas separator Electrodes Packaging unit	5,500,000
2. Operating cost	4,100,000 (up to 75% of the capital cost depending on the process)
(a) Raw material (b) Utilities (c) Operating labour (skilled person and an electrician minimum 3 numbers) (d) Down steam process (purification and concentration)	205,000 (5% of the operating cost) 41,000 (1% of the operating cost) 50,000–75,000/month (24% of the operating cost) 1,025,000 (5% of the operating cost)
Maintenance Laboratory Supervision Miscellaneous Helpers - cleaner, security Shipping, packaging, and transport Insurance	410,000/year (10% of operating cost) 205,000/year (5% of operating cost) 40,000 (5% Maintenance cost) 20,500 (6% of operating cost) 410,000 (10% of operating cost) 1,000,000/year (5% of profit)

Table 7.4 Cost analysis for lactic acid production per year in small-scale industry

Particular	Cost (Rs)
Fixed cost	Up to 8,900,000
Operating and maintenance cost	4,401,000
Production volume	2880–4320 g/L
Depreciation (15%)/year	1,035,000
100 ml of 10% lactic acid cost	Approx. 1500
Net profit	43,200,000
Gross profit	28,864,000

and profit in a fermentation industry withstand the fluctuation over a narrow range and to an extent fermenter yield is ultimately stable for a short period of time. The ratio between the fixed cost and operating cost in the small-scale fermentation industry may remain the same even if a new process or the methods were adapted to upgrade so as to have more yield. Establishing a new reactor may increase the yield in an industry which is already producing more products. This may require more raw material and downstream processing of the final product which leads to a change in relative proportion between the fixed cost and maintenance cost. Hence, the large-scale industry is relatively advantageous as it can produce more yield to make more profit in a competitive market. For more details about cost analysis refer Table 7.4.

7.7 Safety and Administrative Issues During Microbial Fermentation

Execution of these innovations relies upon drawing in neighbourhood partners, giving knowledge about the process and their safety to the frontline workers and convincing the public. So that inquiries about the security, hazard and guideline should be strictly addressed and examined. There is another issue that influences the whole biotechnology-based industry, which is to follow the safety guidelines, educate the workers and risk assessment at regular intervals. Like any innovation, the utilization of microorganisms to do industrial-scale brings up issues of security and hazard. The most important issue to be questioned are

- Do these organisms cause any well-being hazards?
- If the microorganisms are GMO, could their deliberate or accidental delivery represent a danger to the local environment and native species?
- In case of any distinguished hazard, what measures are to be taken to restore the structure?

Acknowledgement of these new advances relies upon connecting with neighbourhood partners as right on time as conceivable so that inquiries of well-being, hazard, and guideline can be transparently examined. All preparation programs pointed towards fostering a labour force for the organism-controlled modern

area ought to incorporate investigation of pertinent well-being just as moral, lawful, and social ramifications (ELSI) issues.

7.8 Uses of Lactic Acid

U.S. Food and Drug Administration approved lactic acid as generally regarded as safe (GRAS) as an additive. The commercial market for lactic acid is of great scope as it is one of the important industrial chemicals. The heat-stable lactic acid esters are used as an emulsifier, food preservatives, fermentation agent, acidulant, flavouring, preventing reducing sugar inversion agent, decontaminant, antioxidant, and cryo-protectant in food industries. In the cosmetic industry, lactic acid is added to the product as moisturizing, humectant, skin-whitening, skin rejuvenating, anti-acne, and anti-coating agents because of its non-toxic and biocompatible nature. Textile industry-low cast technical grade lactic acid is used as mordant for dyeing, and neutralizer for several components in dyestuffs: lactic acid acts as descaling and tanning material in the leather industry. pharmaceutically it is an important ingredient as it undergoes biodegradation in dialysis solution, prostheses, surgical sutures, controlled drug delivery system, immune-stimulant and antibacterial agents (Martinez et al. 2013). In chemical industries, it is used as a mosquito repellent, cleaning agent, descaling, pH neutralizers, eco-friendly solvent, and metal complexing agents. The biodegradable polymers, poly-lactic acid is used to produce fibres, films, propylene glycol, lactate esters, dilactate, etc. (Abedi and Hashemi 2020). Lactic acid esters are used as a green solvent in various fields (Ghaffar et al. 2014).

7.9 Conclusion

The contribution of a microbe to humans is immense. Lactic acid is a naturally occurring organic acid in living organisms. It is one of the important industrially produced lactic acids that has great scope in the commercial market. Industrial-scale production of lactic acid has many challenges. It starts with the utilization of appropriate strain for the production to marketing of the product. Both the production and downstream processing of lactic acid must be carried out with precision and care. One kilogram of lactic acid is sold for Rs. 135–150 in the commercial market in India. Wise management of the lactic acid production industry may yield a reasonable profit for the entrepreneur.

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Chapter 8
Production, Cost Analysis, and Marketing of Acetic Acid (Vinegar)

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Abstract Acetic acid (vinegar), a sour liquid, is a traditional double-fermented product produced from various agricultural origins using yeast and acetic acid bacteria. From the ancient era to the modern era, vinegar has been a rich source of vitamin B1, riboflavin, and mineral salts. Vinegar is used for cooking, cleaning, and as a preservative and condiment. The global vinegar market was US$ 1.3 billion in 2018, reached US$ 2.27 billion in 2022, and will reach up to US$2.62 billion by 2027. Italy (279.0 million USD) is the top vinegar producer and exporter in the world, whereas India exported 152.63 USD million in 2021, which indicates the need to look forward with value-added products. However, in India only 1–2% of perishable fruits have been processed before reaching the consumer's table compared to 40% processed in developed countries. In recent years, the demand of gluten-free and organic vinegar has increased. Hence, although the Indian government released a funded scheme for small-scale start-up businesses to attract, self-dependent and money-making entrepreneurs, in this chapter we try to put efforts for entrepreneurs toward the small-scale quality-quantity-based ecological and economic impacts or boost agri-food and food processing sectors along with technical details and the cost-benefit ratio of vinegar (acetic acid) production.

Keywords Vinegar · Acetic acid bacteria · Yeast · Preservative · Condiments · Agri-food business · Hydraulic press

8.1 Introduction

Since ancient times, vinegar has been a part of the human diet and has traditionally been used in medicine (Luzón-Quintana et al. 2021). Vinegar is a sour liquid produced from raw materials of agricultural origin, that contains high amounts of amylaceous (starchy material), carbohydrates, or sugars. Vinegar production is a two-step microbial fermentation process: first step is an anaerobic process in which

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transform the fermentable sugars to ethanol using yeast cells while the second step involves a biotechnological process, i.e., an aerobic process in which ethanol is oxidized into a key ingredient acetic acid by the action of acetic acid bacteria (Bhat et al. 2014). The acetic acid concentration typically ranges from 4-8% for table vinegar and 18% for pickling. Natural vinegar contains small amounts of citric acid, tartaric acid, and other acids and is a rich source of vitamin B1, riboflavin, and essential amino acids; hence vinegar has become a superior food additive (Hailu et al. 2012). Moreover, it holds a dynamic place in our kitchens and is used to make a variety of dishes, from fermenting pickles, adding flavor, dressing/marinade, aromatizing, and seasoning to various foods (Singh 2020). In addition, vinegar has various applications such as cleaning, medicine, cosmetics, agriculture, therapeutics, and flexibility, making it the most valuable preservative agent and food product (Plioni et al. 2021). Various factors, such as changing diet patterns, increasing disposable income levels, and busy schedules, significantly raise the demand for ready-to-eat food products, which leads to a high demand for vinegar as it keeps the item fresh and nutritious, along with a significant role in enhancing the aroma and unique flavors of food over long periods.

Natural vinegar is advantageous over synthetic vinegar in that it is highly nutritive, eco-friendly, and health-effective; however, due to the higher price, unavailability of substrates, and longer fermentation process, its importance has decreased daily (Othaman et al. 2014). Hence, to overcome this disadvantage, it is necessary to adopt some steps to shorten the process of raw material processing, avoid bacterial contamination, and obtain a highly concentrated vinegar yield resulting in high-quality and low-cost vinegar (Othaman et al. 2014).

India has progressed from an agro-deficit to an agro-surplus. According to a recently published report, interesting thing is that vinegar production is based on raw agricultural materials specifically fruits and grains. Therefore, entrepreneurs can directly link up with the nearby food industry or who deal with food agribusiness, leading to more promising results in terms of the use of second-quality fruits without compromising quality.

From this perspective, fermentation, cost, health effectiveness, and value-added products will play a vital role in reducing pollution potential and enhancing farmer income and the local economy. Hence, this model pattern represents cost-effective vinegar production, packaging, and marketing at a small scale to enhance production by encouraging entrepreneurs to adopt this technology as a food-agribusiness or food processing sector.

8.1.1 Vinegar

From pre-historic to current times, many different types of natural vinegar have been produced from a variety of substrates, as they are superior food additives over synthetic vinegar (Kocher et al. 2006). The vinegar classification is usually based on the raw material used for its production. Vinegar differs from each other based on

Table 8.1 Market price of vinegar

Vinegar	Price (INR) and packaging size	Brand	Uses
Natural apple cider vinegar	162/500 ml	–	Industrial
Apple cider vinegar with garlic, lemon, ginger, and honey	400/500 ml	–	Food
Apple cider vinegar juice	170/500 ml	KR Enterprises	Food
Alpino apple cider vinegar	294/500 ml		Food
Gokul herbal apple cider vinegar	195/500 ml	Gokul Herbals	Food, cooking
Natural organic sugarcane vinegar	150/1 L		Food
Natural unfiltered apple cider vinegar	149/500 ml	Pioneer	Food
Pure grape vinegar	120/cartoon	Sunnah Enterprises	Cooking
Natural aloe vera vinegar	70/500 ml	Rishika Herbals	Home
White cooking vinegar	450/20 kg can		Cooking
White wine vinegar	175/1 L	Bianco	Home
Brio white vinegar	30/bottle	Brio	Home
Red cooking vinegar	27/700 ml	Kollur's	Cooking
White blue elephant rice vinegar	275/200 ml	Blue Elephant	Cooking
Rice vinegar	331/740 ml		Cooking
Organic sugarcane vinegar	80/500 gm	Raghukul	Home, food

Mostly the shelf life of vinegar is of 6–24 months
Note: The price written in the above table is taken from online and offline market price

country, consumer demand, organoleptic features, ingredients, and their usage (Hailu et al. 2012; Luzón-Quintana et al. 2021). The predominant vinegar used was white or distilled vinegar. The aroma and color of vinegar mostly depend on the type of material, the aging period, and the production process. In India, vinegar market price depends on the type, flavor, and volume of vinegar produced. The market prices of vinegar are listed in Table 8.1.

8.1.2 Application of Vinegar

In addition to its uses in the food industries, vinegar has other applications.

1. Medicinal
   Vinegar is highly accepted for its unique functional properties such as antioxidant, antimicrobial, and anti-diabetic properties, control of blood sugar levels, weight loss, and cardiovascular diseases, help in reducing cholesterol levels, replacing unhealthy sodium, and combating diabetes (Johnston and Gaas 2006).
2. Cleaning and hygiene
   Used as a disinfectant, stain remover, and dyes.

3. Agricultural
   Soil enrichment, improved seed germination, root growth stimulation, soil and foliar fertilizer, and insect repellent (Mungkunkamchao et al. 2013).
4. Cosmetics and Other
   Used for hair conditioners, facial astringency, antiperspirants, and photographic materials.

8.1.3 Factors Affecting the Production of Vinegar

1. Use of different yeast strains affected the final vinegar in terms of aroma, volatile compounds, and alcohol content.
2. Temperature and pH, especially acetic acid bacteria play a major role in affecting the yield.
3. Production methods directly affect products qualitatively and quantitatively.
4. The presence of volatile compounds and organic acids contributes to organoleptic properties of vinegar because an elegant flavor and strong aroma are due to the presence of acetic acid, which acts as a precursor for the formation of volatile compounds and organic acids produced during the fermentation and aging process (Ho et al. 2017).

Three main methods are employed for vinegar production (Hailu et al. 2012).

8.1.4 Methods

8.1.4.1 The Orleans Method

The Orleans method was developed in France. It is a well-known, slow, oldest, and continuous process (Hailu et al. 2012). Slower production enhances flavor, aroma, and produces high-quality vinegar. The large (200 L) capacity wooden barrels are used for vinegar fermentation by adding approximately 65–70 L of high-grade vinegar along with 15 L of wine (at regular intervals of week) for optimum growth of bacteria, i.e., natural acetic acid bacteria mostly, *Acetobacter*. These bacteria formed a gelatinous slime layer on the surface of the top liquid, i.e., the liquid–air interface of the medium. Organisms were able to produce cellulose in the presence of oxygen and developed cellulose-bacterial cell mats over time by oxidation and produce the acetic acid from ethanol at approximately 29 °C and the fermentation process was continued for 1–3 months at 70–85 °F. Vinegar is replaced by wine by withdrawing it up to 10–15 L at intervals to add more wine. The problem of pouring additional liquid without disturbing the floating bacterial mat has been overcome using a long glass tube that reaches the bottom of the barrel (Hailu et al. 2012; Bhat et al. 2014).

Disadvantage: Time consumption and high cost per volume with less yield of vinegar production (Hailu et al. 2012).

8.1.4.2 The Generator Method

Because the Orleans process is very slow, another method adopted to speed up the process is the generator method, also called the trickle method. In this method, various sizes of generators (5000–6000 L) are used for fermentation, which are made up of steel or wooden only (Bhat et al. 2014). A bottom of the cylindrical tank is packed with the supporting beech wood shavings. Over these shavings, spray the liquids and add alcoholic solution acidified with acetic acid and other nutrients required for bacterial growth. Inoculate *Acetobacter* inocula and blow air from the bottom of the tank to maintain oxygen availability. Bacteria e grow to form a thick slime coating (vinegar mother) on the surface of the beech wood. The mixtures allowed trickling down from the bottom to over the shavings, resulting in more oxidation of the alcohol until the desired strength of vinegar is not obtained. This process is performed at 27–30 °C and hence cooling coils are provided in order to avoid overheating. This process takes 3–7 days (Hailu et al. 2012; Bhat et al. 2014; Singh 2020).

Disadvantages

1. Maximum loss of ethanol.
2. Accumulation of dead bacteria, and high risk of clogging due to presence of slime of cellulose-producing bacteria (Bhat et al. 2014).

8.1.4.3 Submerged Fermentation

The generator method is expensive; hence, a submerged fermentation process was adopted (Bhat et al. 2014). It is a widely used, advanced, more efficient, and faster production method at an industrial scale. Upgraded parameters such as aeration, stirring, and heating make this process more efficient and advanced (Bhat et al. 2014; Singh 2020). The fermenting tank consists of stainless steel also called 'Acetators' and the parts include foam controller, cooling system, air supplier system, loading (inlet), and unloading (outlet) valve. The fermenter is loaded and inoculated with bacteria, and when acetification reaches its desired level (40–50%), the volume should be unloaded and left-behind vinegar could be used for the next cycle. Centrifugal pumps or air supplier systems are attached at the bottom of the tank through which air bubbles are pumped into the tank, stir the alcohol and acetozyme nutrients are piped into the fermenter. These nutrients stimulate microbial growth. The aeration system smashes up the air bubbles, which can prevent the growth of bacteria (Hailu et al. 2012; Singh 2020). The heater attached to the tank maintains a temperature of 26–38 °C. Vinegar production can occur in discontinuous, semi-continuous, or continuous systems; however, semi-continuous method is

preferable. Submerged fermentation provides a significant, efficient, and continuous aeration system responsible for smashing the air bubbles and preventing cell death. The degree of aeration directly affects the vinegar flavor. Due to aeration, vinegar bacteria float in the liquid and there is no chance of slime of bacteria in the fermenter, that's why the final product will be clean (Singh 2020; Bhat et al. 2014).

8.2 Technological Details

Technological development for vinegar production and selling includes four major steps: (1) raw materials, (2) mass production, (3) fermentation production, (4) bottling and packaging, and (5) marketing. However, raw material processing and production processes are very complex and still face some promising challenges to peel and extract juice processes, limited shelf-life, contamination in mass production, and temperature maintenance problems. The main drawbacks are the high price and lack of awareness about the properties of natural vinegar, long fermentation processes, and low process efficiency; generally, product (vinegar) and investment are ignored by consumers and entrepreneurs, respectively.

8.3 Vinegar Production Process

Processing from raw materials to vinegar can be summarized into six steps: (1) raw material preparation, (2) preparation of starter culture, (3) mass multiplication, (4) alcoholic fermentation, (5) acetous fermentation from ethanol to vinegar, and (6) bottling and packaging (Fig. 8.1).

8.3.1 Raw Materials Preparation

Raw materials preparation is a preliminary but crucial step in vinegar production. Different types of vinegar are produced from raw materials, i.e., the classification is based on the raw material used for vinegar production (Bhat et al. 2014). Most probably, any type of citrus fruit, fruit, grain, or fruit peel can be used for vinegar production because of the acetic nature of vinegars as well as the organoleptic properties of the final product. In vinegar production, the preparation of raw material also includes a process that is necessary for the elaboration of the sugar ratio and protein content (Luzón-Quintana et al. 2021). Waste, low-quality fruits, and by-products generated during the cultivation and food-based industries are also used for vinegar production. Moreover, the alcohol concentration achieved during fermentation is directly proportional to the sugar content of fruits; hence, sugar

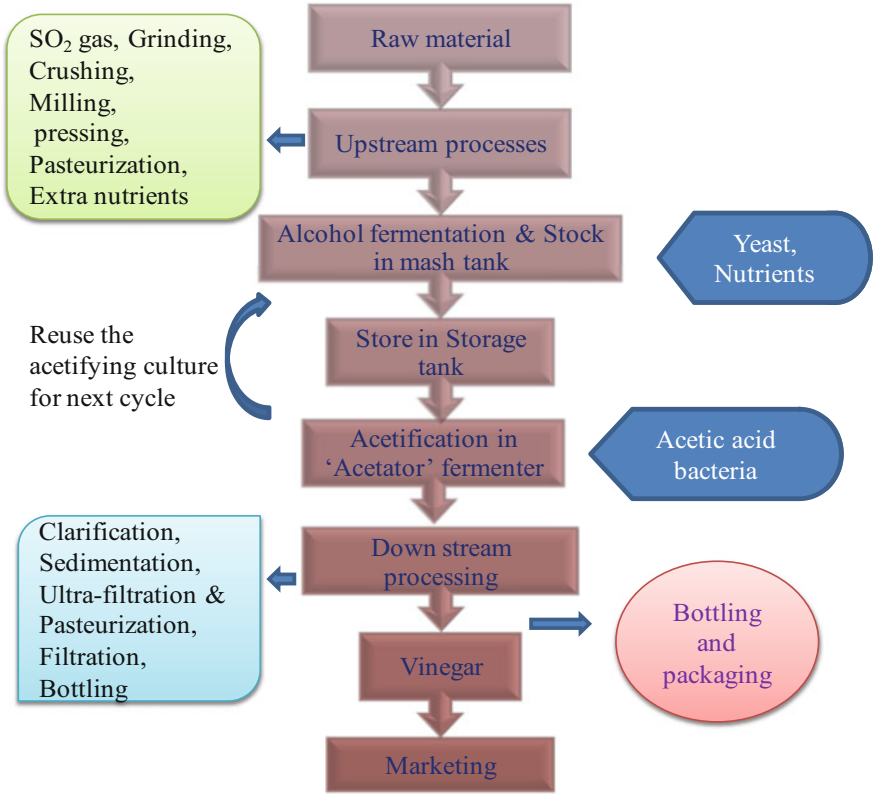

Fig. 8.1 Schematic representation of vinegar production process

content plays a vital role in vinegar and wine production (Luzón-Quintana et al. 2021). The volatile compounds composition of fruits such as alcohols, esters, aldehydes, ketones, terpenoids, and lactones are most important in the vinegar. Many factors affect volatile composition such as environmental conditions, genetic make-up, degree of maturity, post-harvest handling, and storage (Luzón-Quintana et al. 2021). The most common types of vinegar, their uses, and their origins are listed in Table 8.2.

To obtain the juice and fermentable sugar to be acetified, the primary step is the preparation of raw materials. There are two main methods for the preparation of raw materials: (1) juice extraction and (2) pulp extraction. Water requirements are necessary for washing and processing raw materials (costs are described in project detail). Juice can be extracted by upstream processing such as maceration, grinding, crushing, milling**,** and pressing of fruits. The use of an enzyme is another procedure to obtain juice. Normally, the sugar composition is not the same in all raw materials, especially in fruits such as pineapple. Hence, pineapple can be used in its full form, i.e., peel and core, because they are rich in insoluble fiber; therefore, a saccharification procedure should be performed. For enzymatic digestion, cellulolytic,

Table 8.2 Different types of vinegar based on their raw materials

Types of vinegar	Raw materials	Uses	Demand/origin
Balsamic vinegar	White Trebbiano grape + aged in barrels	Cooking	Italy
Cane vinegar	Sugarcane	Pickling, flavoring	Philippine
White vinegar	From distilled alcohol	Cooking	Asia
Cier vinegar	Apples	Cooking	United States, Switzerland, Austria
Distilled vinegar	Grains	Pickling	Asia
Spirit vinegar	Acetic fermentation of dilute alcohol	Flavoring various foods	Israel, Southern and central Europe
Wine vinegar	White, red, or rose wine	Salad dressing	
Rice wine vinegar	Grapes or white, red, or rose wine	As condiments, dressings	East and southeast Asia and China
Sherry vinegar	Grapes + Aged in wooden barrels with sunlight	Drizzle over food, marinades	Spain
Coconut vinegar	Coconut sap and pineapple juice	Preservative, condiments	Malaysia
Malt vinegar	Barley and grain mash	Served with fish and chips	England, South Africa
Fruit vinegar	Fruits	Flavoring, condiments, dressings	Asia, Africa

amylolytic, and invertase enzymes have been used in vinegar production (Luzón-Quintana et al. 2021). Most natural raw materials are rich in nutrients and do not require the addition of additional nutrients. However, in some cases, trace minerals and other compounds must be added for specific reasons (Table 8.2). After extracting the juice, the pasteurization step needs to be performed at 92 °C for 10–15 s in order to kill all microbes. Centrifugation is required to extract the juices. This procedure is also called upstream processes (Fig. 8.2). The juice extracted from fruits should be pumped through plastic pipe into the cooling tank and stored it at 0.6 °C. The initial sugar content is measured by refractometer and pH must be adjusted for the raw material. Concentrate the raw material by adding a sugar solution to reach the respective (°) Brix (Luzón-Quintana et al. 2021). List of extra nutrients added in raw material to increase the quality of production is shown in Table 8.3.

Microbes Involved in Vinegar Production

The fourth and fifth steps are as follows: alcoholic fermentation and acetification after raw material preparation play a vital role in vinegar production. This is because it is carried out by two different microorganisms: first yeast is responsible for alcoholic fermentation, which has significant ethanol tolerance and a high fermentation rate, while *Acetobacter is* required for acetification. Among 19 genera,

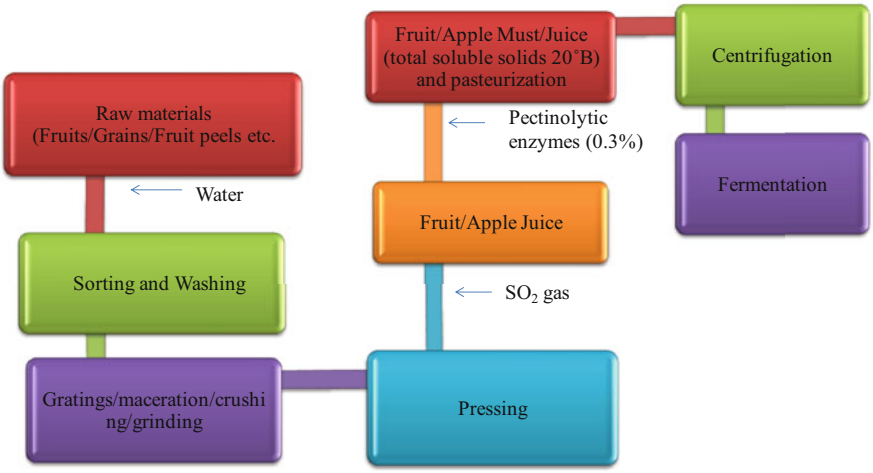

Fig. 8.2 Schematic representation of "upstream processes" in preparation of raw materials

Table 8.3 Lists of extra nutrients to be added in raw material

Fermentation	Extra nutrients	Reason
Apple cider	Ammonium phosphate—100 g/1000 L mash	For satisfactory fermentation
Grape wine	Ammonium phosphate—200 g/1000 L mash	
Distilled vinegar	Glucose, KH_2PO_4, $CaCl_2$, iron, $MgSO_4$, ammonium sulfate, cobaltous chloride, molybdenum, vanadium, copper, manganese, calcium carbonate	

Acetobacter, *Gluconobacter*, and *Gluconacetobacter* are the most frequently reported genera and these strains were predominantly used for industrial vinegar production. They are acedotolerant, thermotolerant and have a great capability for ethanol oxidation (Tanamool and Soemphol 2019).

8.3.2 *Preparation of Starter Culture/Inoculum Media*

Saccharomyces cerevisiae and *Acetobacter* species or *Gluconobacter* (as per production) cultures required for inoculum can be purchased from NCL, Pune, or MTCC, Chandigarh. To maintain the master culture for further use and/or mass multiplication, it is necessary to subculture it on its selective media through good laboratory practices (GLPs).

8.3.2.1 Maintenance of Master/Mother Culture

Pick one loopful culture or single pure colony or dry material (especially for yeast) from the master culture (liquid vial/slant/dry material) and transfer it onto YPD agar (for yeast; Table 8.4) and GYC agar (for *Acetobacter* Table 8.5) (GYC—glucose yeast extract calcium carbonate agar) by the four flame streak method and incubate the plates for 3–4 days at 33–37 °C and 25–33 °C, respectively. Plates containing isolated colonies should be stored at 4 °C for further use.

8.3.2.2 Preparation of Starter Culture

Starter culture/inoculum media can be prepared using YPD (for yeast) and GYC (for *A. aceti*) broth medium (HiMedia). (Note: For vinegar production, standard media must be used for starter culture as there is a high risk of altering the flavor, aroma, and chances of contamination.) The compositions are listed in Tables 8.4 and 8.5. The flask containing the broth should be autoclaved at 15 lb pressure (121 °C) for 15 min, cool it and inoculate it with yeast and *A. aceti* colonies. Incubate both the

Table 8.4 Composition of YPD (yeast peptone dextrose) agar medium

Ingredients	g/L
Yeast extract	10 g
Peptone	20 g
Tryptophan	0.33 g
Dextrose	20 g
Agar	15 g
Distilled water	1000 ml
pH	6.5 ± 0.2

Suspend total 65.33 g ingredients in1000 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lb pressure (121 °C) for 15 min. Cool the medium to 45–50 °C and pour it in sterile Petri dishes

Table 8.5 Composition of GYC/Acetobacter agar medium

Ingredients[a]	g/L
Glucose	3 g
Yeast extract	10 g
$CaCO_3$	10 g
Agar	15 g
Distilled water	1000 ml
pH	7.4 ± 0.2

Suspend total 38 g of total ingredients in 1000 ml distilled water by slight heating and sterilize by autoclaving it for 15 min at 121 °C and at 15 lb pressure. Cool the medium to 45–50 °C and pour it in sterile Petri dishes

[a] Note: due to the presence of calcium carbonate, the prepared medium forms an opalescent solution with a white precipitate

flasks in an incubator shaker at their respective temperatures for 24–48 h. Inoculate both well-grown culture into 5–7 L capacity flasks containing the respective inoculum media and further incubate it for 24–48 h. These serve as an inoculum medium for mass multiplication.

8.3.3 Mass Multiplication

For mass multiplication, prepare the 5 L of YPD and GYC broth media as mentioned above and pour into a fermenter. Yeast culture should be poured in first step (alcohol fermentation), whereas *Acetobacter* bacteria poured in second step (vinegar production). To minimize the cost of production media preparation for large-scale production and increase the growth ability of the culture, there are many cheap media available that could be used as alternative sources for mass multiplication of yeast and *A. aceti*. Sterile distilled water can also be used for yeast. Alternative raw materials for vinegar production include agricultural waste by-products (a combination of pineapple peel extract and banana juice), side streams, waste, and agro-industrial by-products. After adding the inoculum, maintain the optimum conditions (temperature, pH), aeration, and agitation and incubate for 5–20 days. These parameters might be useful in preventing the chances of contamination and decreasing incubation time. Sterile air and water required for aeration, agitation, and temperature maintenance (cooling system). Periodically collect the sample and check the organism purity level.

8.3.4 Alcoholic Fermentation

The concentrated juice is poured into a closed fermenter tank by an inlet valve. The fermentation medium is inoculated with inocula into the tank through the inoculation point and allow to grow for 5–7 days at 33–37 °C temperature including the continuous flow of aeration and agitation of the medium with an agitator-sparger. Periodically collect the sample from the sampling point and analyze the sugar content (° B) (according to AOAC 2000), Brix is the total soluble solids, pH, and alcoholic content through an alcoholmeter until the sugar content reaches a minimum level. Alcohol content increases as sugar content is consumed by yeast. Generally, mash fermentation should not be temperature controlled, but it requires cold conditions but the heat exchanger should be attached to the fermenter. When alcohol production is complete, yeast and fruit pulp settle at the bottom of the tank and form a compact mass. Yeast should be separated from the alcoholic mash by filtration. This mash can be used further for the production of acetic acid.

8.3.5 Acetic Acid Fermentation

For submerged fermentation, transfer the high alcoholic mash into another fermenter (acetator) and adjust the alcohol to approximate 7–8% v/v by adding water. The fermenter is inoculated with 10% *A. aceti* inocula through the inoculum point and allows to grow for 7 days at 33 °C under aerobic condition. The fermenter is fitted with high-grade agitator and efficient heat exchanger inside, whereas oxygen pumps and a cooling system outside the fermenter (in-built). For industrial purposes, the best temperature is 30 °C for 11–12% vinegar. Aeration is provided continuously until the concentration of vinegar reaches its maximum and the alcohol concentration decreases to 0.3% vol. At the end of every fermentation cycle, the respective volume of acetic acid is discharged from the acetator, and the tank is refilled with mash. Hence, one part of the 'mother vinegar' is required to start a new cycle. Collect the samples periodically and analyze the samples for acetic acid concentration, sensory characteristic (size, shape, color, appearance, acidity, viscosity, etc.), and at the end of fermentation, analyze the sample for total phenolic content, and antioxidant content (optional step). The standard acetic acid concentration of the product should be 5–14%, which depends on the initial alcohol content of the raw material (Joshi and Sharma 2009).

This vinegar is crud one hence it is compulsory to pure the vinegar and hence few steps are to be carried out also called as "Downstream processes". Figure 8.3 shows the schematic representation of downstream processes required to obtained the pure and crystal vinegar.

8.3.5.1 Maturation/Aging

The raw vinegar is allowed to mature in wooden barrels for 1–2 months to enhance the flavor and pleasant aroma, because the oxidation of vinegar takes place by air entering through the pores present in the barrels.

Clarification technique can be performed as follows:

1. Sedimentation: Used to achieve crystal clear and stable vinegar, which depends on acidity and age.
2. Fining: This type of rough filtration reduces the total load of suspended material by adding gelation, bentonite, and/or liquid silicon dioxide.

   Protocol: (1) Add 260 g gelatin + 400 g bentonite in 1000 L of vinegar, stir the suspension, and allow it to settle for 1 week before racking; or (2) Add 30 % of 5 L liquid silicon dioxide and 1 kg of gelatin per 5000 L of vinegar and leave it to settle.

   Note: once vinegar is ready it should be kept away from oxygen as it is converted into water and carbon dioxide (Joshi and Sharma 2009).

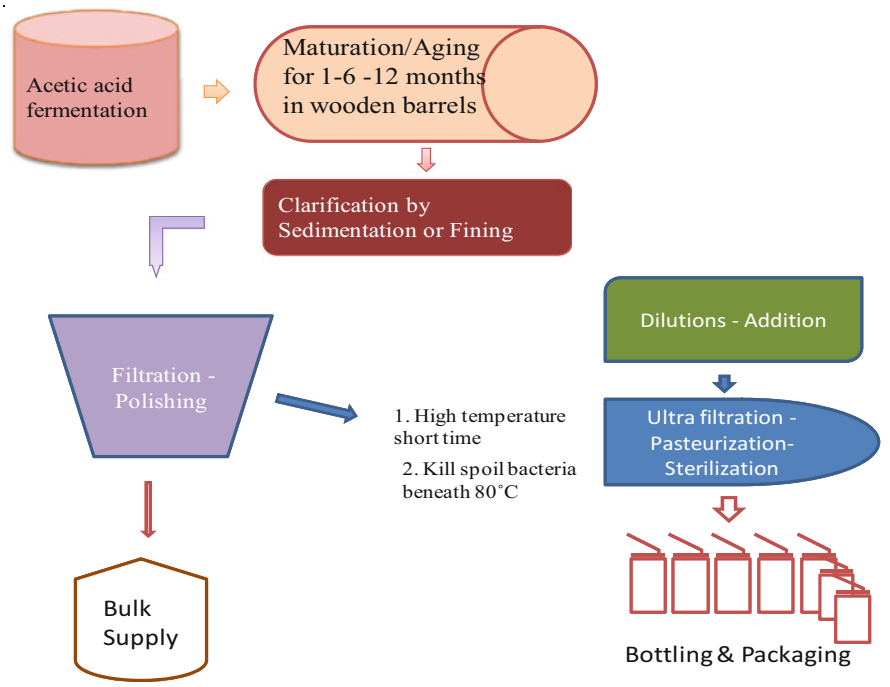

Fig. 8.3 Schematic representation of "downstream processes" of acetic acid fermentation

8.3.5.2 Ultrafiltration and Pasteurization

Ultrafiltration is an upgraded method to replace the normal filtration and sterilization processes**.** After ultrafiltration vinegar is subjected to high-temperature short time (HTST) pasteurization to avoid spoilage**.** The vinegar is passed through a tubular heat exchanger at 66 °C for a short time and allow to cool for bottling. Flash pasteurization is used to kill most wine spoilage bacteria at temperatures below 80 °C within a fraction of a second. Unwanted changes occur due to the presence of enzymes (enzymes added or produced during fermentation) which can be avoided by inactivating the enzymes (Joshi and Sharma 2009).

8.3.6 Bottling and Packaging

Bottling means filling line processes by using vinegar mechanical filler automated connected to a computer in which bottles may be arranged manually or automatically on belt conveyors consisting of plastic or stainless steel. All bottle types ranging from 25 to 1000 ml are filled using this machine. Bottles should be either glasses or plastic, depending on the brand and availability. As soon as the bottles are fixed by a

clamp arm, they should be properly rinsed with water or air, and active ingredients. After the filler (a ring bowl) is filled with vinegar, the bottles can be pre-evacuated under vacuum, or pre-rinsed with nitrogen, and then filled with vinegar by gravity (Strobl 2019).

Furthermore, there are three safe methods for bottling alcohol-free vinegar; hot filling, pasteurization of the filled bottle, and chemical stabilization by sorbic acid or di-methyl-di-carbonate, ascorbic acid, pectin, citric acid, potassium ferricyanide, etc. (Strobl 2019; Joshi and Sharma 2009).

Packaging plays a dynamic role among other processes as it is the consumer's first choice or introduction to the product. Glass bottles are mostly used as containers for vinegar as a packing material, because glass is an inert material that does not react with vinegar. Polyethylene terephthalate (PET), bags, and cartoons packaging have been used as packaging materials. The main functional aspects of product packaging are the description of ingredients and nutritional information, manufacturing date, storage, warning alcohol percentage, and expiry date. Product packaging with proper display is a reflection of the product inside and the brand, which leads to increased consumer satisfaction and likeliness of purchase. Bottling and packaging are vital tools for quality and recycling at which entrepreneurs become better competitors (Strobl 2019).

Precautions During Bottling and Packaging

1. Product should be free from any type of contamination as it may alter the flavor and aroma of vinegar.
2. Vinegar should be produced at 'hygienic standards'.
3. High alcohol content and low pH could allow the growth of microbes.

Checklist of Vinegar Preparing for Market

1. Check the filling level.
2. Proper labeling.
3. Treatment with closed bottles and sealing.

From a transportation perspective, vinegar is packed as a large selling unit and transported in forklift trucks or automated loading systems (Strobl 2019).

8.3.7 Marketing

Marketing is for each product and every product, specifically for vinegar, is certainly not easy. There are many ways to promote and advertise vinegar. Market channels are diversified according to firm and production type. Large-scale retailers are also important customers. Thus, maximum stock goes through this, and the remaining quota may have gone through traditional grocery channels, restaurants, pickling industries, supermarkets, large-scale processors, national, and international markets. Currently, social media is the best marketing approach for any product. Manufacturers can explore vinegar products by introducing innovative flavors, along with an awareness of health benefits and comparative costs.

Key parameters for maximum product selling value

1. Packaging and labeling: Attractive packaging and labeling can also increase the demand for products.
2. Value-added product: Value is added if flavor is added by adding honey, rose petals, etc.
3. Maintenance of quality and purity.

   According to the Federal Food, Drug and Cosmetic Act, it is compulsory that a product to be labeled as the date of delivery, not adulterated, or misbranded.

Product Specification and Quality Standards

Once the new product stock has been out for the first time, product specifications and quality standards are necessary to control its production. These specifications and standards reflect the success or failure of the product.

1. Microbiological safety standards (purity level of inoculum (microbes), including temperature and pH)
2. Product composition
3. Quality of raw materials
4. Nutritional values
5. Sensory characteristics
6. Storage condition and their effects on the shelf life
7. All manufacturers follow the federal guidelines for good manufacturing practices
8. Types of packaging and label design (Axtell and Fellows 2014).

8.4 Project Details

Various facilities are required for the successful establishment and initiation of large-scale vinegar production projects. It is necessary to require a license before establishing and implementing a successful production unit, as this is a food processing agro-sector-based project. These are as follows:

(a) Factory license
(b) GST (Good and Service Tax) registration
(c) FSSAI (Food Safety and Standards Authority of India) license
(d) NOC (No Objection Certificate) from pollution control board etc.

The instrument facilities, laboratory facilities, and downstream processing facilities of vinegar production detailed here can also be significantly applicable for all types of vinegar, but the only difference is in the types of raw material and its upstream processing. Because vinegar is a two-step fermentation process, it is only applicable to vinegar. Here, we describe the approximate project-cost details along with the necessary facilities. Generally, there is 3000/day—60,000 per year bottles

of vinegar production (fruit/apple cider vinegar) plants set up through large-scale fermentation in India depending on their capacity. The present plant described a model pattern for the production unit for 60,000 bottles or 42 tons per annum vinegar, and followed the same pattern for rest of the vinegar.

Land and Location Land is the primary requirement for establishing any type of business. There is a 7000–8500 sq. ft area of land required to build up various facilities from administrative offices to laboratories including fermenter rooms.

The plant should be located at any suitable location keeping in mind the availability of raw materials, power, water, skilled manpower, and marketing convenience. It is advisable that units should be far from residential areas but closer to market areas. It is better if the unit is closer to the food-processing industry. Thus, the cost of the raw material transportation process is minimal.

Built up Area
Many core areas should be designed in infrastructure facilities of production units such as laboratory rooms, microbiology laboratories, administrative rooms, raw material preparation and processing rooms, instrumentation rooms, fermenter rooms, downstream processing facilities, bottling-packaging and dispatching areas, pantry rooms, etc. Currently, land and land areas are also very costly based on location which is why, it is more convenient and ideal to design infrastructure on a floor, excluding fermenter area, to utilize such spaces. Around up to 7500 sq. ft area is sufficient for total infrastructure facilities, and the rest of space should be designed for transportation areas and small gardens. The entire land area can be covered by building a boundary wall.

8.5 Financial Aspects

8.5.1 Fixed Capital (Table 8.6)

Instruments and Machinery Instruments and machinery are required to facilitate vinegar production. Minimum and good quality instruments can fulfill the standard fermentation procedure. The instruments required for the upstream and downstream processes, packaging, and fermentation process are listed in Tables 8.7, 8.8, 8.9, and 8.10.

Table 8.6 Infrastructure cost

Description	Rate	Cost (INR Rs/£ lakh)
Land (8500 sq. ft)	Approx.	25 lakh
Land preparation and infrastructure construction	Approx.	10 + 60 lakh
Total		95

Table 8.7 Instruments, apparatus, and machinery expenses

Instruments	No.	Rate per no.	Total cost (INR Rs./£ lakh)
Electronic weighing balance	1	0.18	0.18
pH meter	2	0.065	0.13
Vertical autoclave (600 × 300 mm)	1	0.77	0.77
Horizontal autoclave (SS, 75–351 L)	1	3.96	3.96
Laminar air flow (4 × 2 × 2)	1	0.65	0.65
Refrigerator (350 L)	1	0.30	0.30
Colony counter	1	0.06	0.06
Orbital incubator shaker	1	1.46	1.46
Rotary shaker	1	0.36	0.36
Deep freeze (110 L)	1	0.35	0.35
Compound microscope (binocular)	1	0.10	0.10
Raw material processing unit			
Distillery unit (semi-automatic, 1000–2000 L/h, voltage—220 to 440 V)	1	0.30	0.30
Gas fired bale out furnace, (capacity: 0.5 tons, power: 36 kW)	1	1.5	1.5
Hydraulic press (40–60 tons with filter bag and splash guard, stainless steel)	1	1.0	1.0
Filter press (>3000 L/h with <100 filter area, Stainless steel)	1	0.95	0.95
Fermenter unit			
Nutrient tank (SS, 316 L, 380 V	2	0.75	1.50
Refractometer (±0.2% Brix)	2	0.025	0.05
Vertical Mash tank (5000–15,000 capacity (120 m^3))	2	1.5	3.0
Bioreactor (acetator) (120 m^3, 500 kL/h, pressure 10 bar)	2	15	30
Wooden barrel (200–250 L)	6	0.045	0.27
Filtration unit (semi-automatic, 1000 L/h capacity, voltage—220 to 240 V)	1	0.74	0.74
Storage tank (1000–1500 L capacity)	2	0.60	1.20
HTST pasteurization unit (100–5000 L capacity)	1	0.89	0.89
Bottling filling, capping and labeling machine (SS, 30 bpm capacity, power consumption: 440 V, 50 Hz, filling head: 4 nozzle)	1	5.0	5.0
Bottle washing unit (SS, semi-automatic, 250 bpm, power consumption: 1.25 HP, 50 Hz material: rubber, 24 no.)	1	0.55	0.55
Air compressor (6–8 or 9–121 Bar, 15 HP capacity)	2	0.48	0.96
Other			
Drinking water cooler/RO system (SS, 50 L/h, Tap-2)	1	0.22	0.22
Cooling system (air conditioner, 1.5 tons, 3 star)	6	0.25	1.50
Vacuum pump (SS, power—110 V AC)	1	0.06	0.06
Generator (60 kVA silent diesel, 3 phase)	1	1.25	1.25
Lab glassware and plastic wares	–	Approx.	1.5
Miscellaneous requirements for lab	–	Approx.	1.5

(continued)

Table 8.7 (continued)

Instruments	No.	Rate per no.	Total cost (INR Rs./£ lakh)
Erection and electrification of machinery and equipment at 10% cost	–	Approx.	0.050
Office furniture and fixtures	–	Approx.	2.5
Grand total cost			64.81

SS stainless steel

Table 8.8 Miscellaneous fixed assets expenses

Particulars (item)	Number	Cost (INR Rs./£ lakh)
Computer	3	1.05
Printer	1	0.048
Fax	1	0.09
Other items	-	0.1
Total cost		1.288

Table 8.9 Pre-operative expenses

Pre-operative cost	Consultancy fee, project report, Deposits with electricity departments, etc.	1.0

Table 8.10 Model format outlay of total fixed capital expenditure

Total fixed capital	Cost (£ lakh)
Infrastructure cost (Table 8.6)	95
Instruments, apparatus, and machinery expenses (Table 8.7)	64.81
Miscellaneous fixed assets expenses (Table 8.8)	1.288
Pre-operative expenses (Table 8.9)	1.0
Grand total fixed capital expenditure	162.098

The cost of the various components will depend on the location of the project

8.5.2 Working Capital

8.5.2.1 Recurring Expenses per Annum (Table 8.11)

8.5.2.2 Laboratory Media and Chemicals

The cost of media and chemicals used for culture preparation and the addition of extra nutrients required for production of acetic acid for 60,000 bottles/42 tons is given in the Table 8.12:

Table 8.11 Manpower specification cost

Designation	No.	Salary per head/ month (Rs./£ lakh)	Total amount/ month (Rs./£ lakh)	Total amount/ annum (Rs./£ lakh
Manager	1	0.30	0.30	3.6
Office Assistant/ administrative person	1	0.25	0.25	3.0
Microbiologist	1	0.20	0.20	2.4
Lab Technician	2	0.18	0.36	4.32
Production Officer	4	0.25	1.0	12
Supervisor	1	0.12	0.12	1.44
Skilled Worker	4	0.05	0.2	2.4
Unskilled Worker	6	0.035	0.21	2.52
Sales Officer	4	0.15	0.60	7.2
Grand total cost			3.24	38.88

Table 8.12 Raw material specification (media and chemicals) and expenses

Medium and specification	Media and chemicals	Quantity	Amt. (Rs.) (INR)	Total cost (£ lakh)/42 ton (INR)
Acetobacter agar medium (GYC) (HM)	Ready to use media	1 kg	8990	0.57
Acetobacter broth medium (HM)		2 kg	17,640	
YPD agar medium (Sigma)		1 kg	9385	
YPD broth medium (HM)		2 kg	20,880	
Extra nutrients can be added in order to satisfactory fermentation in production media as well as after first step, i.e., ethanol production	Glucose	10 kg	2300	0.24
	Ammonium sulfate	10 kg	230	
	$CaCl_2$	10 kg	160	
	KH_2PO_4	10 kg	950	
	$MgSO_4$	10 kg	250	
	Ammonium phosphate	50 kg	6000	
	Cobaltous nitrate	5 kg	4500	
	Copper chloride	10 kg	3500	
	Calcium carbonate	10 kg	450	
	Manganese sulfate	10 kg	650	
	$FeCl_3$	10 kg	450	
	Sucrose	100 kg	4500	
Total acidity	Sodium hydroxide	10 kg	780	0.014
	Phenolphthalein	0.1 kg	590	
SO_2 gas cylinder (2.551g/L)		100 kg (10 kg/bottle)	65,000	0.65
Other lab consumable items	Approx.			0.1
Total cost				1.574

8.5.2.3 Raw Materials Including Packaging Materials

Cost of raw materials required for alcohol production and vinegar packaging is given in Table 8.13

8.5.2.4 Utilities

Power: Owing to the two-step process along with upstream and downstream processes, maximum power is utilized. Approximately 30 kWh r is required. Normally, 3 phases power supply is required. A standby generator is required optionally.

Water: In vinegar production, water is required at every step of production process from preparation of media to acetic acid production. Water is also utilized to wash the bottles as well as fermenter. Approx. 4000–6000 L of water is required per day (Tables 8.14 and 8.15).

Table 8.13 Raw materials (substrates) and packaging material expenses

Particulars	Quantity (tons)	Rate/ton (Lakh)	Total amount (Rs. In lakh)
Fruits	110	0.10	11.00
Glass bottles and caps	60,000	10/no.	06.00
Labels	60,000		00.60
Other raw materials and packaging miscellaneous item			2.5
Grand total cost			20.10

Table 8.14 Utility expenses

Particulars	Cost (INR Rs. In lakh/month	Cost (Rs. In lakh/annum
Power 5000 unit at 5 rs/unit	0.25	3.0
Water (approx.)	0.09	1.08
Total expense		4.08

Table 8.15 Other contingent expense

Particulars	Amount (INR Rs. lakh)
Repair and maintenance	1.5
Consumables and spares, others	0.50
Travel and transport	0.50
Publicity, postage, and telephone	0.30
Insurance at 1%	0.10
Total cost	2.9

8.5.2.5 Working Capital (Tables 8.16, 8.17, and 8.18)

8.5.2.6 Income of 42 Ton/60,000 Bottle Selling of Vinegar

The cost per bottle given in Table 8.19 is average market price for private industries. Private firms may cost more than this, and government organizations and institutions may cost less. In addition, vinegar costing will differ based on the raw material used and type of vinegar produced (Table 8.20).

Table 8.16 Model format for total recurring expense

Total working capital expenditure	Amount (lakh)
Man power specification cost (Table 8.11)	38.88
Raw material specification (media and chemicals) and expenses (Table 8.12)	1.574
Raw materials (substrates) and packaging material expenses (Table 8.13)	20.10
Utility expenses (Table 8.14)	4.08
Other contingent expense (Table 8.15)	2.9
Grand total working capital expenses	67.534

Table 8.17 Total capital investment

Total Capital Cost	Amount (INR Rs./£ lakh)
Grand total fixed capital expenditure	162.098
Grand total working capital expenses	67.534
Grand total	229.632

Table 8.18 Mean of finance

Particulars	Contribution (%)	Total contribution (INR Rs./ £)
Owner's contribution	25	40.5245
Bank loan	50	81.049
Subsidy by government	Project cost at 25% or 40 lakh whichever is less	40.5245

Table 8.19 Approximate income per 60,000 bottles selling of vinegar

Item	Quantity	Rate/bottle (INR Rs.)	Amount (INR Rs. lakh)
Fruit vinegar/apple cider vinegar	60,000 (650 ml)	300	180.00

Table 8.20 Production capacity, total income and expenditure for model project of 60 TPA acetic acid production unit (INR Rs. lakh)

Sr. No.	Particulars	Values
Production capacity		
1	Installed capacity	60 TPA
2	Optimum capacity utilization	70%
3	Capacity utilized in ton	42 TPA/60,000 bottles
4	Income (Rs. lakh/annum)	180.00
Expenditure		
5	Recurring expenses	67.53
6	Administrative expenses	1.2
7	Taxes and insurance	
8	Interest on bank loan (16% annum)	12.97
9	Loss due to contamination (5%)	9.0
19	Depreciation for fixed assets (1%)	1.62
Total expenditure		92.32
Net profit per year (INR Rs.)		87.68

Net benefit ratio = 48.71%

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Chapter 9
Mass Multiplication, Production Cost Analysis and Marketing of Polyhydroxyalkanoates (PHAs)

Sanket Ray

Abstract The polyhydroxyalkanoates (PHAs) are a class of biopolymers with the advantage of biodegradability. The properties of PHAs make them an interesting candidate for replacement of petrochemical-based plastics. The microbial PHA production process flow will give clarity about scope of equipment, machinery and accessories requirement for establishment of full fledge production unit. The PHA production price is much higher than the conventional plastics. The various efforts for mass production and techno-economical studies for PHA production will give better idea for designing or starting the work in the same direction. This chapter aims to cater the information regarding various strategies for cost-competitive large-scale production of PHAs, its cost and global pricing assessment. The information about marketing and product placement of PHAs challenging with cheaper traditional plastic polymers in global market may give insight for future of bioplastic industry.

Keywords PHAs · PHB · Biopolymer · Biodegradable · Bioplastic · Petrochemical-plastics

9.1 Introduction

9.1.1 General

The invention of petrochemical-based plastic has changed our lifestyle to a great extent. Due to its diverse properties profile, it has replaced almost all the materials in one or another way. Modernization and increased population have drastically increased the daily usage of petrochemical-based polymers. Consequently, plastic waste management has become a global problem as it takes hundreds of years to decompose in nature. By 2060, the 155–265 Mt/y mismanaged plastic waste shall be produced globally (Kaza et al. 2018; Lebreton and Andrady 2019). Problems

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associated with mismanaged plastic waste have attracted interest and researchers in the search of biodegradable polymers which can be a substitute for conventional plastics. Polyhydroxyalkanoates (PHAs) are biodegradable polymers with similar properties to petroleum-based plastics. The PHAs are polyesters of 3-hydroxy fatty acid monomers which are produced by microorganisms belonging to genera such as *Cupriavidus*, *Bacillus*, *Escherichia* (recombinant), *Halobacterium*, *Nitrococcus*, *Rhizobium*, *Staphylococcus*, *Pannonibacter*, *Thiobacillus* and *Vibrio* are few among many (Koller et al. 2010; Ray et al. 2016). The cyanobacterium *Synechocystis* sp. is also reported for the accumulation of PHA granules in cell cytoplasm (Markl et al. 2018). PHA is typically produced as a polymer of 10^3 to 10^4 monomers, accumulating as inclusions of 0.2–0.5 μm in diameter. These inclusions or granules are produced and stored by both Gram-positive and Gram-negative bacteria without hazardous effects on the hosts. While the presence of these intracellular granules in bacterial cells was recognized early by many microbiologists, the composition of these particles was first clarified by Lemoigne (1923).

PHAs are a family of optically active biological polyesters containing (R)-3HA monomer units (Anderson and Dawes 1990). Polyhydroxybutrate [P(3HB) or PHB] is the most well-known candidate for the PHA class containing (R)-3HB repeating units. Depending on the microorganism and growth conditions, the monomers are polymerized into high molecular weight polymers ranging from 20,000 to 3,000,000 Da (Byrom 1993). Depending on the number of carbon atoms in the monomer of PHAs, they can be divided into two groups—short-chain length (SCL), which consists of 3–5 carbon atoms, and medium-chain length (MCL), which consists of 6–14 carbon atoms (Anderson and Dawes 1990). This difference is mainly due to the substrate specificity of the PHA synthase that can accept 3HAs of a specific range of carbon length (Madison and Huisman 1999). The PHA synthase of *Alcaligenes eutrophus* can polymerize 3HAs consisting of 3–5 carbon atoms, whereas that present in *Pseudomonas putida* can only accept 3HAs of 8–10 carbon atoms. Initially, it was believed that PHA is a crystalline solid in the in vivo state (Alper et al. 1963). But later on, it was demonstrated for the first-time using solution-state NMR techniques that the bulk of P(3HB) in vivo is not crystalline and indeed is a mobile amorphous polymer.

9.2 PHA Production

The fed-batch microbial PHA production process is basically divided into two phases (Fig. 9.1a), which include upstream fermentation and downstream recovery of PHAs. The large-scale upstream PHA production processes are carried out in multiple vessels including inoculum development (Fermenter I), PHA production bioreactor (Fermenter II), Filtration and centrifugation of cell biomass (Separation Unit I), Washing Unit (Reactor I), secondary biomass washing (Reactor II) and cell biomass concentrator (Fig. 9.1a). The inoculum development (Fermenter II) is usually being done to propagate industrial strain which is being introduced into

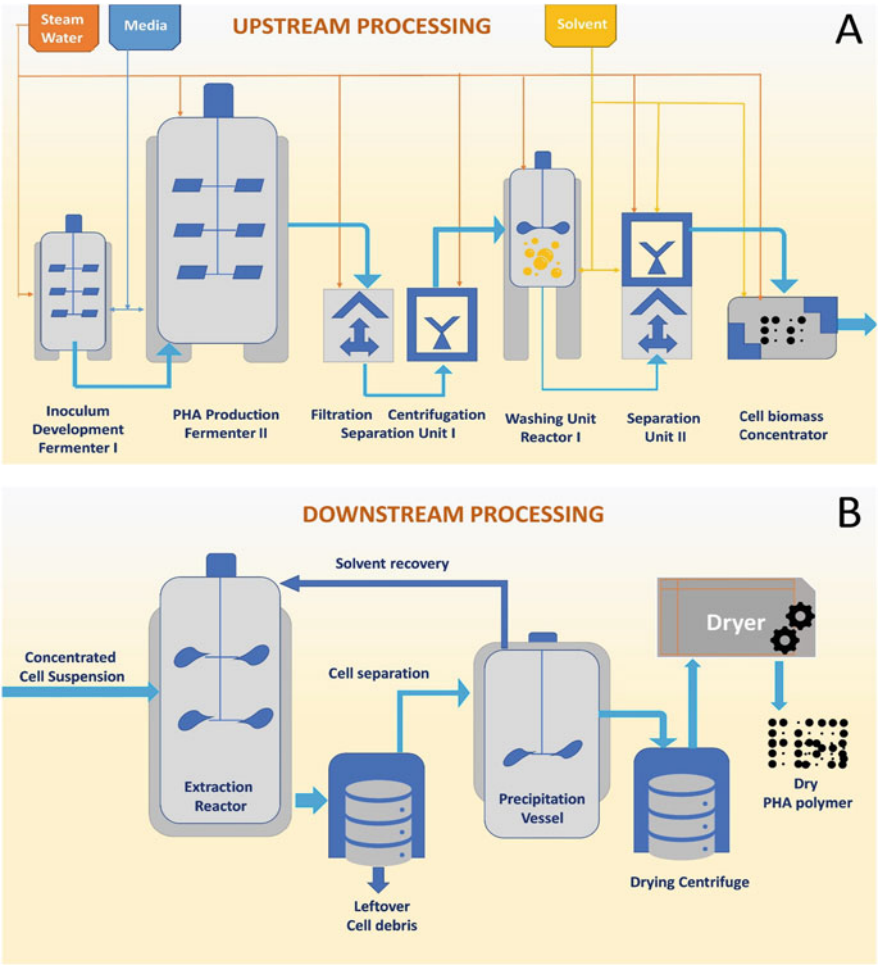

Fig. 9.1 Process flow of (**a**) upstream fermentation and (**b**) downstream processing of large-scale microbial PHA production

Fermenter II where actual PHA accumulation is achieved using an appropriate production medium at controlled condition. Various parameters such as cell biomass, PHA production yield, %PHA accumulation, pH, temperature, carbon and nitrogen substrate utilization, DO, and aeration are being observed and controlled throughout the fermentation batch. The cell biomass is harvested from the fermented broth in two-stage separation unit I using industrial filtration and centrifugation. To remove unspent fermentation media residues, traces and impurity the cell biomass washing pre-treatment using acetone and water mixture is carried out in Washing Unit (Reactor I). After pre-treatment, the cell biomass is collected using filtration and centrifugation process at separation unit II. The concentrated cell biomass containing PHA is subjected to solvent extraction and purification at phase II process. The

cell-free supernatant is being treated and repurposed as per good laboratory practices and regulations. The concentrated cell suspension is subjected to extraction using hot chloroform under agitated condition for 6–12 h in extraction reactor. The accumulated PHA in cell biomass dissolved in chloroform and submerged in solvent phase. The extraction liquor passed through industrial centrifugation for removal of cell debris. The cell-free liquor which contains PHAs is subjected to precipitation vessel, where chilled methanol is added gradually to precipitate polymers. The chloroform and methanol will be recovered through distillation and reuse during downstream processing of PHAs. The solvent residues will be recovered by subsequent stages at drying centrifuge and vacuum dryer (Fig. 9.1b).

9.2.1 PHA Productivity and Bacterial Fermentation

PHA productivity is defined as the amount of PHA produced by unit volume in unit time. Various factors such as PHA production time, cell-biomass multiplication, % PHA content, and productivity are important criteria to monitor the efficiency of large-scale PHA production process. For the production of the high amount of PHA per year, the process with lower productivity requires larger equipment (Choi and Lee 2000). Very few species of bacteria producing PHA have been used at an industrial scale for PHA production. This include *C. necator*, *A. lata* and recombinant *E. coli* (Khanna and Srivastava 2005). On the contrary, there are some bacterial strains such as *C. testosteronei* (Thakor et al. 2005) and *A. chroococcum* which can accumulate high amount of PHA and hence could be used for the large-scale production of biopolymer (Table 9.1). Several studies have demonstrated the PHB production using different microbial strains, either in batch, fed batch or continuous cultures. Batch fermentation for PHA production is having the advantage of its low operation cost and flexibility (Peña et al. 2014).

9.2.2 Influence of Carbon Source on PHAs Production

The major recurring expenses in the production of PHAs are determined by the cost of the fermentation substrate, the polymer extraction from the cells and the treatment of fermentation and extraction wastes (Chen 2010). Of all these factors, the cost of the carbon source has the greatest influence on the price of PHAs. Considering these new alternatives have been proposed to reduce the costs of raw materials. It is important to note that the selection of carbon sources should not focus only on the market prices but also on the availability and on global price (Chanprateep 2010).

Most of the carbon substrates supplied for PHA production are pure alkanes, fatty acids or carbohydrates. Complex substrates, such as castor or euphorbia oil, gave mcl-PHA of (C6–C14) with *P. aeruginosa* (Jincy and Sindhu 2013). However, the use of sub-products or wastes has hardly been explored at all, which may well be due

Table 9.1 Comparison of PHB production, content and dry cell weight (DCW) using different carbon sources by different microorganisms

Organism	Carbon source	DCW ($g\ l^{-1}$)	PHB content (%)	References
Alcaligenes latus	Sucrose	10.78	48.0	Zafar et al. (2012)
Cupriavidus necator DSM545	Glucose	164.00	76.2	Mozumder et al. (2014)
	Waste glycerol	104.70	62.7	
	Pure glycerol	82.50	62.0	Cavalheiro et al. (2009)
	Waste glycerol	76.20	50.0	
Azotobacter chrocooccum H23	Acetate	7.36	82.9	Pozo et al. (2002)
	Starch	54.00	46.0	Kim et al. (2002)
E. coli recombinant *GCSC 6576*	Whey	31.00	80.0	
A. latus	Sugar beet juice	7.80	28.4	Wang et al. (2013)
Azatobacter beijerinckii	Corn steep liquor	16.00	25.0	Purushothaman et al. (2001)

to the complexity of their composition. *Pseudomonas resinovorans* accumulated 15% of the cellular dry weight of mcl-PHA from tallow (Cromwick et al. 1996). Short-chain length PHA was produced by *R. eutropha* or *Pseudomonas* when incubated with the oil remaining from a rhamnose-producing process (Füchtenbusch et al. 2000). Non-related substrates, such as molasses or brewery malt processing wastes, were converted into PHB by various *Azotobacter* strains or *A. latus*, respectively (Choi and Lee 2000; Page and Cornish 1993). Biosynthesis of mcl-PHA by *Comamonas testosteroni* was achieved by cultivation on vegetable oils (Thakor et al. 2005).

PHA production from pure carbon substrates such as alkanes, fatty acids and carbohydrates has been reported by many scientists but the cost of such carbon sources, fermentation process and the downstream processing of the polymer contributes to the high cost of manufacturing process of polymer which is 5–10 times more than petroleum-based polymers. Carbon sources add about 50% cost of the production of the PHA (Fasaei et al. 2018). Researchers have also analysed that PHB production costs of 100,000 tonne/year would decrease from \$4.91 to \$3.72 kg^{-1} upon utilizing surplus hydrolysed corn starch (USD \$0.22 kg^{-1}) instead of glucose (USD \$0.49 kg^{-1}) (Choi and Lee 1999). Therefore, in order to use these polymers as commodity plastics, the cost of production has to be sufficiently lowered without affecting the useful properties of the polymer.

9.2.3 *Influence of Nitrogen Source on PHAs Production*

PHA accumulation in bacterial cell cytoplasm is mostly achieved through nitrogen starved and rich carbon substrate presence in fermentation mediums. Several reports suggest a very small amount of complex nitrogen source such as tryptone could enhance PHB production by recombinant *E. coli* (Lebreton and Andrady 2019). Several complex nitrogen sources such as yeast extract, fish peptone, meat extract, casamino acids, corn steep liquor, soybean hydrolysate and cotton seed hydrolysate were found to be reducing the lag phase of PHA fermentation (Page and Cornish 1993; Lebreton and Andrady 2019; Chen et al. 2006; Purushothaman et al. 2001; Koller et al. 2005).

The whey from the dairy industry contains several essential amino acids, sugars, vitamins and fatty acids which could be repurposed for cost-effective nitrogen supplement for large-scale PHA production. The recombinant *E. coli* utilized dairy whey for the PHA production of 168 gl^{-1} and a volumetric PHA productivity of 4.6 gl^{-1} h^{-1} (Ahn et al. 2000).

9.3 PHA Production Cost Analysis

According to Price et al. (2022), the cyanobacterial poly(3-hydroxybutyrate) (PHB) production plant with a capacity of 10,000 tons/year requires the capital cost of $193.5 M USD with an additional operating cost $147.3 M USD/year. The overall revenue of the said production plant was estimated at around $40 M USD/year. For the breakeven minimum PHB selling price for a zero 20 years Net Present Value (NPV) was $18,339 USD/ton which is almost 4.58 times higher than the current market price of PHB ($4000/ton). The major cost contributing aspect is land, equipment, cultivation and downstream processing of PHB (Table 9.2). To minimize the cyanobacterial PHB production cost, several processes tweak up such as increasing the size of raceway pond, solar-based electricity, on site anaerobic digester and usage of flocculants.

9.4 Marketing and Positioning of PHAs

Day by day, due to increasing concern and awareness regarding the climate change, the bio-based economy tends to shift from conventional fossil-based products. The international agencies, forums, governments, and policymakers are determined to accelerate the biopolymer products integration in market at multiple verticals. Researchers have throttled the efforts for the development of diverse applications of PHAs in various fields such as agriculture, food and beverages packaging, optically active chiral compound synthesis, novel medicine, clinical implants and

Table 9.2 The cost contributing factors and their impact on overall PHB production techno-economics

Cost contributing factors	Cost analysis	Scale
Equipment (Fasaei et al. 2018)	• A standard scaling equation was applied below where Cost (A) and Cost (B) are the price of the process equipment at Size (A) and Size (B), respectively • The scaling factor *n* was derived from engineering databases and supplier catalogues or literature • In this case, the majority of process equipment had a scale factor of 0.6 $\frac{Cost(A)}{Cost\ (B)} = \left[\frac{Size(A)}{Size\ (B)}\right]^{n}$	High impact
Cultivation	• Cultivation cost is recurring cost which further includes media preparation, processing, manpower, and utility	Medium impact
Downstream processing of PHB	• Harvesting, extraction and drying • Adds equipment cost as it is being carried out in large vessel with control system • High amount of usage of water, acetone/chloroform for extraction • High electricity consumption for vacuum drying and distillation	High impact
Addition costs	• Waste water treatments, maintenance, regulatory audit and commissioning and taxes	Low to medium impact

next-generation fuels. At present, PHAs are the most promising biopolymers substitute for petrochemical-based plastics. Already various policies of phasing out or strict ban on "single-use plastics" are being implemented around the globe, which results in increasing the demand of biobased alternatives for packaging purpose (Wagner 2017; Adam et al. 2020). As a consequence, global bioplastic and biopolymers market may march towards $14.9 Billion (USD) by 2024 (https://www.researchandmarkets.com/reports/4804726/bioplastics-and-biopolymers-global-market). To tackle the demand, many major market players such as Arkema SA, BASF SE, BIOTEC GmbH & Co. KG, Mitsubishi Chemical Corporation and many such have already positioned their patented products in the commercial bioplastic market. The bioplastic market is going to be an exciting field as many start-ups are hustling to capture the arena with innovative products made using sustainable resources. The various institutions and government agencies have also provided fund, and reduction on Tax to encourage development, acceptance and implementation of microbial bioplastics.

9.5 Conclusion

The major hurdle for the commercialization of PHAs at wide horizon of market is its high production cost. Efforts are being made for searching for efficient producers as well as scale-up of the exciting fermentation process with cost-competitive substrate to lower the production cost. Furthermore, for developing a wide range of applications, the bioplastic could chemically graft polymerized with various monomers to obtain tailormade semi-synthetic polymers with diverse properties. The genetic engineering, metabolic engineering and biotechnological advancement could leverage the new possibilities in biodegradable plastic material.

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Chapter 10
Small, Large-Scale Production and Cost-Benefit Analysis and Marketing of Agar from *Gelidium*

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Abstract Agar is a hydrocolloid present in red seaweeds that have piqued commercial attention over decades with many uses in food, cosmetics, and medicine. This polysaccharide is isolated from the cell wall of the red seaweed by boiling for many hours. There are several industrial and pilot-scale techniques for producing quality agar from seaweed. As these seaweeds have commercial value, they can be cultivated through many methods for the high yield of seaweed. This review presents the extraction, cultivation, and agar production from the red seaweed *Gelidium*. Furthermore, this study emphasized the cost analysis for the global agar market, which

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includes agar segment, opportunities, growth dynamics, trends, challenges, and regional assessment.

Keywords *Gelidium* · Agar market · Challenges · Growth dynamics · Agar segment

10.1 Introduction

The ocean spans over 70% of the earth's area and is a habitat of tremendous biodiversity, with over 10,00,000 marine organisms and approximately 1 billion distinct types of marine microorganisms (Costello et al. 2010). Most of the chemicals utilized for modern applications are obtained from natural resources. With its enormous richness and number of organisms, the marine environment promises an inexhaustible store of substances that might be exploited and used for the benefit of human well-being. The latest scientific issue is highly interested in active chemicals derived from marine creatures for medical applications. Over the past half a century, various bioactive compounds from the marine environment have been researched, characterized, and identified from bacteria, animals, and algal species (Papaleo et al. 2013; Gugliandolo et al. 2015). Marine-derived small molecules (MDSM) are chemicals obtained from marine organisms with a broad spectrum of biological functions. Several chemicals have been derived from marine macroorganisms, such as sponges, ascidians, bryozoans, and mollusks. Marine macro- and microorganisms provided a wealth of chemo diversity and a novel biological investigation scaffold (Vaske and Crews 2014). It has been reported that sea algae have indeed been employed for diverse applications since prehistoric times. Seaweeds are now harvested and employed in various industries, including direct human intake (traditionally in eastern countries, but more lately in western countries) (Dias et al. 2012). Some examples of algae include *Porphyra* spp., *Monostroma* spp., *Enteromorpha* spp., *Laminaria japonica*, *Undaria pinnatifida*, *Hizikia fusiforme*, *Cladosiphon okamuranus*, *Caulerpa lentillifera*, *Palmaria palmata*, *Chondrus crispus*, *Alaria esculenta*, *Gracilaria* spp., and many others (McHugh 2003). Algae have been used as fertilizers, soil conditioning, animal foods such as in aquaculture farms (Zhu et al. 2016; Mendoza Rodriguez et al. 2017), biofuels, and biofilters in wastewater treatment to remove inorganic and heavy metals (Mehta and Gaur 2005), food packaging materials, cosmetic industry, and bioactive molecules with antioxidant activities. Seaweeds are macroscopic algae that thrive on rocky coastlines and in the ocean and shallow ocean areas. Seaweeds are considered a sustainable and clean source of energy, chemicals, foods, and medications, with numerous nutritious, industrial, medicinal, and agricultural applications. Seaweed has been labeled as "Medicinal food of the twenty-first century" because they are employed as laxatives, tablets, and in the treatment of goiter, cancer, bone replacement surgery, and coronary operations. There are about 46 seaweed-based enterprises in India, 21 for agar production and 25 for alginate manufacturing. However, they are not operating at total capacity owing to a lack of raw material. Seaweed features prominently along the shores of Tamil Nadu, Gujarat, and the Lakshadweep and

Andaman and Nicobar islands. Rich seaweed beds can be found in Tamil Nadu, Andhra Pradesh, Odisha, Goa, Mumbai, Ratnagiri, Karwar, Varkala, Vizhinjam, and Pulicat.

Approximately 844 species of seaweed have been identified in the Indian ocean, with a capital supply of 58,715 tons (wet weight). India has 434 red algae, 194 brown algae, and 216 species of green algae, out of a total of 844 seaweed species. Agar is made from the red algae *Gelidium, Gelidiella acerosa, Gracilaria edulis, Gracilaria crassa, Gracilaria foliifera*, and *Gracilaria verrucose,* while the brown algae *Sargassum* spp., *Turbinaria* spp. are used to make alginates and liquid seaweed fertilizer. The existing supply of seaweed is insufficient to fulfill the raw material needs of the Indian seaweed Industry. Seaweed stock is typically harvested from the seabed in the shallow ocean along Tamil Nadu's southern coast. Furthermore, natural resources have been depleted due to constant, indiscriminate, and uncontrolled exploitation. Seaweed farming is a very profitable activity that uses low-cost, low-maintenance equipment and has a quick grow-out cycle.

10.2 Agar

Agar is one of the most frequently employed phycocolloids in the food industry, biotechnology industry, and cosmetics as a gel-forming factor. It is indeed derived from macrophytes, which are red seaweeds. Agar is a polymer that is the principal structural element in the cell walls of certain *Rhodophyceae* seaweed species. Brown algae (Ochrophyta-Phaeophyceae), red algae (Rhodophyta), and green algae (Chlorophyta). Algae from *Gelidium* and *Gracilaria* are the most common sources of agar (Marinho-Soriano 2001). *Gelidium sesquipedale* is still the most extensively utilized main ingredient for agar extraction in Spain and Morocco, and it usually generates agars with greater gel-forming ability than those obtained from *Gracilaria* species (Carmona et al. 1998; Pereira-Pacheco et al. 2007). Agarose and agaropectin are the two primary elements of agar. Agarose, a jelly ingredient of agar, consists of repeating units of overlapping β-d-galactopyranosyl and 3,6-anhydro-α-l-galactopyranosyl groups. In particular, polysaccharide chains with 3,6-anhydro-alpha-l-galactose elements were partly or replaced by alpha-1-galactose-6-sulfate units (Sousa et al. 2012). Agaropectin contains 5–10% sulfate esters and other chemicals such as methoxyl and pyruvic acid (Araki 1966). The nature of the substituents, quantity, and distribution will influence the physiochemical characteristics of agar. When agar becomes dissolved in heated water in certain settings, it becomes a mildly viscous liquid that could transform into a thermo-reversible gel when the temperature is reduced below a certain point. The agarose elements are essential for agar gelation. A two-step procedure is reported to be responsible for agar gel formation. The first stage transforms from a variable, disorganized coil configuration in solution to a rigid, organized arrangement that forms the gel network's connection sites. Furthermore, these helices can join to create thick bundles in particular circumstances, resulting in the production of powerful gels.

Agar is widely utilized in industrial, pharmaceutical, cosmetic, biotechnological, and biological applications. Thanks to its distinctive physiochemical qualities, like stabilizing, biodegradability, excellent water retention proportion, and thickening agents (Porse and Rudolph 2017).

10.2.1 Gelidium

Brown algae, red algae, and green algae are three types of seaweeds found in the ocean. But the study found that red algae, like *Gelidium,* have a higher carbohydrate level. It could be broken into simpler molecules like galactose and glucose, making them a promising biomass material for various applications. This algal biomass species had previously been employed to produce valuable compounds such as bio-ethanol and soluble sugars in prior investigations (importantly glucose, 3-hydroxymethylfurfural, formic acid, and clavulanic acid). Many scientists subjected algal biomass to rigorous treatment settings to generate a liquid fraction of organic products instead of solid cellulosic components. The producer of major agar is from *Gelidium* and *Gracilaria* species, and notably *Pterocladia*, and *Gelidiella*. *Gelidium* and *Pterocladia* were widely used to produce the highest quality agar, generating higher revenue. *Gelidium* types are found along the Japanese coast, but they have been reduced due to modern development and contamination. Bacteriological agar, sugar-receptive agar, and food-grade agar are the three main types of agar. Since their components can efficiently satisfy the demands for gel consistency and temperature variation (emulsification and gelling temperatures). For the production of bacteriological agar, *Gelidium* and *Pterocladia* are responsible. The beneficial properties include gelling temperatures of 32–36 °C and liquefying temperatures of 85–86 °C. Some *Gelidium* species identified in the eastern pacific provide sugar-responsive agar to a significant level. The gel stability of this type of agar has been retained as the sugar expands and the gel becomes flexible.

Natural communities of the red algae genus *Gelidium* are used to produce technical agars worldwide, and they are the industry's most important supply of raw materials because *Gelidium* aquaculture is not practical on a big scale (Melo et al. 1991). But although *Gelidium* agar accounts for just around 1.6% of the global phycocolloid supply, it has high gelling strength, and low gelling temperatures make it challenging to find the substitute with the agars derived from other species (Porse and Rudolph 2017). *Gelidium* marine biomass seems to be plentiful across the globe, but especially on the shallow shores of numerous east Asian nations such as Malaysia, Singapore, Japan, Korea, and China, in which the level of water is 3–10 ft. below the sea level. The yield of red algae has risen rapidly, from 5.3 million tons in 2006 to 10.8 million tons in 2011 (Kim et al. 2015). *Gracilaria* spp. (53%) and *Gelidium* spp. (44%) are the most common source of agar in the industry (Porse and Rudolph 2017). The agar material was isolated from *Gelidium*, the primary means of generating agar.

The food business creates a vast number of solid wastes that have been currently unvalidated and are commonly released into the environment without consideration (Seo et al. 2010). It contains a considerable amount of cellulose; this solid residue could be fully utilized in manufacturing nanocellulose. Seo and associates initially reported using two separate species of red algae, including *Gelidium amansii* and *Gelidium corneum*, for the pulp generation in the paper sector in 2010 (Seo et al. 2010). *Gelidiaceae* and *Gelidiellaceae* are the two families that make up the *Gelidales* order. There seem to be 13 *Gelidium* species in the Chinese ecosystem within each essential and distribution feature. Seaweeds are the main ingredient for various items with an estimated yearly amount of USD 5.5–6 billion. The worldwide seaweed industrial sector utilizes 7.5–8 million tons of wet seaweed annually, which comes from either found in nature seaweed or farmed crops, both of which are rapidly rising as the need for natural sources outperforms production. Wild, commercial seaweed harvesting takes place in about 35 nations worldwide.

10.2.2 Taxonomic Details of Gelidium Amansii

Kingdom: Plantae
Subkingdom: Biliphyta
Phylum: Rhodophyta
Subphylum: Eurhodophytina
Class: Florideophyceae
Subclass: Rhodymeniophycidae
Order: Gelidiales
Family: Gelidiaceae
Genus: *Gelidium*
Species: *Gelidium amansii*

10.3 Small- and Large-Scale Production of Agar

10.3.1 Extraction of Agar from Seaweed

Agar must have a variety of qualities depending on its intended application (i.e., different gel strengths, purity, color, and so on). While high-quality agar with gel strength of more than 700 g/cm^2 is often required for microbiological uses (Armisen 1995), medium to lesser gel strengths (30–200 g/cm^2) may indeed be preferable for food-related applications. The chemical properties and the molecular weight of the extracted agar greatly influence the seaweed species employed and the extraction methodology used as they will alter the chemical element and the molecular mass of the agar (Pereira-Pacheco et al. 2007). The commercial agar extraction techniques start with alkaline pretreatment, then the duration and power high temperatures and

intense pressure operations, high heat filtration techniques, and several freeze-thawing rounds to remove the waters. Extracting time and temperature on agar characteristics in *Gracilaria* species have been studied.

Agar can be extracted in several methods, but the most common process involves dissolving the agar in hot water, separating the agar from the cell wall debris by filtering, and isolating the agar from the dilute solution. For the separation of agar, many ways have been developed. The classic approach involves cutting the gel into strips and freezing it overnight before thawing it out in the sun the next day. The strips lose water with each freezing and cooling cycle due to the high syneresis created in an agar gel until a dry strip is formed. Agar can be manufactured in a variety of methods in the industry. In a simple scaled-up version of the conventional approach, one way includes freezing agar solutions in ice tanks. A recent approach, which only works for agar varieties with strong synereses, such as *Gracilaria*, is wrapping gel blocks in garments and pushing the water out. Large static concrete weights are commonly used for pressing. The pressed agar is often pressed again in hydraulic presses to reduce the water content before drying. Feeding shattered agar gel into massive filter presses and utilizing the pressure from the feed pumps to drive water out of the matrix is one form of the gel pressing the process. Hispanagar invented this technology in the 1960s, and it is now the most common way to press agar.

Another way is to dry the removed agar using a roller. This approach benefits using a wide range of agar species, including *Gelidium*, which is difficult to press. Alkali treatment can increase agar gel strength by eliminating some of the ester sulfates from the agar chain. Alkali treatment improves syneresis and makes agar pressing simpler. Agar is traditionally sold in strips or blocks. Agar powder is typically milled from commercial agar. Gel preparation procedures in modern carrageenan processing techniques are based on the agar gel press method. Not all agar weed types can be pressed, just as carrageenan, and some of the weeds preferred for their low syneresis, such as *Gelidium* kinds, are extremely difficult to press. In microbial plates, very low syneresis agar is preferred.

Many cultural techniques have been adopted to produce seaweeds as a cost-effective and ecologically sustainable solution to conserve natural sources. The crushed *Gelidium* seaweed has been employed to generate agar-based extracts, which only partly relied on the commercial procedure that entails a pretreatment of alkali of the whole dehydrated seaweed proceeded by a 120 °C hot water separation. The different ultrasound-assisted and microwave-assisted retrieval procedures were also analyzed to check how they matched up against the standard process. For the pretreatment of alkali, 40 g of dry powdered *Gelidium* powder was immersed in 1 L of 5% NaOH at 80 °C for 1.5 h. After that, the suspension was shaken using a muslin cloth to extract the solid fraction from the culture supernatant. The solid portion has been washed several times using running tap water to remove any leftover NaOH until the filtrate's pH becomes 7–8. This purified algal material was used for separation after the required agar extraction procedure. This will help them in strengthen their product portfolio.

The extraction step consisted of:

1. In an oil bath, heat the mixture to 90 °C for 4 h while stirring at 240 rpm.
2. An ultrasound-assisted separation was performed in a jacket wall container with a standard temperature of 90 °C for 30 min or 1 h, with an ultrasound probe with 100% power at 25 kHz.
3. A microwave-assisted separation utilizing lab-designed microwave equipment with a maximum power of 480 W for a total of 12 min of treatment divided into eight portions, with 1.5 min in each sector. 2 portions (3 min) of pre-heating time to attain >90 °C and six portions (9 min) of extraction time at 95–100 °C were included in the treatment.

While the solutions were still hot following the extraction operations, the agar-containing solutions were removed from the solid residues by filtering with a muslin cloth. The filtered extract was deposited in aluminum trays, refrigerator to room temperature, stored in a freezer overnight at −21 °C, and then dried for 36 h in a freeze dryer after being freeze-thawed to remove water-soluble impurities. The extraction efficiencies for purified preparations (obtained from alkaline pretreatment) were 2–5% for all the investigated procedures. However, compared to levels described in the literature for a range of agarophytes, which vary from 30–35% for certain *Gelidium* species, such outputs are negligible (Nil et al. 2016; Sousa-Pinto et al. 1996). A similarity of the yield potential disclosed with those found in the publications will not be suitable owing to the excessive variations in the extraction methods (extraction temperature, time, water-seaweed proportion, and particle size) and the hardware used to undertake the extraction, washing and filtration process. The variations in the contents could be caused by a variety of seaweeds, growing, and harvesting conditions.

A previous work using similar extraction and recovery circumstances showed significantly lower extraction yields (2–3%) for agar recovered from the *Gelidium* seaweed (Martínez-Sanz et al. 2019). A stronger alkaline treatment was done (1 g seaweed/10 mL NaOH solution at 90 °C for 2 h), and the time taken was halved. Considering the improvements in extraction procedures, the low yields reported here suggest that the alkaline treatment and extraction parameters have to be modified before they are employed for commercialization. This treatment should also improve agar recovery (Praiboon et al. 2006). Alkali treatment is most often used in the industry to loosen the seaweed cell walls, assisting agar extraction in the successive agar separation stages, thereby reducing the non-agar elements and most notably separating alkaline sulfate with the increase in 3,6-anhydro L-galactose content, which was providing agar gel strength properties (Nishinari and Watase 1983). If the settings are too extreme, phase separation can occur simultaneously, leading to the mechanical efficiencies in the alkali solution and throughout the thawing process. The degraded agar portion spreads into the aqueous solution, significantly decreasing the extraction yield (Martínez-Sanz et al. 2020; Kumar and Fotedar 2009; Ahmad et al. 2011; Lemus et al. 1991).

The data imply that some agar was broken down or lost during the alkaline treatment, resulting in considerably lower extraction efficiency for purified extracts than the semi-purified extracts. It must be recognized that among many semi-purified

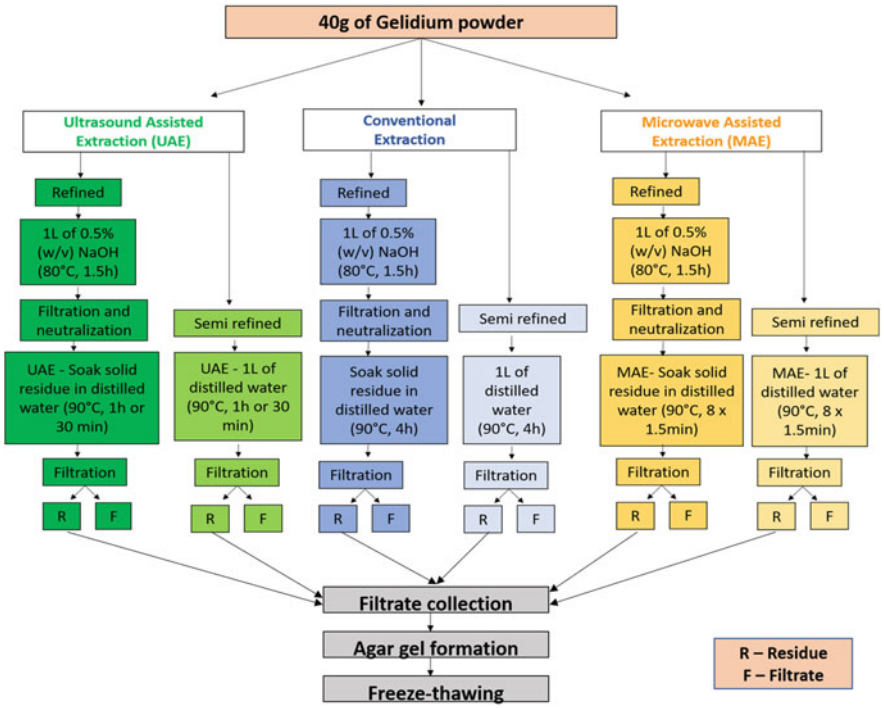

Fig. 10.1 The different extraction techniques of agar from *Gelidium*

extracts, the microwave-assisted recovery produced the lowest yields; however, the ultrasound-assisted recovery produced even better output than that of the standard extraction procedure with the fourfold reduction in extraction time. Ultrasound-assisted recovery method was discovered to be a potential extraction method (Martínez-Sanz et al. 2020). Most importantly, production tends to increase when the extraction time is increased, hinting that productivity could be increased even more if the extraction parameters are optimized and changed (Fig. 10.1).

10.3.2 Cultivation of Seaweed

Seaweed production, whether sexual or asexual, is complicated and requires specialized knowledge to carry out. Creating propagules is critical for ensuring a steady material supply for vegetative growth. The morphological factors such as identical or distinct gametophytes and sporophyte stages need to be determined to select the appropriate morphology to farm. Several seaweed species naturally decompose upon reproduction or reaction to different seasons, with extreme or quick modifications to behavioral changes. The spore propagules are grown in the lab and connected straight to ropes, nets, or threads, which are then linked to the ropes and nets and

then sown for the growing season. It is possible to employ high-quality, homogenous propagules from the strong and healthy parental lines. The creation of alternative resources to generate vital products such as food, fuel, cosmetics, and medicines is required worldwide due to heavy requirements on numerous natural resources. Large-scale seaweed farming production in Europe seems to have the opportunity to influence future resource demands significantly, but it must be done in a way that does not compromise the usage of the quality of indigenous marine ecosystems. Seaweed farming on a large scale level has been performed for centuries in Asia, but it has only recently become a commercial industry in Europe. The following factors will be used to choose suitable seaweed cultivation sites:

- Seaweed with a salinity of at least 30 parts per thousand (ppt)
- A sandy or rocky bottom with clear water
- The optimum temperature is 26–30 °C
- At low tide, the region should have a minimum water depth of 1.0 m, and mild water currents are desirable

10.3.3 Main Seaweed Cultivation Techniques

10.3.3.1 Line Cultivation

The planting is done using the primary cultivation methods, which are largely centered on using ropes and nets after collecting or creating propagules. Using the appropriate amount or density of propagules for seeding methods in ropes or nets might attain the required result. The planting entails deploying them at sea at a specific depth and arranging them in a present physical configuration depending on an optimum plant density per region. The density is determined by defining the number of plants inside and between the ropes and nets. Ropes and Nets provide a suitable medium for seaweed culture to grow in a range of layout length and diameter for various scenarios through floating, submerging, affordable, and widely distributed. Seaweed is connected to ropes of different lengths (from 10 to 50 m) and positioned in a linear agreement with differing separation distances based on the scale of seaweed yield, which differs according to the following Off-bottom—sowing close to the bottom anywhere around the shore, ideally with a minimum of 0.3 m of water on the top, at least tidal wave. This strategy is employed to cultivate tiny and often farmed seaweeds. A submerged hanging line is planted in the middle of water along the coast, submerged over several meters at large waves and displayed at lower waves. Growing seaweeds partially immersed on a floating line.

10.3.3.2 Net Farming

Net farming is identical to line cultivation in that seaweed propagules were linked to nets positioned at a specific water level, generally floating at the top or partially immersed. Sowing occurs at the surface by a floating frame structure constructed of bamboo or other items. Although stocking densities should be decided by species requirements and operational concerns based on the stocking density, growth and productivity have been demonstrated. The following are the approximate stocking and harvesting details

- Float rafting dimensions (made of bamboo poles having 7.5 to 10 cm diameter)
- Seed material required per raft: 50–60 kg/raft
- Seaweed harvested from one raft: 250 kg/raft
- Net produce from one raft (after deducting seed material): 200 kg/raft
- Price of dried seaweed (Rs. Per kg): 35

10.3.3.3 Tank or Pond Farming

For the sensitive species for new markets and intense manufacturing, the seaweeds cultivated in tanks within the regulated circumstances are essential. Bouncing provides stability for the seaweed that is tethered or free-floating. Seaweed aquaponics uses culture for sewage biofiltration in ponds, canals, or tanks for shrimps and fish farming to eliminate the extra minerals from the water.

10.3.3.4 Minor or Experimental Methods

Seaweeds linked to artificial platforms are planted directly on the ocean floor, stimulating native benthic vegetation like kelp forests and seaweed plains. Free-floating rafts: similar to line or net culture, but in geographical patterns with or without rigid frameworks that keep the structure and therefore it does not require holding and are allowed to float freely.

10.4 Cost and Market Analysis

In terms of industrial commercialization, seaweed hydrocolloids have sparked a great deal of interest. The phycocolloid business is growing at 2–3% per year, with worldwide annual output reaching 1,00,000 tons and a net selling price of more than USD 1.1 billion. Thus, according to Rhein-Knudsen et al. (2015) and Veeragurunathan et al. (2019), agar had the highest selling value per kilogram (18 USD/kg), followed by alginates (12 USD) and carrageenans (10.4%). The need for *Gelidium* agar has already outpaced the supply. *Gelidium's* bacteriological

agar consumption increased from 250 tons to around 700 tons. It is a greater phycocolloid, with a 2019 market worth USD 173 million (Guerrero et al. 2014). Economic factors are essential in seaweed farming; however, they are rarely discussed in cultivation publications. The chosen type of farming is determined by the methodology that allows the farmers to make the most profit. That, in return, is dependent on the farmer's investments to take full advantage of the biomass produced.

Nonetheless, no systematic cost-benefit study for seaweed farming has been done. The quality and amount of agar and other products derived from the seaweed determine the commercial worth. The economic feasibility of agarophytic biomass for value addition has been established by the price and value of the products. In 2020, Agar-agar was the world's 3320th most traded product, with a total trade of 259 M dollars. Between 2019 and 2020, the export of Agar-agar decreased by −6.88%, from 279 M Dollars to 259 M Dollars. Trade-in Agar-agar represents 0.0015% of total world trade. In 2020 the top exporters of Agar-agar were China ($69 M), Spain ($42.2 M), Chile ($31.8 M), Morocco ($27.7 M), and Italy ($13.7 M). In 2020 the top importers of Agar-agar were Japan ($41.2 M), the United States ($33.2 M), Russia ($18.1 M), Italy ($17.5 M), and China ($13.7 M). In 2018 the average tariff for Agar-agar was 4.15%, making it the 4920th lowest tariff using the HS6 product classification. The countries with the highest import tariff for Agar-agar are Poland (77.7%), Tunisia (36%), Bahamas (31.2%), Ethiopia (29.7%), and Bhutan (29.4%). The countries with lowest tariff Angolo (0%), Egypt (0%), Kenya (0%), Mauritius (0%), and Rwanda (0%).

10.4.1 Agar Market Overview

The agar market segment was valued at 239 million dollars in 2020, and it is expected to increase at a CARG of 4.8% between 2021 and 2026. Agar is a jelly-like material derived from algae. Agarose is a polymer that forms structural support in the cell walls of certain algae species and is released when they are boiled. They belong to the Rhodophyta phylum; these algae are sometimes known as macrophytes. Agar is a safe substance commonly used in several cuisines as a vegan substitute for gelatin. The market is being driven by increasing demand for agar as a thickening agent in ice creams, cakes, and many other sweets and clearing components in the brewing and wine-making industries. It is also used as an emulsifier in milk products and in preserving meat, fish, and poultry. The technical developments and increased knowledge about the advantages of agar are expected to propel the agar market forward during the projected timeframe 2021–2026 (Arc 2021; Verified Market Research 2020; Intelligence 2021).

10.4.2 Agar Market Segment

The business is split into strips, powder, flakes, and many others based on the product type. Due to the rising demand from the producers that create pure vegetarian, vegan, and kosher meals in significant numbers, the strips category accounted for the fastest market share in 2020, and it is predicted to grow swiftly at a CARG of 6.6% over the projection period 2021–2026. The food and beverage industry will dominate the agar market in 2020. The gum's dominance is due to its use as a gelling agent in aerated confectionery items such as marshmallows, and fruit candy, making it an excellent option. Moreover, the product's excellent heat tolerance and stabilizing properties have increased the demand for ice creams, pie fillings, and other desserts in the pastry and baking sectors. Consumer knowledge of the medical benefits of baked products, such as lower fat content and better digestion, is expected to fuel market expansion. The cosmetics application sector saw the most robust growth in 2020, expanding at a CARG of 6.5% between 2021 and 2026. The increase in the use of agar in skincare products is because the seaweed from which agar is collected is high in minerals such as calcium, magnesium, copper, and iron. It moisturizes the skin while hardening and adhering other components together. Geographically, Asian Pacific led the agar market in 2020, with a 38% share, followed by Europe and North America. A growing inclination toward vegan goods and the presence of a significant number of agar manufacturing sectors that engage significantly in research are expected to boost the agar market. Because of the significant demand from the confectionery industries, the European region is expected to witness agar industry growth (Arc 2021; Verified Market Research 2020; Intelligence 2021).

10.4.3 Agar Market Opportunities

Growing recognition of the health advantage of agar, which includes numerous minerals, and vitamins such as phosphorous, calcium, iron, iodine, and others, has been anticipated to fuel market expansion. Agar's market is predicted to rise due to the rising need from the food and beverage sector as a thickener and stabilizing ingredient in a variety of food items. The increased demand for agar as a preserve in meat and poultry products contributes to market expansion. Furthermore, increased use of gelatin alternative, thanks to its good diffusion properties, purity, and low adhesiveness, seems likely to boost agar market expansion. In terms of revenue, Europe dominated the worldwide agar market in 2018, with a 42% market share, followed by North America and the Asia Pacific, respectively. A key reason for stymieing agar market growth is the restricted supply of agar generated from scarce seaweed.

Additionally, agar production and supply are restricted in a specific territory, which is forecasted to hamper the market's growth. Various agar firms have

discontinued their distribution of goods due to the scarcity of raw ingredients. As a result, this issue is expected to limit the expansion of the agar market. The increased usage of agar in culture media and dentistry as a hydrocolloid adhesive layer and rising demand from the pharmaceutical sector for suspensions, emulsification, pills, and injections in surgical lubricants seems to propel the agar market to new heights. Agar is also used in the cosmetic sector to make moisturizers and other beauty products. It is more widely used as a significant ingredient in cosmetic items such as lipsticks, ointments, creams, soaps, and lotions (Arc 2021; Verified Market Research 2020; Intelligence 2021).

10.4.4 Agar Market: Growth Dynamics

Agar is a kind of seaweed that is widely utilized in various sectors. Agar is used in the food and beverage sector to make various items such as ice creams, bakery goods, dairy goods, confectioneries, and drinks. As a result, higher demand from the makers of such items is expected to aid in the growth of the worldwide agar market between 2020 and 2030. In the following years, the worldwide market is expected to see a steady increase in revenue. The increased understanding of the various uses and health advantages of employing agar is a major factor in this achievement. Agar is generally recognized as a hunger suppressant due to over 80% fiber.

Consequently, agar is often taken by those trying to lose weight. This element is boosting the worldwide agar market's demand pathways. In all parts of the globe, agar is regarded as one of the essential cooking materials. It is frequently used for gelatin in a variety of dishes. The usage of agar as a thickening agent in fruit preserves, soups, ice creams, and various other goods. This feature supports vendors in the worldwide agar market in generating large scales. The worldwide agar market has seen several technical breakthroughs in recent years. In this sector, the metal scraper is an example of modern technology. The innovative equipment makes it simple to harvest agar, increasing the total agar production. This indicates that the worldwide agar market will expand rapidly in the next few years. The worldwide agar market's major competitors are facing very high competition. One of the significant causes for this reason is the presence of several operating businesses. Several manufacturers in the worldwide agar market are focusing on introducing novel products to stay competitive. This move will aid in the expansion of their product line. Other competitors are increasing their efforts to improve their manufacturing capacity (Arc 2021; Verified Market Research 2020; Intelligence 2021).

10.4.5 Agar Market Trends

Over the market expansion, the growing trend of vegan dietary preferences, together with the developing food and beverage sector, is expected to boost the market expansion of agar. Furthermore, the rising demand for confectionery food items is projected to boost the future of the agar market. Another critical factor projected to drive the market expansion is the increasing consumption of a healthier diet. Given agar is perfect for customers looking to lose weight and keep an excellent health. It has a low saturated fat and cholesterol level, which is likely to promote market growth in the coming years (Arc 2021; Verified Market Research 2020; Intelligence 2021).

10.4.6 Agar Market Challenge

Seaweed cell walls contain agar, obtained from the low altitudes in water sources and then processed to generate a jelly-like substance. Agar was first created by boiling water and extracting it, but as technology evolves, it is now widely manufactured on a large scale using sophisticated technology, which ultimately leads to enhanced quality of the finished product. Greater consumer awareness of healthy ingredients and foods and increasing approval processes for using agar as a flavor enhancer have been predicted to propel the market upward over 2021–2026. Given various circumstances, shipping and mobility constraints have led to a lack of agricultural goods for various sectors, reducing agar market sales and profitability. Export and import restrictions have disrupted the global supply chain, making it challenging to get seaweed from other countries, resulting in a shortage of raw materials needed for manufacturing. During the projected 2020–2025, these issues are expected to be a key obstacle to the global agar market's development. *Gelidium's* need for bacteriological agar and agarose has risen from 250 and 15 tons to around 700 and 50 tons, respectively (Santos 1993). Callaway (2015) recently pointed out that demand for *Gelidium* agar is currently stronger than availability. The shortage of bacteriological and technical agars had pushed wholesale costs to a peak of roughly USD 35–45 per kg, approximately double the price before the shortage occurred (Arc 2021; Verified Market Research 2020; Intelligence 2021).

10.4.7 Agar Market: Regional Assessment

Morocco is indeed the world's fifth-largest agar supplier. The yearly exporting volume of agar in Morocco ranged from 905 tons to 1066 tons between 2012 and 2016. Locally produced agar has a limit of 1247 tons per year that can be exported. *Gelidium corneum* is the most common species used in local agar production.

According to the current statistical information on the *Gelidium corneum* population, this species is overexploited. In addition, the number of *Gelidium* growers without the need for a harvesting license had become out of line. The collecting season was frequently neglected. An active commitment to basic research is needed to examine different algal sources from the Moroccan coast with vital industrial interests. The Iberian Peninsula and Morocco are the most populous geographical areas. The highest production comes from Spain (7100 tons in 1989, compared to 5500–6000 tons in an ordinary year); but, most of the Spanish *Gelidium* has been highly polluted with some other seaweeds so that an average year harvest would be closer to 3000 tons when compared to the harvest in nearby Portugal, where 2000 of pure *Gelidium* were harvested (Arc 2021; Verified Market Research 2020; Intelligence 2021).

10.4.8 Production Cost Analysis

The cultivation cost for *Gelidium* production is shown in Table 10.1. From the table, we suggest the entrepreneur interested in cultivating *Gelidium* can profit up to Rs.9,50,000 in the first and subsequently Rs. 24,50,000 in the following years.

Table 10.1 Probable unit cost for the cultivation of *Gelidium* spp. through tube net/raft cultivation method

Component	Cost/unit
Cost of one bamboo raft (3 m × 3 m) or tube net, and input costs	Rs. 2000
Crop duration per cycle	45
Capital cost (for 1000 raft) @ Rs.1500/− per raft	Rs. 15,00,000
Recurring cost for first cycle (for 1000 rafts including seed stock cost) @Rs.500/− per raft	Rs. 5,00,000
Recurring cost from second to sixth cycle (for 1000 rafts excluding seed stock cost)	Rs. 12,50,000
Total cost	Rs. 32,50,000
Price of dried seaweed (Rs per kg)	Rs. 35
Annual seaweed production (after retaining 50 kg seed stock for next crop; total seaweed production from 1000 rafts; 6 cycles) (wet weight in kg)	12,00,000 kg
Total dried seaweed production (from 1000 raft; 6 cycles; @10% of wet weight) (dry weight in kg)	1,20,000 kg
Price of dried seaweed (Rs. per kg)	Rs. 35
Gross revenue	Rs. 42,00,000
Net revenue first year (gross revenue - Total cost)	Rs. 9,50,000
Net revenue from second year onwards	Rs. 24,50,000

10.5 Future Perspective of the Seaweed Industry

The global demand for seaweed and its products has raised the interest in their production and the desire of stakeholders to invest more broadly in the manufacturing of numerous algal species that could meet the requirement of the various economic sectors. Seaweed is essential for reducing the need to feed a growing population on a globe where agriculture crops would run out of land, as seaweed cultivation does not compete for inland agricultural land, freshwater, or agricultural fertilizers. There is a demand to cultivate and harvest more seaweed to meet the increased demand for seaweed and seaweed-based goods. Due to commercial overexploitation, the scarcity of seaweed poses a threat to wild seaweed populations, thereby producing major environmental concerns. As a result, more reliable aquaculture systems in various settings are required (inland, nearshore, offshore). Several sectors can only use chemicals from seaweed, and Science is progressing in understanding how the metabolism of seaweeds works for identifying the potential metabolite. Following that, seaweed aquaculture technology has advanced tremendously in the last 70 years, primarily in Asia, America, and Europe. However, there are still numerous obstacles to overcome, one of which is that it is still difficult to adopt cost-effective and sustainable methods in certain parts of the world. The main elements that have to be considered in seaweed aquaculture include disease resistance, fast-growing seaweed species, and a high concentration of desired molecules methodologies and technologies. The development of more robust and cost-effective aquaculture systems that can withstand storm events and maintain cultivation for a more extended period. Progress in new cultivation technologies that are more efficient and environmentally friendly is essential. Thus a multidisciplinary team is needed to reduce the hazards involved in the seaweed aquaculture. There is a risk of over-dosing aquaculture with chemical fertilizers or other substances that can decrease water quality and harm the ecosystem, and caution is required to avoid over-exploitation of ecosystem sustainability. Thus, IMTA appears to be the best solution for sustainability and profit.

10.6 Conclusion

Traditional extraction technologies have been extensively researched and successfully employed for commercial purposes. The foundation for enhancing the agar extraction process is to understand how each phase impacts the quality and quantity of agar. Alkali treatment alone reduced the weight of algae but slowed their disintegration, resulting in a lesser yield. The increase in the production can be achieved by acidifying algal hardening following alkali treatment. Although enzyme treatment alone will not provide agar with high purity, following acidification and bleaching will yield agar with poor gel strength and high sulfate concentration. Macroalgae production has an enormous commercial opportunity and the

opportunity to improve the long-term viability of fish farming operations through technology solutions. It also provides possibilities for advancement and social change in underdeveloped coastal areas worldwide, where overfishing is a common problem. Yet, history has consistently stated that extensive farming and domestication often lead to irreparable biodiversity losses. For the long-term viability of algae farming as a socially acceptable, ecologically sustainable industry, a massive effort in basic research is needed to evaluate the genetic sources and design sufficient genetic conserving regulations, which are now lacking. Besides direct localized implications such as reduced light, nutritional content, and elevated seawater temperature, it is also critical to begin examining the long-term environmental impacts of algae production.

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Chapter 11
Mass Production of Valuable Pro-Vitamin a Pigment from a Microbe, Cost Analysis and Targeting It for Health Benefiting Purpose

Daiji Brahma, Debjani Dutta, and Sibabrata Mukherjee

Abstract Microbial pigment production has become popular these days due to its numerous application in various fields like pharmaceutical, food, and cosmetic industries. Color of an object attributes its quality that attracts our attention. Increasing awareness of toxicity of synthetic colors has surfaced consumers' preference for natural food color additives. Microbial pigments are preferred these days owing to their stability, cost-effectiveness, feasibility, availability throughout the year, and sustainability toward environmental aspects. In food industry, food colorants usage from compound is highly regulated. Safety of microbial pigments is determined through their harmless, nonpathogenic, and biodegradable nature. Bacteria are the most explored microorganism due to their adaptable nature. Hence, research on bacterial pigments should be a sustainable approach for cost reduction and increasing applicability for industrial production. Yield and productivity determines the economics and viability of a bioprocess. Organic chemistry and metabolic engineering advancement have enabled microbial mass production. Development of new tools through recombinant DNA technology has improved yield through genetic manipulation of biosynthetic pathway. There is great scope of metabolic engineering of bacteria for industrial application which may solve certain metabolic diseases and environmental problems. These days, nanotechnology has been applied in food industry for pigment formulation to increase stability, shelf life, leading to better delivery systems for food and feed.

Keywords Microbial pigment · Carotenoids · Food color · Cost-effective · Bacteria

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11.1 Introduction

Recently, natural products produced by microorganisms have gained much attention for application in pharmaceutical, food, cosmetics, textiles, etc. These natural products from microorganisms include bioactive compounds (Ahmed et al. 2020). Bioactive compounds are the metabolites and by-products of microorganisms like pigments, polyphenols, fatty acids, vitamins, organic acids, etc. (Ramesh et al. 2019). Pigments are the naturally or chemically synthesized colored compounds. Before the modern era, natural organic pigments were part of ornaments, cosmetics, and textile dyeing (Parmar and Singh 2018). The first chemically synthesized pigment, Prussian Blue, was made in Germany in 1704 by Diesbach. The widely accepted invention of artificial pigments due to their stability, cost, and coloring ability led to a surge in the manufacture of artificial colors (Barnett et al. 2006). However, synthetic pigments showed their scarce nutrients and hazardous nature toward health and the environment, which got banned due to their potentially carcinogenic and teratogenic nature. These have caused rising demand for highly safe, naturally occurring edible colors in recent years (Wang et al. 2006). Color is the essential quality of any object that attracts our attention. Previously, consumers were unaware of the pigment type used in food and cosmetics (Dufosse 2006). However, recently the concern about the toxic nature of synthetic colors has moved consumer preference toward natural food additives, expanding the demand of natural pigments (Babu and Shenolikar 1995). Therefore, natural pigment production was focused on overcoming the unlimited usage of synthetic pigments from a few years. These have necessitated the development of natural pigments and accentuated optimization technologies to meet their worldwide demand (Es-Safi 2004). Though plants, vegetables, and roots are good sources of pigments, microorganisms are receiving much attention these days due to their stability, low cost, and availability for cultivation throughout the year (Pattnaik et al. 1997; Usman et al. 2017). Natural pigments derived from microbes are employed in research due to their availability, feasibility, and sustainability toward environmental aspects (Ramesh et al. 2019). Researchers have recognized critical applications of microbial pigments in cosmetics, food, pharmaceuticals, and textiles that exhibit cytotoxic, antioxidant, antimicrobial, anticancer, and antitumor activities that deserve importance in current years (Sen et al. 2019).

Natural products have become fundamental aspects of research to discover new natural colorants for multi-purpose applications. The source of food colorants are highly regulated by The United States Food and Drug Administration (FDA), the European Food Standards Authority (EFSA), and The World Health Organization (WHO), hence safe dosages of colors in food, drugs, and cosmetic items have been advocated (Oplatowska-Stachowiak and Elliott 2015, Wrolstad and Culver 2012). The pigment sources exempted from colorants certification include microbes, plants, minerals, animals, and synthesized compounds identical to natural products (Simon et al. 2017). The non-allergic, non-poisonous, biodegradable, eco-friendly, and harmless nature of the natural product indicates the safety of compounds

(Aberoumand 2011; Burrows 2009). Microbial pigments used as food colors include carotenoids, flavins, indigoids, melanins, pheomelanin, canthaxanthin, astaxanthin, and β-carotene (Sen et al. 2019). Bacteria is the most explored microorganism due to its adaptable nature. The classical pigment-producing microbes are mostly tolerant to pH, different carbon and nitrogen source, temperature, and minerals, producing a reasonable yield (Kumar et al. 2015).

In the current situation, work on bacterial pigments should be a primary concern owing to its research and developmental stage to reduce the cost of pigment production from various sources, thereby increasing applicability for industrial production. Fermentation is a rapid production process compared to other chemical processes (He et al. 2017). The crucial factors driving the economic feasibility of the process are compound yield and productivity. Breakthrough in organic chemistry and metabolic engineering fields has led to huge production of microbial compounds (Kumar and Prasad 2011). The target metabolite production could be increased through genetic engineering within cells to upregulate compound synthesis. Recombinant DNA technology and other related technologies have contributed to yield improvements through genetic manipulation of biosynthetic pathways (Sen et al. 2019). Optimization of various fermentation parameters could enhance metabolite production. Metabolic engineering of bacteria showed a great scope of industrial application, potentially solving certain metabolic diseases and environmental problems (Kumar and Prasad 2011). Appropriate microbial fermentation strategies, optimization study, and cost-effective extraction processes have been applied so far. Recent technology such as nanotechnology has also been applied for pigment formulation to increase stability, shelf life, and better delivery systems (He et al. 2017). This book chapter focuses on the potential usage of microbial pigment in food industries, advantages, and overcoming challenges faced during production and application. The developmental strategy for increasing yield has been elucidated. Analysis of pigment production, product development, and marketing has been discussed.

11.2 Different Sources of Natural Pigments

Pigments derived from natural sources are called natural pigments. The sources of natural pigments are broadly classified as plants, animals, minerals, and microbial pigments.

11.2.1 Plants

Plants are the primary source of natural pigments (Saxena and Raja 2014). The plant's unique features capture the light energy and convert it into sugars via the photosynthesis process. Different parts of plant-derived pigments from leaves,

stems, bark, flowers, fruits, etc., have wide applications as food ingredients and medicines. The essential pigments found in plants include chlorophyll, carotenoids, anthocyanins, flavonoids, etc. (Saxena and Raja 2014).

11.2.1.1 Chlorophyll

One of the plants' primary pigments and green color appearance is due to a pigment called chlorophyll. It synthesizes glucose, a plant's food source through the photosynthesis process (Hynninen and Hynninen and Leppakases 2002). The blue and red light of solar radiation is absorbed by chlorophyll at 430 nm and 660 nm, reflecting the green spectrum (Inanc 2011; Pareek et al. 2018). Based on their structural function, chlorophylls are classified as chlorophyll-a found in all higher plants, algae, and cyanobacteria; chlorophyll-b of higher plants, and green algae; some marine and photosynthetic chlorophyll-c; the red algae synthesized chlorophyll-d and chlorophyll-e which is found only in algae. Chlorophyll a and b are the primary photosynthetic pigments (Pareek et al. 2018).

11.2.1.2 Carotenoids

They are hydrophobic red, orange, and yellow-colored pigments classified into xanthophylls (oxygen-containing) and carotenes (oxygen-free) (Britton et al. 2008; Bendich and Olson 1989). Higher plants, some algae and bacteria synthesized carotenoids and were also widely distributed in animals via diet. Carotenoids possess antioxidant properties through scavenging oxygen radicals released from chloroplasts during photosynthesis. It protects cellular constituents like DNA and protein from free radical damage. The commonly found carotenoids in food are lycopene, beta-carotene, alpha-carotene, beta-cryptoxanthin, zeaxanthin, lutein, and astaxanthin (Miller et al. 1996). The pro-vitamin A carotenoids are beta-cryptoxanthin, alpha-carotene, and beta-carotene. Apricots, persimmons, oranges, and a few citrus fruits are good sources of pro-vitamin A carotenoid (Lea 1988).

11.2.1.3 Anthocyanins

Anthocyanins are the most significant group of hydrophilic pigments (Harborne 1998). They impart purple, red, blue, and orange colors to various sources of fruits and vegetables such as blueberries, cherries, raspberries, etc. (Mazza 2007). Belonging to a family of flavonoid compounds, they can form flavylium cations (Mazza 2007). There are about 17 anthocyanidins found in nature. The common anthocyanin pigments in the human diet are cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin (Jaganath and Jaganath and Crozier 2010). Epidemiologists' study suggests that anthocyanin consumption could reduce the risk of few disease owing to their antioxidant and anti-inflammatory properties (Wang and

Wang and Lin 2000). Also, anthocyanins administration could enhance and improve night vision or overall vision (Lila 2004; Miguel 2011).

11.2.1.4 Flavonoids

Flavonoids are biologically active low molecular weight secondary metabolites produced by plants. The reports show that over 10,000 structural variants of flavonoids are available. Based on their pH, flavonoids appear red, blue, purple, and other dark colors. In plants, flavonoids play essential roles in auxin transportation, root and shoot development, pollination, reactive oxygen species modulation, and symbiotic bacteria signaling in the legume rhizobium symbiosis. This pigment possesses medicinal properties against bacteria, fungi, and virus and is an essential dietary component for humans and animals which also have a role in developing new pharmaceuticals (Weston and Mathesius 2013; Cutler et al. 2007; Hassan and Mathesius 2012). The most potent flavonoids having antimicrobial properties are isoflavonoids, flavanones, and pterocarpans (Armero et al. 2001; Naoumkina et al. 2010).

11.2.2 Minerals

Mineral pigments like cinnabar, red and yellow ocher, malachite, etc., have history for coloration purposes. Since old times, rubber, linoleum, plastics, ceramics, glass, colored pencils, and other products were painted with mineral pigments. Popular mineral pigments are ultramarine, arsenic, cadmium, carbon, cobalt, chromium, lead, manganese, mercury, molybdenum, titanium, etc. (Habashi 2016).

11.2.3 Animal

Insects are the primary animal source of natural dyes importantly due to various pigment molecules in the various physical structures. The main pigments are aphins, pterins, anthraquinones, ommochromes, melanins, carotenoids, and flavonoids which are hydrophilic and hydrophobic in nature; hydrophilic insect pigments include papiliochromes, anthocyanins, and flavonoids (Shamim et al. 2014). Anthraquinones belong to a huge class of dyes and pigments mainly produced by coccids (Morgan 2010).

Aphins is a pigment with varying colors like green, red, brown, or black found in aphids. Pterins are cyclic compounds containing nitrogen atom belonging to a class called pteridines (Brown 1988). Ommochrome pigments work as screening pigments in insect's eyes by cutting out stray light (Futahashi et al. 2012). Melanin pigments found in insect cuticles of Blattodea, Diptera, Coleoptera, and Lepidoptera

strengthen the cuticle and protects against UV damage. Carotenoids pigment like carotene couple with proteins to give blue, green, blue-green, and red colors in integument and hemolymph of insects. Some of the carotene derivatives found in insects are lycopene, β-carotene, zeaxanthin, violaxanthin, astaxanthin, xanthophylls, and β-carotene monoepoxide (Chapman 2013).

11.2.4 Microbial and Fungal Origin

Microbes and fungal origin pigments produced as secondary metabolites have been pretty popular in recent times. *Bacillus, Brevibacterium, Flavobacterium, Pseudomonas*, and *Rhodococcus* spp. are well-known pigment-producing bacteria (Joshi et al. 2003). Microbial pigment sources offer an advantage, like cost-effective substrates under controlled conditions. Beta-cryptoxanthin pigment, a pro-vitamin A carotenoid, was produced by *Kocuria marina* DAGII using low-cost dairy waste (Mitra and Dutta 2018). *Monascus purpureus pigments* are used to color some traditional oriental food items. *Trichoderma* sp. has been used to color silk and wool (Gupta et al. 2013). Dyes derived from mushroom species like *Cortinarius* are excellent dyes which have become popular since the 1970s (Saxena and Raja 2014). Yeast, fungi, bacteria, and algae-derived pigments are carotenoids, anthraquinone, and chlorophyll (Joshi et al. 2003). As discussed earlier, microbial-derived carotenoids play a fundamental role due to pro-vitamin, antioxidant, or possible tumors-inhibiting agents constituting valuable compounds for several industrial applications (Olson 1989, El-Banna et al. 2012a, b). *Carotenoids* are lipophilic naturally colored pigments belonging to the class of tetraterpenoid pigmented compounds naturally produced by diverse organisms, including plants, fungi, and bacteria (Zoz et al. 2015). The growing scientific evidence and reports on the potential health benefits of carotenoid pigments caused increased interest in commercialization during the past years (Libkind et al. 2004). Industrial carotenoid pigments like carotene, astaxanthin, and torularhodin are used as natural food colorants or food additives (Ungureanu et al. 2011). Several algae such as *Dunaliella, Dictyococcus*, and *Haematococcus;* many eubacteria and archaebacteria; filamentous fungi like Ascomycetes; yeasts such as *Cryptococcus, Phaffia, Rhodosporidium, Rhodotorula, Sporidiobolus*, and *Sporobolomyces* have been reported to produce carotenoids (Ungureanu et al. 2011). Bacteria are a good source of carotenoid pigment. *Streptomyces chrestomyceticus* subsp. *rubescens* has been employed to produce lycopene, while zeaxanthin and lutein are produced from *Flavobacterium* sp. (Andrewes et al. 1976; Potera 2010). Valuable sources of beta-carotene used earlier were either Ascomycetes (*Penicillium sclerotiorum* and *Neurospora crassa*), or Deuteromycetes (*Rhodotorula aurantiaca*), yielding beta-carotene in less amount. Many yeasts like (*Rhodotorula, Yarrowia lipolytica, Cryptococcus* sp., *Phaffia rhodozyma*) are good sources of microbial pigments. Astaxanthin, a red pigment, is found in animals but rarely in microorganisms like *Pseudomonas rhodozyma*. The manufacturing and selling of

natural carotene were first done by Mis Betatene Limited, founded in 1985 (Joshi et al. 2003).

11.2.4.1 Crucial Factors for Pigment Production by Microorganisms

A classical pigment producing microorganisms should satisfy the following criteria during pigment production: Utilization ability of different nitrogen and carbon source; tolerance to pH, temperature and mineral concentration; having moderate growth conditions; reasonable color yield; non-toxic and non-pathogenic nature of microbe; easily separable from the cell mass and adaptable to culture and saline conditions (Andrewes et al. 1976).

11.3 Importance of Natural Pigments over Synthetic Pigments

The utilization of synthetic food colors is a cause of the onset of cancers and chronic diseases in recent years (Potera 2010). Several animal models revealed carcinogenic artificial colors, including Brilliant Blue, Indigotine, Fast green FCF, Allura red AC, Tartrazine, and Yellow 6. Caffeine, the widely used colorant in soft drinks, causes heart defects (Ranaweera et al. 2020). Earlier, consumers were unaware of the synthetic pigments in food coloring, but recently, synthetic pigments' toxic and health hazard nature has inclined people toward natural pigments preference (Delgado-Vargas et al. 2000). Food and Drug Administration (FDA) has classified the food colorants as certifiable and exempt from certification. Certifiable pigments are manufactured and subdivided as synthetic pigments and lakes. Exempt from certification, pigments are derived from natural sources such as vegetables, minerals, or animals and manufactured counterparts of natural derivatives (Delgado-Vargas et al. 2000). Fresh fruits and vegetables are rich sources of many diverse nutrients, including vitamins/antioxidants, trace minerals/micronutrients, and potent chemoprotectant properties (Aruoma 1994; Bagchi et al. 2000). However, plant pigments have drawbacks like instability against light, heat or adverse pH, low water solubility, and non-availability throughout the year. Hence, microbial pigments are essential due to their numerous clinical characteristics, stability, and highly abundant nature (Kumar et al. 2015).

11.4 Production of Natural Pigments by Microbial Fermentation

As mentioned earlier, sustainable production of microbial pigments requires cost-effective processes to increase their applicability for industrial production. Microbial growth and pigment production require growth medium containing substrates which support mass multiplication and enhanced pigment production. Fermentation is a fast and productive process compared to other chemical processes controlled by certain factors:

11.4.1 Type of Fermentation

Different types of fermentation used for microbial growth and pigment production are solid-state fermentation and submerged fermentation.

11.4.1.1 Solid-State Fermentation

For centuries, solid-state fermentation (SSF) has been applied for large- and small-scale food processing and production of traditional fermented foods like soybean and fermented rice and brewing of the Japanese rice wine (sake)) (Krishna 2005). The selected microbes of SSF grow on low moisture content solid substrates. SSF is an essential technique in biotechnology for traditionally processing fermented foods like mold-ripened cheese (Musaalbakri and Colin 2017). The solid substrates utilized for SSF are rice straw, sugarcane bagasse, wheat bran, etc. The advantage of solid substrate usage in SSF is the easy recycling of nutrient-rich waste materials as substrates. This technique allows slow and steady substrate utilization, uses the same substrate for prolonged periods, and supports the controlled release of nutrients. SSF is done for fungi and microorganisms, which need low moisture. It is inapplicable for organisms requiring high moisture content (Babu and Satyanarayana 1996).

11.4.1.2 Submerged Fermentation (SmF)/Liquid Fermentation (LF)

Submerged fermentation utilizes liquid substrates containing bioactive compounds produced during the fermentation process. There is rapid utilization of substrates which requires constant replacement or nutrients supplement. This fermentation technique is performed for microorganisms which require high water content such as bacteria. Product purification is easier in SmF process and is mostly used for the extraction of secondary metabolites in liquid form (Subramaniyam and Vimala 2012). Till now, submerged fermenters are mostly used in the industry because it saves space and are amenable to engineering control and design.

11.4.1.3 Different Modes of Operation of Submerged Fermentation for Microbial Pigment Production

Batch, fed-batch, and continuous process are the different operation modes of submerged fermentation. Batch fermentation involves the microorganism introduction to a specific medium volume in a fermenter followed by utilization of nutrients and decomposition of by-products upon microbial growth. The environment of the culture changes continuously, and at the end of the run broth is collected (Annam 2020). Merits of batch operation are smooth operation and less contamination. Demerits of batch fermentation are production of less cell densities and extended downtime between batches. Fed-batch is a standard mode of operation in the bioprocess industry (Annam 2020). In fed-batch, microorganisms are introduced to grow under a batch regime for a specific time; fermenter is fed by nutrients in increments throughout the remaining duration.

At the end of each cycle, the suspension of the entire culture is collected. Substrate limitation and time profile are designed to determine the start feed and avoid non-excessive substrate while fully supporting microbial growth. Fresh nutrient addition leads to extensive biomass deposits during the log phase benefiting the targeted high product yield (Li and Sha 2016). There is simultaneous addition of fresh medium in the fermenter during the continuous mode, while harvesting the utilized medium on the other hand maintains the culture volume. Hence, the maximum working volume of the vessel does not limit the amount of fresh medium added to the culture in the course of the process. Furthermore, keeping the working volume constant simplifies culture scale-up based on a constant power-to-volume strategy (Li and Sha 2016). However, the long cultivation time could complicate the downstream processing (Ying and Sha 2020).

11.4.1.4 Factors Influencing SSF and SMF

Different factors influencing SSF and SMF are microorganism, temperature, pH, aeration rate, particle size, and agitation.

Microorganism

Suitable microorganism selection is crucial for the fermentation process. SSF process is primarily for the fermentation activity of pure microbial cultures like fungi and bacteria (Krishna 2005). Application of a single microorganism in the industrial SSF process has merits of improved substrate utilization rate and control over product formation (Subramaniyam and Vimala 2012). The solid substrate based on unprocessed residues of food and agro-industry crops is an essential source in SSF, supporting the growth of microorganisms (Li et al. 2014). On the other hand, soluble sugars, molasses, and liquid media are the common substrates used in SMF. Studies

have shown that co-utilization of both binary substrates yields higher growth rate than that with each source in submerged fermentation (Hermsen et al. 2015). A study performed by Mitra et al. (2017) in our lab suggests that the cultivation medium containing binary sources was more desirable for biomass and beta-cryptoxanthin production by *Kocuria marina* DAGII than the single substrate (Mitra et al. 2017).

Temperature

Temperature is one of the main factors of microbial pigment production. Each microbe species can grow within a range of temperatures by resisting the damaging effect of heat or cold on its molecular components. Hence, the temperature range is divided into minimum, maximum, and optimum. At optimum growth temperature, the rate of cellular reproduction is most rapid (Peričin et al. 2012). Most carotenoid pigment-producing microbes grow at mesophilic temperatures between 20 and 45 °C. The temperature regulator can be used in submerged fermentation. It is difficult to control the temperature in the solid-state fermentation process due to heat accumulation by microbial activity, necessitating heat removal from the system to prevent overheating and disturbance of microorganisms growth and product generation (Borucki Castro et al. 2007). In high-scale production, the generation of heat could lead to substantial moisture losses disturbing the fungal growth in SSF. Hence, the air is blown into the system by taking care of the flow rate to remove the produced heat through a gas outlet (Nagamani et al. 2012). Installation of cooling system can also be done to overcome this problem.

pH

Neutral to slightly alkaline pH favors lycopene formation, whereas acidic pH favors β-carotene synthesis. A study reported that the optimum pH required for canthaxanthin production by *Dietzia natronolimnaea* HS-1 is 7.66. Beta-cryptoxanthin, a pro-vitamin A carotenoid pigment, was produced by *Kocuria marina* DAGII at pH 7.9 (Mitra et al. 2017). Canthaxanthin production from microbe *Dietzia maris* had *a* slightly acidic pH value of 6.7 (Bera 2020). Beta-carotene production by microalga *Dunaliella salina* at pH 7.5 was also reported (Dipak and Dipak and Lele 2005). According to several study reports, most of the microbe favor carotenoid pigment production in the pH ranging from neutral to slight alkaline. It is tough to measure and control pH in SSF due to the nature of solid substrate, less water content, heterogeneity systems, and lack of suitable on-line pH measurement methods. There is no authentic electrode to measure pH in the solid medium until now. Hence, microbes capable of utilizing a wide range of pH are often applied for SSF (Peričin et al. 2012).

Aeration Rate

Microorganisms usually vary in their oxygen requirements. In SSF, aeration provides oxygen demand in aerobic fermentation and heat and mass transport in a heterogeneous system (Adinarayana et al. 2004). Flow rate and air quality are the two crucial factors of aeration. The high flow rate of dry air may affect the moisture of fermented substrate, which could be avoided by applying saturated air to control the temperature and moisture levels of the solid medium (Batal et al. 2005). Different types of automated aerated systems are used in submerged fermentation to control the aeration rate.

Particle Size

Solid substrate particle size facilitates their shape, surface area, and porosity (Batal et al. 2005). Smaller particle size provides a greater surface area per volume, allowing adherence of microorganisms with the nutrients, affecting oxygen diffusion (Batal et al. 2005). Larger particle size has a less area per volume ratio providing excellent oxygen diffusion but affects nutrient contact. Hence suitable size is recommended to facilitate both microbial growth and oxygen and nutrients demand.

Agitation/Mixing

Agitation helps to overcome complexity problems in SSF through gradient disruption and evenly distributed airflow, improving the microbial growth conditions along the fermented bed (Adinarayana et al. 2004). Intermittent agitation is employed for a slower agitation speed (Singhania et al. 2006). Agitation in SSF process is done for bacteria or yeast since the cells are in loose contact with the solid substrate surface. Agitation is not mandatory in SmF since aeration provides enough agitation in the case of airlift fermenter and medium with low viscosity. The ratio of dimension between the vessel height/diameter ratio should be 5:1 for aeration to provide agitation (Nurudeen et al. 2015).

Design of Bioreactors

Aerated stirred tank and tank batch fermenter are the commonly used fermenter for SMF. A *classical fermenter* is an upright closed cylindrical tank fitted with one or more baffles attached to the side of the wall, a water jacket and coil for heating and cooling, sparger for forcible aeration, impellers for mechanical agitation, injector for feed introduction collector for sample collection, and outlets for exhaust gases (Batal et al. 2005). Sparger introduces air into the fermenter providing sufficient oxygen for an organism. Fine bubble aerators must be used to facilitate oxygen transfer to a greater extent (Singhania et al. 2006; Nurudeen et al. 2015). Modern fermenters have

highly automated monitoring, controlling, and recording system for pH, oxidation-reduction potential, dissolved oxygen, effluent O_2 and CO_2, and chemical components.

11.4.2 Isolation and Identification of Pigment-Producing Microbe

Bacteria are applicable for large-scale production of carotenoids than algae and fungi due to their unicellular nature, relatively high growth rate, smooth handling, and easy processing. Different microbes are capable of synthesizing carotenoid pigment. Hence, identifying isolated microbes helps to characterize and understand the nature of microbes. Techniques involved during isolation and identification of pigment-producing microbe are:

11.4.2.1 Screening and Strain Development

Sample Collection

Sampling of microbes through specimen collection, preservation, culturing, and microscopic examination is the first step of screening and strain development of microorganism. Numerous isolation of carotenoid pigment-producing bacteria has been done using environmental samples like water, soil, mud, and sediment. Collection of sample is done in sterile condition and refrigerated prior to use (Lecomte et al. 2011). Enrichment of samples is done in selective enrichment culture broth for multiplication of bacteria from the sample (Mitra et al. 2017).

Isolation, Selection, and Screening of Microbes

Isolation of microbial colony separates and classifies them based on the growth pattern. Different bacteria grow differently based on nutrient medium, and factors like temperature, pH, oxygen availability, etc. Nutrient broth is common enrichment media which supports the growth of various microorganisms. The growth factors for microbes were maintained almost similar to that of the sampling site. Isolation was done by serial dilution method and agar spreading method. Microbial culture plate should be incubated and routinely check for growth. Post incubation, pigmented isolated colonies containing plate is chosen to be master plates and store at refrigerated condition for later use (Dhere et al. 2020).

Primary Screening

Primary screening of microbe is performed by picking and streaking isolated colored colonies on agar plate from master plates followed by incubation process allowing the growth of microorganisms. The primary screening of isolates identify non-diffusible pigment-producing microbial cultures on plates which were separated and transferred on nutrient agar slants and preserved at freezing condition for further studies. The bacterial species were subjected to gram staining for morphological identification subjected to biochemical tests (Senthamil and Iyer 2003).

Secondary Screening

It is done for qualitative tolerance to pH and salt (NaCl %). The isolates growing on nutrient agar with a wide range of pH were considered as tolerant cultures and streaked on nutrient agar with higher NaCl (1–6%). Cultures showing good pigmentation with pH and NaCl tolerance while retaining their bright pigmentation were screened secondarily (Mendpara et al. 2013).

11.4.3 Bacterial Identification through Gene Sequencing Technique and Strain-Level Analysis

The two essential methods for bacteria metagenome sequencing are 16S rRNA-based sequencing and whole metagenome shotgun sequencing (WGS) (Janda and Abbott 2007). The merits of the 16S rRNA method are the ability to provide a complete overview of the community, cost-effective, and time-efficient method. Moreover, these 16S rRNAs have certain limitations, including limited information about the microbial community single region detection of 16 s rRNA in the genome, underestimating microbes' diversity. Compared with 16 s rRNA sequencing, the whole bacterial genome could be examined with accurate species and diversity levels detection through WGS technology (Li et al. 2021). The several drawbacks of WGS technology are short sequencing reads (mostly <200 bp) and significant phenotypic differences between highly related strains of the same species, which are difficult to distinguish by WGS. *Third-generation sequencing technology (TGS)*, also known as long-read sequencing, detects the isolated genomic DNA without amplification and produces long reads (average 10–20 kb) (Li et al. 2021). TGS can produce genome assemblies of unprecedented quality by detecting much longer fragments than 16S rRNA and WGS. However, unfortunately, the application of TGS in microbiota genomes is unfamiliar, although It has been applied extensively in eukaryotic genome detection.

11.4.4 Genome Database and Phylogenetic Analysis

A genome database is a repository of DNA sequences from different plants and animals generated by molecular biologists and geneticists. It helps interpret sequence data and determine the order of individual nucleotides of a complete DNA sequence through supportive databases. Prokaryotic genome data were acquired from databases in NCBI (Carroll et al. 2002). A total of 266,319 prokaryote genomes have been identified until now. A total of 108,506 prokaryote genomes were associated with humans (Li et al. 2021). Phylogenetics studies evolutionary relatedness among organisms through a two-dimensional tree representation of relatedness among different biological species (Singha et al. 2014). The three forms of a phylogenetic tree, Phylogram, Dendrogram, and Cladogram, are built mainly by either distance-based methods or character-based methods. The commonly used distance-based methods include the unweighted paired group method with arithmetic mean, neighbor-joining, minimum evolution, and Fitch-Margoliash. Character-based method derives trees that optimize the distribution of the actual data pattern for each character. The most commonly used character-based method includes Maximum Parsimony and Maximum Likelihood methods. The neighbor Joining method is superior to other tree-building methods that can handle many sequences with bootstrap tests with ease and are currently in use. A tree-building method is considered a "consistent estimator" if the method tends to give the correct topology as the number of experimental sequences tends to infinity (Singha et al. 2014).

11.5 Strain Development of Microbe Using Metabolic Engineering

Reducing production costs and increasing yield is the strategy behind the hyper-production of microbial strains through metabolic engineering. Metabolic engineering involves improving cellular properties by modifying specific biochemical reactions and pathways (Park et al. 2007). *Genetic engineering* is a useful technique that allows the manipulation of well-defined metabolic pathways of microorganisms to enhance carotenoid productivity yields (Ye and Bhatia 2012). Several researchers have successfully modified yeasts like *Saccharomyces cerevisiae* and *Candida utilis*, through metabolic engineering by inserting carotenogenic genes from *Erwinia uredovora, Agrobacterium aurantiacum*, and *Xanthophyllomyces dendrorhus* to produce β-carotene, lycopene, and astaxanthin (Misawa and Shimada 1998; Miura et al. 1998; Bhataya et al. 2009; Ungureanu et al. 2013). *Pichia pastoris* is another non-carotenogenic yeast which has also been studied for carotenoid production (Araya-Garay et al. 2012). These examples demonstrate that genetic engineering can be employed to increase carotenoid production yields, which could meet the worldwide demand.

11.6 Process Optimization to Obtain High Yield Pigment Production

The range of different significant factors for microbial pigment production like pH, media concentration, NaCl %, temperature, and moisture could be optimized through the 'one factor at a time' (OFAT) approach. Though OFAT is a tedious method, it could be used as a preliminary experiment to efficiently set the range of the factors, making the results more reasonable and credible (Hamid and Said 2018).

11.6.1 Statistical Approach for Microbial Pigment Production Optimization

The range of factors from the OFAT study obtained during microbial pigment production can be applied to perform statistical analysis using response surface methodology (RSM) through design expert software to solve the complexity (Dikshit and Tallapragada 2016). RSM is the most convenient approach mainly employed to reduce the requirements of experimental series. It evaluates the most significant single factors and determines the optimum conditions for the multivariable system (Sani et al. 2013). Several studies have applied RSM to optimize microbial pigment (Prajapati et al. 2013). It had been applied for the statistical designing of process parameters for biomass and canthaxanthin production by *Dietzia maris* NITD previously in our lab. The maximum pigment yield of 122 mg/L and biomass yield of 7.39 g/L was obtained under optimized process parameters (Goswami et al. 2012). The central composite design of RSM helps to quantify the relationships between one or more measured responses and the vital input factors. This optimization process follows three significant steps: carrying out statistically designed experiments, evaluating the coefficients in a mathematical model with the prediction of response, and examining the adequacy of the model. Coded values of +1, 0, and −1 corresponded to high, medium, and low levels of factors, respectively. CCD involves examining the simultaneous, systematic, and efficient variation of essential components that are used to identify possible interactions, higher-order effects and determine the optimum operational conditions. However, RSM using CCD is helpful for a small number of variables (up to five) but is impractical for many variables due to a high number of experimental runs required (Hamid and Said 2018). The statistical significance of the model equation was evaluated by the F-test analysis of variance (ANOVA). The ANOVA helps determine the statistics for responses, multiple regression analyses of the response surface design, and the significance of the model. The optimal level of each variable for maximum pigment production can be determined through 3D surface plot design as a function of two factors at a time, holding all other factors at a fixed level. This design helps to understand both the main and the interaction of the two factors. The

response values for the variables can be predicted from the 3D plots (Hamid and Said 2018).

11.7 Downstream Processing of Microbial Pigment

Downstream processing refers to unit operations that increase the purity of the target product through isolation, purification, and concentration of a product. It determines the economic feasibility of the process (Cheryan 2009). Microbial production of carotenoids is primarily intracellular which requires downstream operations for carotenoid recovery and processing. This includes pigment extraction through centrifugation steps, followed by clarification steps for removal of unwanted bulk contaminants to obtain the purified pigment. The most important step in downstream processing from a commercial point of view is to maximize product recovery by minimizing the production cost (Khanra et al. 2018).

After the pigment production, the downstream process follows separation of cell pellets containing the pigments from the supernatant through centrifugation/filtration steps, which include washing the cell pellets to remove the unwanted impurities. Once the impurities and supernatant are removed from cell pellets, different physical, chemical, and biological cell-disruption methods are applied to the cell pellets to release intracellular carotenoids (Mussagy et al. 2019). After the disruption of cell pellets using several methods, the released intracellular carotenoids are extracted by using a solvent of interest. The obtained pigment is then separated from cell debris using a PTFE membrane syringe filter (Saini and Saini and Keum 2018). The disruption of bacterial cells is more accessible than yeast and micro-algal cells due to the fragile nature of bacterial cells than yeast and micro-algal cells. Due to the strong association of carotenoids with other intracellular macromolecules most intense methods like cryogenic grinding are required for extraction of carotenoids (Saini and Keum 2018). Conventional extraction processes use volatile organic solvents (VOCs) as solubilizing agents. Methanol has been widely used as an extraction solvent for different carotenoids.

11.7.1 Alternative Method for VOCs Pigment Extraction

VOCs-based extraction processes possess several health hazard and environmental risks although they have a record of high extraction yield (Salar-García et al. 2017). The approach to overcome this problem is to replace the VOCs with green, biocompatible, and less toxic solvents (Yara-Varón et al. 2016) and to reduce the usage of solvent required by combining chemical extraction with novel physical or biocatalytic procedures. Hence, numerous extraction techniques have been introduced which are better in terms of time required for extraction process, efficiency, and solvent consumption. Some of the techniques employed are the microwave-assisted

extractions (MAE), pressurized solvent extraction (PSE) high-pressure processing, ultrasound, electrical methods, enzyme-assisted extraction, and surfactant-assisted extraction (Mussagy et al. 2019). Although many techniques have been incorporated, most of the academic still follow VOCs-based process for carotenoid extraction due to the high solubilizing potential and cell-disrupting capability of VOCs through cell membrane permeabilization. Some of the best solvents used for extraction of non-polar carotenoids include tetrahydrofuran, ether, and hexane. Solvents used for extraction of polar pigments are dimethyl sulfoxide (DMSO), acetone, and ethanol (Mussagy et al. 2019).

11.8 Characterization and Quantification of Carotenoids

TLC method is used to determine the purity of the compound by identifying the mixture's components. Rf (retention factor) plays a crucial role in pigment quantification in TLC. A drop of pigment sample is put on the TLC plate under the marked area below. The plate is then put on the mobile phase, in the vertical position allowing the sample's components to move along the plate. Rf is determined by calculating the distance traveled by the substance divided by the distance traveled by the solvent (Siva et al. 2011). Samples identification could be performed through comparison of the distance traveled by standard to the distance traveled by the test sample. Movement of the sample along the TLC separates the different fractions of the carotenoid pigment. This technique has been used to separate different fractions of carotenoid pigment from *Sporobolomyces* sp. using benzene and petroleum ether as a mobile phase (Manimala and Murugesan 2018). The Rf value of the yellow fraction is similar to that of standard β-carotene spot and a resemblance in their absorption spectra, which shows the presence of beta-carotene pigment. Liquid chromatography-Mass spectrometry (LC-MS) is an analytical technique that combines liquid chromatography's physical separation capabilities. LC-MS is useful for rapidly detecting the compound and its structural identification, scanning more than 300 compounds present in the sample. For analysis of pigment by LC-MS, models like LCQ Fleet Ion Trap LC/MS (Thermo Scientific, USA) equipped with automatic sample injector, Photodiode array detector (PDA), TSQ Quantum Access with Surveyor plus HPLC system APCI Mass selectivity detector are used (Subramani et al. 2014). The mass spectrum was recorded in the positive ion mode, ranging from m/z 50 to 1500. Fourier Transform Infrared Spectroscopy (FTIR) determine the different functional groups present in purified pigment through FTIR spectrophotometer. The sample is usually dissolved in dichloromethane and deposited as a film on KBr plates and was scanned between 400 and 4000 wave numbers (cm^{-1}) on spectrum GX FTIR, Perkin Elmer (Sindhu et al. 2015). Quantification of mixture of pigments in a sample can be performed by using HPLC (high-performance liquid chromatography). HPLC is used to separate the components of mixture of substances. Polar and non-polar columns are used according to the nature of the sample to be analyzed. All-trans-HPLC did β-carotene (TBC) quantification by Sanchez

et al. Peaks were identified by comparing retention time and spectral characteristics against a pure standard and available literature. Quantity was determined by the integration of peak area against a standard curve prepared with known concentrations of all-trans-β-carotene. Total carotenoid content and trans-β-carotene were estimated on a fresh (TCC-FW and TBC-FW) and dry weight (TCC-DW and TBC-DW) basis (Darwin et al. 2011). Water, methanol, and acetone are the commonly used solvents for quantification of carotenoids by the HPLC method (Mussagy et al. 2019).

11.9 Production of Microbial Pigment at Industrial Scale

The advantages of working with microbial pigments could be observed and well known from many research work as well as literature surveys. There is a rapid growing interest among researchers for the production of various types of microbial pigments. Despite the valuable properties of microbial pigments, the industrial-scale production of pigments is not economical which requires development of low-cost processes. Different studies have introduced the usage of low-cost materials and substrates to reduce the cost of microbial pigment production at industrial scale. These include substituting polypropylene plastic bags with Erlenmeyer flasks for solid-state fermentation (SSF) which is more efficient, easily applicable, and less cost-consuming than traditional SSFs published so far (Pongrawee et al. 2015). Cheap substrates could be applied for submerged fermentation of the microbial pigment since SmF processes are mostly used for commercial products (Subramaniyam and Vimala 2012). Till now, potential of different agricultural and diary wastes was employed to reduce the cost of substrate during microbial pigment production. The different agro-residues used as cheap substrates for the growth of microbial pigment include rice bran, wheat bran, coconut oil cake, sesame oil cake, tamarind seed powder, groundnut oil cake, cassava bagasse, sugarcane bagasse, and rice flour (Gordana et al. 2021). Since the microbial pigment production is still at development process, therefore cost-effective and low time-consuming method should be focused to successfully carry out at the microbial pigment production at industrial scale.

11.10 Stability of Extracted Microbial Pigment

Microbial pigments are sensitive to factors like air, heat, light, etc., causing major disadvantage of microbial pigments in food application (Joshi et al. 2003). Purified carotenoids in organic solvents react easily with atmospheric oxygen. Twenty oxidation products of carotenoids were identified by FT-IR, GC-MS, and HPLC techniques. A number of isomers and degradation products such as 13-cis, 9-cis, and di-cis isomers, beta-apo-13-carotenone, beta-carotene 5,8-epoxide, and betacarotene

5,8-endo-peroxide were visualized by using UV-Vis spectroscopy, HPLC, and LC-MS techniques (Henry et al. 2000). Hence, carotenoid pigment should be prevented from oxidation to prevent degradation. Formation of a number of cis isomers and oxidation products of beta carotene were found when heated up to 180 ° C for two hours in presence of air (Marty and Berset 1990). Exposure to acids can produce ion pairs, which form carotenoid carbocation upon dissociation (Konovalov and Kispert 1999). Also, iron can directly interact with carotenoids to synthesize product degradation. The common iron oxidizing agent used to study the degradation of carotenoids is ferric chloride (Konovalova et al. 2001).

11.11 Applications of Carotenoids through Delivery System

Carotenoid pigment has been widely studied for application into various industries like food, pharmaceutical industry, and cosmetics industry. However, before application into large-scale process, understanding the carotenoid degradation mechanism is crucial for adopting strategy and developing technology for incorporation into functional foods. For successful incorporation of carotenoids into the target compounds without degrading the bioactive compound, different techniques and delivery systems have been developed so far. Some of the delivery systems have been discussed below:

11.11.1 Conventional Emulsions

These emulsions are produced under high pressure by blending oil and aqueous phases along with emulsifiers which results in oil droplets coated with surfactant forming an interfacial layer between the oil and aqueous phases (McClements et al. 2007). Carotenoids could be incorporated through oil phase of oil-in-water emulsion due to their lipophilic nature. This technique has been effective in encapsulating bioactive compounds into food products providing physical and oxidative stable (Chee et al. 2005). This emulsion is used for omega-3 fatty acids delivery systems in ice cream and yogurt. Environmental stress during food processing can cause unstable emulsion (McClements et al. 2007).

11.11.2 Multilayer Emulsions

Multilayer emulsions are produced by forming repetitive layers using the principle of electrostatic attraction around an oil droplet (Guzey et al. 2004). The thicker interfacial membrane of the emulsions could protect carotenoids from pro-oxidants in the aqueous phase and improve bioavailability by reducing carotenoid

degradation during the digestive process prior to adsorption (McClements et al. 2007). Successful oxidative stability of omega-3 fatty acids has been obtained through the application of multilayer emulsion (Gu et al. 2007). Due to the requirement of additional process and ingredients, multilayer emulsions have higher production costs than conventional emulsions.

11.11.3 Solid-Lipid Particles (SLPs)

The methods used for conventional emulsions are used to produce SLPs. The temperature higher than the lipid's melting point is applied for the homogenization of solid lipid particles (Helgason et al. 2008). Encapsulation of carotenoids into the solid-lipid particles provides physical barrier from aqueous pro-oxidants (McClements et al. 2007). However, heat required for solid-lipid particles could potentially cause thermal degradation of carotenoids concerning the bioavailability of incorporated carotenoids.

11.11.4 Liposomes

Liposomes are the bilayer membrane formed from aqueous dispersion of phospholipids It is biocompatible in nature through incorporation of both hydrophobic and hydrophilic compounds. The phospholipids are arranged into spherical cell membrane-like lipid bilayers with hydrophilic end toward the aqueous medium and hydrophobic "tails" tucked toward each other to create pockets of entrapped water along with water-soluble compounds and the potential to protect them from hostile digestive conditions potentially facilitating gastrointestinal (GI) uptake (Kulkarni et al. 2011). At the same time, the hydrophobic fatty acid core of the bilayers could host hydrophobic compounds, creating a small spherical package that could carry both hydrophilic and hydrophobic compounds. It was demonstrated that the lipid bilayer of liposome provides the physical-chemical barrier for incorporated molecules against pro-oxidant making them water-dispersible and possible to be dispersed in aqueous food formulation (Shade 2016). Liposomes have been employed for encapsulating substances in food formulation, such as enzymes, antimicrobial agents, vitamins, and functional peptides. Carotenoids such as β-carotene and lutein have been encapsulated using liposomes so far (Kulkarni et al. 2011). Different layers of liposomes could be formed. Those are the unilamellar, bilamellar, and multilamellar vesicles liposome. The evolution of research showed that smaller size of liposome is better. Application of shear forces generates smaller and uniform-sized liposomes which effectively increase absorption kinetics and efficient circulation time during intracellular delivery of encapsulated compounds (Shade 2016).

11.12 Applications of Carotenoid in Different Industry So Far

11.12.1 Food Industry

Application of microbial pigments in food industry serves benefits in terms of cost as well as health. Microbial pigments like carotenoid have antioxidant and anticancer antibiotic property. Also being source of vitamin A, it has several health benefits like improving poor vision, skin texture, increasing bone density, etc. Some of the microbial fermentation-derived pigments, applied in food industry, are found in food market these days (Kumar et al. 2015). These pigments are derived for filamentous fungi which include Monascus pigments, Arpink red TM from *Penicillium oxalicum*, riboflavin from *Ashbya gossypii*, lycopene, and β-carotene from *Blakeslea trispora*. Monascus red pigments, generally produced as MFR (Monascus Fermented Rice) improve the organoleptic characteristics of the food products. These pigments contain monocolins, which reduce the LDL-cholesterol and increase HDL-cholesterol. Monascus pigment has been implemented as a coloring agent in food which provides an additional advantage of specific flavor in the products (Kumar et al. 2015).

11.12.2 Pharmaceutical Industry

Many microbial pigments are used for pharmaceutical industry-developed products. Microorganism pigments have significant potential clinical applications and currently research works are going to treat diseases like cancer, leukemia, diabetes mellitus, etc. Anthocyanins pigment are engaged in a wide range of biological activities by preventing the risk of certain disease through decreasing and modulating immune response insult (Katsube et al. 2003). Prodigiosin is a potential pigment which was reported as active component for prevention and treatment of diabetes mellitus (Hwanmook et al. 2006). The violet pigment violacein isolated mainly from bacteria *Chromobacterium violaceum*, exhibited important anti-tumoral, anti-parasitary, anti-viral, anti-bacterial, and antioxidant activities (Konzen et al. 2006).

11.12.3 Cosmetics Industry

Microbial pigments are now targeted toward application in cosmetic industries apart from food and pharmaceutical industries. Many biological molecules have been used to produce various perfumes, lotions, creams, and facial scrubs (Kumar et al. 2015). The merits of using microbial ingredients are due to their biocompatible nature, clarified process, persistent product quality, and environmental footprint. Bacteria

could secret biologically active compounds with significant commercial values like oligosaccharides, exopolysaccharides (EPS), biosurfactants, enzymes, peptides, vitamins, etc., which have replaced chemical compounds in various cosmetic products for beautification and health improvement purpose (Gupta et al. 2019).

11.13 Cost Analysis for Microbial Pigment Production

Cost-benefit analysis is a widely used economic evaluation method to provide a consistent procedure for evaluating the decision. The project costs are compared to the benefits of the intervention which allows considering all costs and benefits over time. This analysis involves project evaluation, and determines whether or not a project should be accepted (Holland 2012). Evaluating a project from its significant point of view is necessary while predicting its total effect on the project economy. Hence a comparison of the project with economy and without economy should be performed. A primary purpose of using cost-benefit analysis techniques is to allow decisions at the level of the enterprise in the public sector (Holland 2012). Research in microbial pigment production for various applications in food, cosmetics, and pharmaceutical industries has proliferated in Biotechnology. The eco-friendly, cost-effective, and sustainable nature of microbial pigment has pushed researchers to develop an idea about targeting this bioactive compound for specific applications as mentioned above.

Although microbial pigment production is still at the Research and Development stage, much work needs to be performed to understand its full potential at an industrial scale (Kumar et al. 2015). However, several study reports related to its antioxidant, anticancer, and anti-inflammatory could help predict its possibility of the outcome soon. As mentioned earlier, the fear of the health hazard nature of synthetic pigment has caused consumers in the market to shift their preference toward natural pigment. Microbes are a natural source of pigments abundant in soil, water, and marine sediment. Merits of using microbial sources over other natural sources include their high abundance, easy cultivation throughout the year, feasibility, sustainability, etc. (Wang et al. 2006). Hence, targeting microbial pigment for industrial-scale production for various applications could potentially serve to meet the market demand. However, when we talked about setting up any project or program for industrial application, cost-benefit plays a vital role in project evaluation, based on which decision is taken. Therefore, in this case, benefit-cost ratio analysis is done by comparing the values (costs and benefits) of an activity to determine whether the program produces more benefit than its cost applied. The cost and benefits of industrial carotenoid pigment production have been tabulated in Table 11.1, which show the possibility of more benefits than the cost for microbial pigment production of carotenoid. Now, when we discuss about lab scale production of microbial pigment, the foremost requirement for microbial growth is the nutrient source, environment, and pH. Effective microbial pigment production in lab scale requires proper set of protocol, optimized parameters, and well-designed equipments

Table 11.1 Cost-benefit of microbial carotenoid pigment production

Cost	Benefits
Direct cost • Land for industrial site • Raw materials for microbial pigment production • Instruments, equipments • Labor • Labor wages *Indirect cost* • Quality assurance of product • Telecommunications • Facilities like rent, utilities, building expenses • General office work supplies • Supervisor/manager • Insurance	• Industrial set up and project development will create employment opportunities for people living within and outside the industrial site. Also it will help to meet the global demand of product through project start up
	• Cost-effective waste raw materials like cheese whey rich in glucose and maltose can be substituted to fermentation media available in market. This will help to reduce the cost of media required for carotenoid pigment production
	• Efficient production of carotenoid pigment in controlled conditions. The processed conditions can be optimized to enhance the yield of product. Better yield and productivity may settle the cost of few equipments required for production
	• Build a skilled and strong teamwork with distributed work load, which will ultimately enhance the production process, development and contribute toward growth and development of industry in future. More effort towards production may increase the outcome of the production process
	• Source of livelihood
	• Maintain desired level of quality, prevent errors or defects in manufactured goods or services • Consumer's trust • High quality will increase the market demand of product • Good communication skill between manufacturer and consumer • Product transparency • Trust assurance • Indirect contribution toward product processing, manufacturing, development, and packaging • Detailed work records; employment opportunities; systematic work plan • Lead role in product manufacturing and development process • High work through-put through supervision of teamwork • Safety assurance to employees by providing benefits related to medical condition like injuries and accidents

to facilitate and carry out the process. Hence, prior to microbial pigment production, cost analysis of the overall procedure becomes an important part to make certain feasibility of a designed method of production. To show that carotenoid pigment obtained from microbes could be a revolution for pigment production industry, let's give some comparison. Unlike microbes, pro-vitamin A carotenoids such as alpha-carotene, beta-carotene, and beta-cryptoxanthin are obtained from vegetable and fruit sources like carrots, persimmons, papaya, etc., which are seasonable in nature. Currently, as per the market report in India, the cost price of carrot is between 35–50/kg, persimmons 250–500/kg, and papaya 30–50/kg which varies with geographical location. Also, the idea behind utilizing the waste products of the mentioned vegetable and fruit sources requires a well-established food industry capable of

Table 11.2 Comparison of total cost of carotenoid production from microbial and vegetable source in lab scale

Lab requirements	Cost of production	
	Microbial production of carotenoid pigment	Carotenoid production from fruit and vegetable sources
Chemicals (solvents, reagents)	1000 (INR)/L	1000(INR)/kg (may vary)
Media (carbon and nitrogen source); some nutrient source could be substituted with waste products	100 (INR)/L; under optimized conditions	No media is required
Vegetable sources	No vegetable source	Depends upon the price; quantity required and quality of raw material
Consumables (tissue, cotton, filter)	70 (INR)/L	100 (INR)/kg
Laboratory equipments		
Apparatus (flask, measuring cylinder, petri plates, beaker, pipette, tips, etc.)	10,000 (INR)	10,000 (INR)
Instruments		
Weighing balance	11,000 (INR)	11,000 (INR)
Magnetic stirrer	10,000 (INR)	10,000 (INR)
Ph meter	5000 (INR)	5000 (INR)
Autoclave	50,000 (INR)	50,000 (INR)
Laminar hood	1,00,000 (INR)	–
BOD incubator	50,000 (INR)	–
Centrifuge	1,00,000	1,00,000
Water-bath	10,000	10,000
Hot air oven	–	20,000
Probe sonicator with sound proof	–	1,50,000
Blender	–	5000
Total cost of production	3,47,170 (INR) (approx)	3,72,100 (INR) (approx)

producing sufficient amount of poorly managed bio-waste products which is quite challenging. The well-established extraction method of carotenoid pigment from above-mentioned fruit and vegetable sources undergoes a set of optimized protocols and well-equipped instruments. On the other hand, microbes like bacteria are capable of producing high amount of pigment under optimized conditions using a minimal amount of substrates. The waste products rich in nutrient source required for microbial growth could also be used to substitute the media obtained from market. The comparison of requirements of basic laboratory equipments and total production cost of carotenoids in lab scale from microbes and vegetable sources have been shown in Table 11.2. The cost of all the requirements has been approximated to its maximum value as per our knowledge. From Table 11.2 we can understand the different types of laboratory equipments and the cost required for the extraction process. The differences in cost of production between the microbial

Table 11.3 Income and expenditure statement of lab scale carotenoid pigment production from microbial source in a single batch

Statement	For a batch (in rupees)	Note
Non-recurring expenditure		
Repairing of instruments	30,000	May occur once in every 3–4 years
Instrument servicing and installation	20,000	
Total	50,000	
Recurring expenditure		
Electricity bills	200	Electricity bills calculated for a day
Cost of raw materials	20	
Other utilities	100	
Total	320	
Revenue		
Sales of products	1,51,200	One batch fermentation requires 1 week for the production and extraction process which is capable of working with maximum of 2.5 L culture volume in a single batch; it yields 3.6 mg (approx) carotenoid pigment from 2.5 L culture volume
Cost of 1 mg of pro-vitamin A carotenoid in market	50,000	
Profit	1,01,200	
Total operating expenses	50,320	
Net income	50,880	(Excluding taxes and interest expense)

and vegetable sources can be noted owing that microbial pigment could serve as advantageous and suitable to work with. The income and expenditure statement of lab scale carotenoid pigment production from microbial source in a single batch has been shown in Table 11.3. From the Table 11.3 statement, the possibility of income incurred during the microbial pigment production could be observed which suggests that further work should be carried out for microbial pigment production for industrial application in near future.

11.14 Marketing of Microbial Carotenoid Pigment

These days, we are very familiar with the term marketing, which is a set of communication processes with the target consumers. These communication processes help to develop a marketable and beneficial offering to fulfill the target consumers' unfulfilled requirements thereby making a profit of ourselves in return. Marketing has five objectives which are satisfying the customers by fulfilling their desires, needs, and wants through earning profit for sustainable growth of the business, developing product demand through communication with target audience, establishing a unique company brand through creating a public image toward company by fulfilling consistent brand promise. The four principles of marketing

are product, price, place, and promotion. Product is an important component of marketing which could be goods, services, ideas, etc. The life cycle of product has an important position in marketing strategy. The life cycle of product is defined as the stages of product development, market acceptance till removal of a product. Products which are bought by consumers for their usage and final consumption are known as consumer products. Whereas products which are purchased for reselling purpose or producing other items for sales are known as industrial products. Life cycle of a product is an important tool for analysis and planning of the marketing mix activity representing the finite market life of most products. The finite market life could be short as in the case of fashion goods or long as in the case of certain types of industrial equipment. Thorough research, adaptability, flexibility, and focused approach are some of the characteristics of successful new product development. A good product life cycle in the market depends upon the characteristics mentioned above. Developing a microbial carotenoid product development could be a revolution to the market; however, at the same time, it could be a challenging task. Several products containing different types of carotenoid pigments have been already launched in the market. Understanding the characteristics of microbial pigment like stability, degradability, susceptible to certain environmental factors and developing a technology to protect the product could extend its shelf life during the product life cycle in the market. Also thorough research on toxicity, and carcinogenicity using clinical trial study could potentially solve the complexity of product developed from microbial pigment.

11.15 Conclusion

The rising trend of usage of biological and eco-friendly products has caused sharp demand of naturally derived pigments food, pharmaceuticals, and cosmetics industries. Thus different industries are continuously putting their effort to use microbial pigments owing to their cheap alternatives toward existing synthetic compounds. However, a lot of improvement is required in terms of productivity, cost-effectiveness, and scaling up the products for industrial product development and marketing which is lacking behind. Advances in biotechnology through approaches like metabolic engineering, nanotechnology, and recombinant DNA technology of organism could considerably enhance the production of microbially derived compounds. The wide beneficial property of microbial pigments could serve as the best replacement for synthetic products available in the market. However, considering the adverse effects of microbial pigments on health, further research based on safe consumption of microbial pigments are need to be carried out through thorough study, methodical and rigorous assessment through clinical research and testing before validation of true product potential.

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Conflict of Interest The authors declare there is no conflict of interest while carrying out the work.

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Chapter 12
Pseudomonas Species-Derived Chitinase Mass Multiplication, Production Cost Analysis, and Marketing: As a Biocontrol Agent for Crop Protection

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Abstract Chitin is an insoluble structural polysaccharide present in the exoskeleton of insects. One potential biopesticide is enzyme chitinase, which degrades chitin-soluble and insoluble oligosaccharides. Recently, chitinase enzymes as a biopesticide are developing to control insect and fungal pests—an alternative approach with enzyme-based biopesticides to avoid the chemical pesticides against pests and pathogens. The success in exploring chitinase from microbial sources, especially for agriculture, has a high volume low cost, and it also depends on the availability and active formulation of the product at a reasonable cost. The roles of chitinolytic enzymes as a biocontrol agent, different mechanisms to mass culture the chitinase and the formulations, and making the enzyme stable for the long term is the wide area to research. The chitin degradation, and identification of chitinase from different microbial sources with varying specificities, may make them more useful in commercial processes soon. In this chapter, *Pseudomonas* sp.-derived chitinase enzymes, essential in the agricultural field, benefit the plant by PGPR activity and mass multiplications of bacteria and the enzyme, production, and formulations in low cost, requirements to register the biopesticide and marketing were discussed in this chapter.

Keywords *Pseudomonas* species · Mass production · Formulation · Shelf-life · Commercialization · Bio-control agent

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12.1 Introduction

The increasing demand for food for the increasing human population is one big global concern. It is estimated that by 2050 the world population will reach 9 billion, which demands ~60% extra food from the present; this food demand directly causes the farmers to produce the needed food. Increased use of chemicals causes environmental problems, pest resistance, and human health issues (Arora 2018). Crops largely depend on the microbes, and plant-microbe interactions happen in the soil (Khare and Arora 2015). The soil rhizosphere has several microbes that promote the plant's growth and protect the plant from pathogens. For the last two decades, plant growth-promoting bacteria (PGPB) have been the best choice to use as biocontrol agents to enhance plant growth and disease management. PGPB and its metabolites and enzymes play a significant role in agriculture as biocontrol agents; many PGPR-based formulations are arising nowadays. It has a good effect and has advantages against chemical pesticides because they are non-toxic and naturally occurring. PGPB has many advantages, like it involves the production of antibiotics, cell wall-degrading enzymes, biosurfactant, and volatile compounds, also induces systematic resistance in plants (Pérez-Montaño et al. 2014).

Among the bacteria used as biocontrol agents, the number of given chitinase activity are *Streptomyces*, *Bacillus*, and *Pseudomonas (*Avis et al. 2001). Chitinase enzyme is produced by PGPR bacteria, which is a significant group of hydrolytic enzymes secreted, involved in the enzymatic hydrolysis of chitin. Chitin is an essential fungal cell wall component (Muzzarelli et al. 1997). It has already been reported that *Bacillus* and *Pseudomonas* sp. produce the chitinase enzyme (Faramarzi et al. 2009; Rishad et al. 2016). The primary thing that cell wall-degrading enzymes do is affect pathogens' structural integrity (Budi et al. 2000).

12.2 General Characteristics of *Pseudomonas* Species

Pseudomonas is an anaerobic, gram-negative, Gammaproteobacteria belonging to Pseudomonadaceae, containing 191 diversified species. Pseudomonads are known for their metabolic versatility and genetic plasticity, which are ever-present in soil ecosystems (Lugtenberg and Kamilova 2009; Pathma et al. 2011; Jain and Pandey 2016; Kumar et al. 2016). The rhizosphere is the most abundant habitat for the influential group of microbes. Microbes interact like symbionts and play a curious role in host plants. Pseudomonas is present in both the root and tissue of plants, readily available in nature. The beneficiary or non-beneficiary of microbes to plants was based on the type of microbes. Microbial symbionts in the rhizosphere may benefit plant species by producing phytohormone, availability of nutrients, and abiotic tolerance. García-Gutiérrez et al. (2012) stated that *P. fluorescens* and *Bacillus* spp. individually and in combined form is equally effective against bacterial and fungal pathogens. The *Pseudomonas* species gets the attention of researchers to

produce more biocontrol agents as it acts as PGPB and contributes to the plants to induce systematic resistance (ISR). Pseudomonas strained Psf5 and resulted in significant increases in the plant stand and dry weight of root in tomato crop. Goswami et al. (2013) reported that the growth had been enhanced after inoculating *Pseudomonas* spp. in chickpea. The recent report shows that *Pseudomonas putida* bacteria enhanced the cucumber plant's growth and development (Nawaz and Bano 2020). Many researchers used *P. Fluorescens* to enhance the yield of the crop. Generally, fluorescent pseudomonads affect the growth and development of insects; *Pseudomonas maltophilia* affects the growth of *Helicoverpa zea* in the larval stage; as a result, the adult emergence is 60% reduced (Bong and Sikorowski 1991).

The bacteria have other mechanisms to inhibit the growth of pathogen by secreting antibiotics, toxins, enzymes that degrade cell wall, surface-active compounds biosurfactants, and the secondary metabolites production that will ultimately induce the systemic resistance against the pathogen (Haas and Défago 2005; Kumar et al. 2015a, b; Van Loon et al. 1998). Many chitinolytic bacteria have plant-growth-promoting properties; *Pseudomonas* sp. in in vitro antifungal chitinolytic activity shows enhanced nodulation in chickpea (Sindhu and Dadarwal 2001). *P. aeruginosa* strains have some additional activity isolated from the soil using SCSP as C/N source showed two kinds of activity such as chitinases/lysozymes having antibacterial and cell lysis against many bacterial species (Wang et al. 1995(Wang et al. 1995); Wang and Chang 1997).

12.3 Chitinase and Its Role

The primary and most used mechanism for degrading soil-borne pathogens was from bacterial enzymes, i.e., chitinase, which degrades the cell wall of fungi and other species. *Pseudomonas* is the well-known bacterial genus for chitinase production (Chalidah et al. 2018).

Examples of cell wall degrading enzymes are β-1,3-glucanase, chitinase, cellulase, and protease. Chitin degradation aims to kill the insect pest, chitinases from PGPR bacteria act as biocontrol agents. Chitin ($C_8H_{13}O_5N$), an insoluble, essential structural part of insect cuticles, guts lining, maintains insects' structural integrity (Bhattacharya et al. 2007). Chitinase degrades chitin into its monomeric or oligomeric components; if this enzyme is sprayed on the insect, it penetrates the gut tissue. On disturbance of gut tissue, the feeding abnormalities occur, and it disrupts the chitin-containing cuticle region and causes abnormal molting in insects (Chandrasekaran et al. 2012). Some bacteria reported for chitinase production, such as *Streptomyces* (Blaak and Schrempf 1995), *Serratia marcescens* (Synstad et al. 2008), *Aeromonas punctata* and *A. hydrophila* (Kuddus and Ahmad 2013), and Bacillus (Rishad et al. 2016). *Serratia marcescens* controls the growth of *Sclerotinia minor, B. cinerea, Rhizoctonia solani*, and *F. oxysporum* (Someya et al. 2000; El-Tarabily et al. 2000). A high amount of genetic alternation is required to secrete a sufficient quantity of chitinases in the microbes/biocontrol agent, and the stability

of those chitinases in the field would still be arguable. The molecular weight of enzyme chitinase varies from 20 to 90 kDa (Hamid et al. 2013).

Proteases secreted by PGPR biocontrol strains produce an inhibitory effect on the hyphal growth of fungi (Bahadur et al. 2016; Masood and Bano 2016; Meena et al. 2016; Teotia et al. 2016). *P. aeruginosa* produced chitinolytic activity, and *P. fluorescens* has been found (Nielsen and Sørensen 1997). Among the filamentous and basidiomycetes fungi, 6% of the organism's dry weight is chitin. The cell wall morphogenesis and differentiation are highly dependent on chitin. *Pseudomonas*, the chitinase producer, promises a tremendous antifungal activity by lysing the fungal cell wall rich in chitin, altering growth and development. The diversity and distribution of many chitinolytic bacteria as biocontrol agents made the researchers focus on it. Accumulation of bacterial chitinases in plant systems, responses against infestation by the virus (Bol et al. 1990), bacteria (Robert et al. 2002), fungi (Krishnaveni et al. 1999), or insects (Krishnaveni et al. 1999) is well recorded.

12.4 Methods for the Mass Multiplication of *Pseudomonas species*

Additional nitrogen and carbon sources in the medium other than chitin help to increase the available source of energy for the microorganisms and produce the enzyme for chitin degradation. The addition of ammonium phosphate to the medium produced the highest chitinase activity compared to other treatments, and the results show the activity of 0.031 U·mL-1 on day 3 of fermentation.

12.5 Formulation Development

*12.5.1 Different Methods of Extraction of Chitinase from Bacteria (*Pseudomonas Species*)*

Pseudomonas spp. the chitinase-producing bacteria were isolated from soil and maintained on nutrient agar plates at 5 °C. The growth of the bacteria was maintained in a medium containing 0.1% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$ (pH 7) and supplemented with different chitin-containing seafood processing wastes as the carbon/nitrogen (C/N) sources. The chitin-containing seafood wastes include shrimp shells powder (SSP), shrimp and crab shell powder (SCSP), etc. The resultant medium should be cultured for 1–5 days at 30 °C in 150 rpm, and centrifugation was done at 12,000× at 4 °C for 20 min, the supernatants were collected for chitinase activity (Wang et al. 2010).

The method described by Cattelan et al. (1999) in colloidal chitin medium composed of crab shells (0.8 g); NH_4NO_3 (0.78 g); K_2HPO_4 (0.20 g); $MgSO_4{\cdot}7H_2O$

(0.20 g); $CaCl_2$ (0.06 g); NaCl (0.10 g); $Na_2MoO_4{\cdot}2H_2O$ (0.002 g); $ZnSO_4{\cdot}7H_2O$ (0.00024 g); $CuSO_4{\cdot}5H_2O$ (0.00004 g); $CoSO_4{\cdot}7H_2O$ (0.010 g); $MnSO_4{\cdot}4H_2O$ (0.003 g); $Na_2FeEDTA$ (0.028 g); H_3BO_3 (0.005 g); and, agar 15 g (Sigma-Aldrich Chemical Co., USA) in 1 liter of distilled water. $MgSO_4$ and $CaCl_2$ were autoclaved separately and later added to the medium. To the autoclaved medium biotin (5 μg/l^{-1}) and ρ-aminobenzoic acid (10 μg/l^{-1}) were sterilized and added.

The isolates were spotted on the chitin medium and incubated at 28 °C for 72 h. A clear zone around the colony indicates the chitin-solubilization by chitinase (Quesssaoui et al. 2017).

12.6 Liquid Fermentation of Chitinase Enzyme

Microbial chitinase can be produced by liquid fermentation processes like batch, continuous and fed-batch fermentation and is commercially available at a high cost (Dahiya et al. 2006). The chitinase enzyme production is controlled by physical factors (aeration, pH, and temperature) and different growth mediums (Miyashita et al. 1991). The chitin containing seafood waste like SCSP, SSP induce the production of antimicrobial chitinases by different microbes like *P. aeruginosa* (Wang and Chang 1997), *B. amyloliquefaciens* (Wang et al. 2002), and *B. subtilis* (Wang et al. 2006). The advantage found on soil isolated *P. aeruginosa* using SCSP as sole carbon and nitrogen source showed two kinds of activities chitinases producing and lysozymes having antibacterial and cell lysis activities against some bacterial species (Wang et al. 1995; Wang and Chang 1997).

12.7 Solid-State Fermentation and Submerged Fermentation

In solid-state fermentation and submerged fermentation, some substrates were used in *Verticillium lecanii* culture, those substrates obtained by lactic acid fermentation of shrimp head wastes containing chitin, proteins, lipids, and minerals (Shirai et al. 2001). The growth time of *Verticillium lecanii* was reduced when added sucrose or sugar cane pith bagasse (Matsumoto et al. 2001, 2004). The mixture of shrimp waste silage and sugar cane pith bagasse in solid-state fermentation increased the enzyme yield (Matsumoto et al. 2004). Some marine organisms produce chitinous material, a good source of inducers for microbial chitinase production. Dahiya et al. (2005) used solid-state fermentation to optimize chitinases in *Enterobacter* sp. with a statistically designed experiment.

12.8 Zeolite-Based Formulation

Synergistic action of two strains formulated with zeolite-based chitosan in a ratio of 1:1 or 4:1 showed better results in controlling the disease than treating with individual strains (Singh et al. 1999).

12.9 Talc-Based Formulation

Pseudomonas fluorescens formulated using talc (with and without chitin) were tested against rice diseases such as sheath blight and leaf folder. Under greenhouse and field conditions, the talc-based formulation was applied through different stages of plant-like in seeds, roots, soil, and foliar spraying, which showed a significant reduction in sheath blight and leaf-folder disease (Compare et al. 2002).

12.10 Colloidal Chitin Formulation

Supplementing the bacterial formulation with 1% colloidal chitin reduced the leaf spot disease in groundnut, a substrate for chitinase (Huang et al. 2005; Kishore et al. 2005). Chitin-mediated composts showed increased chitinase activity; eventually, the composting process fastens (Poulsen et al. 2008), inducing antifungal activity. In a study by Yasir et al. (2009), he added chitinolytic bacteria to vermicompost and found decreased spore germination of *Fusarium moniliforme*.

12.11 Effects of pH, Temperature, Chemicals, and Surfactant on the Chitinase Activity

Generally, the production of enzymes is controlled by physical factors such as aeration, pH, and incubation temperature and by the growth media components used (Miyashita et al. 1991).

The optimum pH of chitinase was studied by analyzing the samples in different pH; the sample is dialyzed against a buffer solution of different pH ranging from 3 to 11. Chitinase pH value ranges from 6 to 9, confirming that it could survive in the insect's gut wherein the pH is alkaline. Also, Gomaa's (2012) report suggests that pH 7–8 is where the maximum chitinase production occurs in *B. thuringiensis* and *B. licheniformis*.

To determine the optimum temperature of the chitinase, the values were measured at various temperatures ranging from 25 to 90 °C. The stability of the chitinase at different temperatures was measured by incubating it for 30 mins. The effects of

chemicals on chitinase activity were measured by adding chemicals to chitinase in a phosphate buffer solution (pH 7) for 10 min at 37 °C and measuring the chitinase activity. The effect of surfactant on chitinase activity was measured by incubating the enzyme with 0.25 ml of surfactant solutions at 25 °C for 30 min (Wang et al. 2009).

12.12 Shelf Life of Pseudomonas Chitinase Formulations

Selection of the proper carrier materials is vital to formulate chitinase; as it is an enzyme, it should sustain a high amount of microbial inoculants for a long time. Sustainability of enzymes for a long time in the microbial diversity determines the product is in good condition. Many materials can be used as carrier materials like peat, lignite, vermiculite, charcoal, press mud, manure, and talc (David et al. 2018).

12.13 Delivery of Pseudomonas Chitinase for Disease Management

Fungi and insects' structure is made up of chitin which acts as a protective barrier from predators/pathogens, induction of chitinases in plants is the primary defense response. Recently *Bacillus* sp. shows antagonistic activity against many fungi because this bacterium can secrete hydrolytic enzymes like chitinases, chitosanases, laminarinases, and cellulases (Nielsen and Sørensen 1997; Takayanagi et al. 1991). Many plant pathogens have been controlled using *S. marcescens*, such as *Botrytis cinerea*, *Rhizoctonia solani, Fusarium oxysporum*, and *Sclerotinia minor* (Brurberg et al. 2001). In another study, the chitinase activity of *Bacillus thuringiensis* showed insecticidal activity against the pests like *C. occidentalis* and *P. xylostella* (Brar et al. 2008; Smirnoff 1974). Some microbial chitinases were formulated with propane-2-ol and polyoxyethylene lauryl ether and sprayed on the field of rice; this eventually decreased the disease caused by *Pyricularia oryzae* (Tanaka et al. 1970). The hydrolytic enzymes produced by *Trichoderma* sp. can degrade the cell wall, *Pseudomonas syringae*, which produced syringomycins against *B. cinerea* (Koga 2004). They resulted in a decrease in powdery mass on fruits of strawberry and its leaf, and for more than 15 days, those powdery mass was not seen on the plant after the treatment.

12.14 The Cost of Enzyme Chitinase

The chitinolytic enzymes cost low on agricultural practices and more on human-related issues and industrial application (Patil et al. 2000; Dahiya et al. 2006). The γ-proteobacteria *Pseudomonas* spp. is well known for its antagonistic activity against fungi and secretes chitinase. The commercial feasibility of using chitinolytic enzymes in the agricultural field for disease and pest control, the central part that needed to be considered like the volume, production cost of enzyme, formulation, storage stability in different field conditions. There is no literature on the high-scale production of chitinase enzymes. The comparative efficacy of enzyme-based formulation is higher than other biopesticides; it needs to be optimized in large-scale production of enzymes with different strategies to make the enzyme formulation more feasible for agricultural consumption (Chavan and Deshpande 2013) (Table 12.1).

One batch fermentation in solid-state fermenter (40 L) takes 72 h. Total cost of production (40 L) in laboratory scale- 32,000 INR Approx per fermentation. Total cost of production (5000 L) in industrial scale- 4,000,000 INR Approx per fermentation (Table 12.2).

Production cost = 255,500,000 INR per year in Industrial scale per fermenter.

The selling cost of the product 1 L—750 INR.

Table 12.1 The cost of production of chitinase in laboratory scale

Requirements for chitinase production	Chemicals and equipments
Chemicals	Ammonium sulphate, sodium hydroxide, hydrochloric acid, methanol, DNSA, iodine, di-potassium hydrogen orthophosphate anhydrous, calcium chloride, chitin, dextrose, *N*-acetyl glucosamine, barium chloride, sulfuric acid, PBS, powder chitin, colloidal chitin, carboxy methyl cellulose
Media	Nutrient agar, colloidal chitin agar, glucose phosphate broth, nutrient gelatin broth
Basic laboratory equipments	SSF bioreactor, flasks, UV-spectrophotometer, biosafety cabinet, microwave oven, microcentrifuge, Colony counter, hot air oven, weighing balance, BOD incubator, −20 °C refrigerator, glass wares, centrifuge, SDS PAGE, pH meter
Production in lab scale	700 INR/ L Approx.

Table 12.2 Net profit and other details of the products (one fermenter/year)

Non-recurring expenditure (one-off or extraordinary events)	500,000 INR
Recurring expenditure (required for operating the business)	1000,000 INR
Revenue generation (process of planning, marketing, and selling)	2,000,000 INR/YEAR
Net profit (the amount of money your business earns after deducting all operating, interest, and tax expenses over a given period of time)	6,000,000 INR/YEAR

12.15 Enzyme-Based Formulations Overcome the Limitations of Biological Control Agents

To design enzymes, computational methods should be used that suit the specific requirements for the enzyme activity as a biocontrol agent. This relates to the basic understanding of antagonist–pathogen interactions and the biotechnological tools involved in designing an enzyme product that should be feasible for the economy. The chitinase can be produced from various systems, like purified recombinant optimized enzymes, from engineered microorganisms, transgenic plants, which promise to control fungal pathogens in many crop fields. Engineering different chitinases together from different species or chitinase with the genes encoding for antifungal agents help to look forward to producing a more stable product against pathogenic fungi. Developing suitable applications the enzyme-based formulations is very important, and it needs to be explored (Neeraja et al. 2010).

12.16 The Demand for Bacterial Hydrolytic Enzymes in the Industrial Sector Is Ever-Increasing

Hydrolytic enzymes in different parts of the world are ever-increasing, like in industries and environmental-based technologies. Enzymes such as chitinases, cellulases, pectinases, amylases, glucanases, and glucoamylases have a variety of industrial applications and also showed a high degree of success (Neeraja et al. 2010). Chitinase is used as an antifungal agent with antifungal drugs to treat fungal infections (Oranusi and Trinci 1985). Chitinase targets the insect cell wall and the insect's gut and initiates metamorphosis (Rathore and Gupta 2015). Chitinase is used to detect human fungal infections (Vega and Kalkum 2012). Chitinase is used to degrade the fungal cell wall, release tannase from the cell wall, and increase yield (Barthomeuf et al. 1994).

12.17 Standard Protocols for Bio-Control Agents Requires

Biopesticide regulatory mechanisms differ from country to country. The mechanism is based on the country's local needs in the region. To register biopesticide in India, there is a two-tier system, Tier I (9(3b)) is what approves our biopesticide as a provisional registration for 2 years with conditions, and Tier II (9(3)) is for permanent registration of biopesticide after you submit the additional data. Indian tier-I registration for biopesticides is sufficient to apply for permanent registration in other countries. Nevertheless, in contrast to India, the United States does not take Indian tier-II registration as permanent registration in the United States as it requires additional data even after meeting tier II in India. CIBRC India requires additional

data after tier-II like the US system because of the safety measures against humans during the 348 and 349 meetings in 2014. This specifies the complexity in registering the biopesticide among various countries and their regulatory bodies. In many countries, laboratories that do not insist on good laboratory practice (GLP) cannot register for biopesticide. The data generated through laboratories adopting universal good laboratory practices (GLP) and following a uniform protocol are eligible for registering biopesticide. Small and medium enterprises (SMEs) that do not follow uniform protocol are getting into difficulty when their product cannot be registered (David et al. 2018). The most regulated biopesticides are derived from microbes and biochemical components which are naturally occurring. The biopesticides act for G.M. plants has been brought to act in the United States and some European countries (McHughen and Smyth 2012). The Environmental Protection Agency (EPA) allowed using genetically modified organisms (GMOs) to be active as biopesticides. Using kairomones, allomones, pheromones, anemones, predators, parasites, and parasitoids as pest management is not covered under regulation (David et al. 2018).

12.18 Future Prospects

There is an urgent need to develop biopesticides in different formulations such as powder/liquid/granular with longer shelf life. Across the country, many researchers are establishing new biopesticides with microbes and their hydrolytic enzymes against different pathogens. For such enzymes to reach from laboratory to agricultural land, they need to develop a cost-effective, stable formulation, longer shelf life, performance under field conditions, registered the product by the institution, and an essential thing to commercialize the product with the entrepreneurs. Few isolates of *P. fluorescens* biopesticide have been approved and registered in India. There is a need to commercialize the enzyme-based biopesticide as it has many advantages over the other biopesticides. Production of enzymes costs more, and many have done the laboratory scale production, those needs to get into the large-scale production. The thing to note down on formulating the enzyme-based biopesticides is that they should stay longer in the plant to control the disease – shelf life, production cost, and the performance in different field conditions. Many pieces of research are needed to enhance the enzyme production, and significant scaling them and formulating that enzyme into product and get that to the market in the stable form. Industrial knowledge with biotechnology techniques together works to make up the chitinase as a biopesticide.

12.19 Conclusion

The target-specific action of chitinases on inhibition of harmful pest/pathogen with an economical cost is needed. Soon, protein engineering techniques may help generate more chitinase as a biocontrol agent, which is stable in any environmental conditions and more production. The biopesticide products that will result in new scientific advances may increase the adoption of different policies in many countries.

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Chapter 13
Production, Cost Analysis, and Marketing of Bioorganic Liquid Fertilizers and Plant Nutrition Enhancers

Veysel Turan, Sevcan Aydın, and Osman Sönmez

Abstract Due to increasing chemical fertilizer costs, the importance of reducing the use of chemical fertilizers in agricultural areas is increasing day by day. Moreover, the European Union and many countries encourage the use of enriched plant growth and liquid fertilizers instead of chemical fertilizers. Furthermore, bioorganic liquid fertilizers produced with different contents have emerged as an alternative plant nutrition tool as soil conditioner and improver in recent years. The development of new production processes of organic fertilizers and plant nutrition enhancer can contribute to the circular economy. In addition, some agricultural wastes can be a raw material source in fertilizer production due to their rich nutrient content. As a result, bioorganic liquid fertilizers and plant nutrition enhancer applications that promote plant growth, which are developed as an alternative to chemical fertilizers, should contribute to sustainable land management by increasing their trials both in terms of low cost and in field and greenhouse conditions.

Keywords Bioorganic liquid fertilizer · Soil conditioner · Production process · Chemical fertilizer

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13.1 Introduction

The rapid increase in the growing human population around the world, with the increase in food demands, poses a risk for food security and agriculture in developing countries. This increase in the world population is expected to reach 10 billion by the year 2050, and as a result, the demanded agricultural production in 2013 should be increased by 50 percent (FAO 2018). In parallel with this, the use of chemical fertilizers becomes costly and unaffordable for farmers in the short term, posing both a potential environmental threat and climate change effects (Struik and Kuyper 2017; Kopittke et al. 2019; Shah et al. 2021; Syed et al. 2021). The widespread and unwise use of synthetic chemical fertilizers leads to a decrease in soil microbial diversity and soil fertility as a result, which is difficult to recycle and causes damage (Bai et al. 2020; Dincă et al. 2022). Therefore, researchers are experimenting with innovative bio-based organic fertilizers as an alternative to chemical fertilizers (Chojnacka et al. 2020; Ye et al. 2020; Naher et al. 2021). Bioorganic liquid fertilizers have come to the fore in recent years as an alternative to soil conditioner and plant nutrition tool and to chemical fertilizers in order to meet the needs of plants (Nhu et al. 2018; Duraid and AL-Taey 2021). Furthermore, it is necessary to develop fertilization methods and technologies by using some organic matter liquid fertilizers, to increase biodiversity, to eliminate the deficiencies caused by agricultural yield and inadequacy of plant nutrients (Sirbu et al. 2018; Köninger et al. 2021). Hence, it is possible to bring the most suitable and best agricultural practices to climatic conditions. Bioorganic liquid fertilizers and mixtures containing different microbial mixtures and promoting plant nutrition are obtained (Wang et al. 2020). These are biological tools that maintain sustainable soil fertility, soil microflora, and plant health and can be an effective alternative to chemical fertilizers (Kumar et al. 2022). Similarly, it can be used in various doses as an additive to commercial fertilizers (Bhardwaj et al. 2014; Nhu et al. 2018). Hence, it is possible to bring the most suitable and best agricultural practices to different land management and climatic conditions.

13.2 Production of Bioorganic Liquid Fertilizers and Plant Nutrition Enhancer

In recent years, the use of mineral fertilizers in agriculture has increased significantly to support the growing global demand for food security. Since the use of chemical fertilizers negatively affects both soil and plant health, the need for the use of organic fertilizers from edible wastes is increasing. Therefore, the development of new production processes of organic fertilizers can contribute to the circular economy (Chojnacka et al. 2020; Ye et al. 2020; Fernández-Delgado et al. 2022). Furthermore, bioorganic liquid fertilizers consist of beneficial microorganisms and essential plant nutrients for the recycling of organic matter (Phibunwatthanawong and

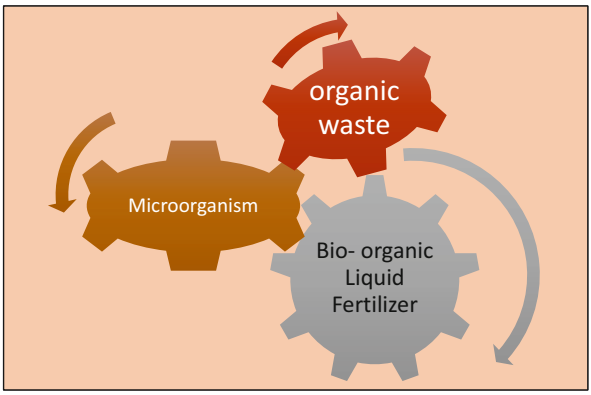

Fig. 13.1 Production process of bioorganic liquid fertilizer

Riddech 2019). In addition, microorganisms have an important role in the breakdown of substances during the fermentation process for organic residue (Bondarenko et al. 2021; Shaji et al. 2021).

As opposed to organic composts, bioorganic liquid fertilizers can be manipulated by the fermentation process, balanced micro- and macroelement content, and various microorganisms and can be effective in the transport of nutrients that plants can take (Syed et al. 2021). Consequently, it is necessary to develop new technologies for the processing of liquid fertilizers and for the production of liquid organic fertilizers with greater added value.

Some agricultural wastes are a rich source of raw materials for obtaining liquid fertilizers, as they contain high carbon source and many nutrients (Sadh et al. 2018). For example, sugarcane leaves, a waste product of sugarcane harvesting, are a source of organic matter. Similarly, agricultural wastes resulting from banana production are the waste products of the banana harvest (Phibunwatthanawong and Riddech 2019; Tao et al. 2020; Alzate Acevedo et al. 2021).

The production process of bioorganic fertilizers and the development of new technologies can increase the functional content and benefits of organic fertilizers (Fig. 13.1) (Jalalipour et al. 2020; Bondarenko et al. 2021). Recently, many methods have been used to obtain bioorganic liquid fertilizers. One of them is to obtain liquid fertilizer from organic wastes by conventional methods, and another is microwave-assisted extraction method (Priyam et al. 2019; Amran et al. 2021; Bhat 2021; Izydorczyk et al. 2021; Fernández-Delgado et al. 2022).

13.3 Cost Analyses and Marketing of Bioorganic Liquid Fertilizers and Plant Nutrition Enhancers

In recent years, bioorganic liquid fertilizers have attracted the attention of both researchers and policymakers, and in this framework, innovative studies have been carried out in order to obtain more efficiency from the unit area. Moreover, there has

been an increase in agricultural areas where organic products are produced in the world. Thus, the importance of using bioorganic liquid fertilizers has emerged in order to obtain sufficient yield in these areas. Of course, when considering the market share of these products, a very good cost planning should be done together. So far, many enterprises and organizations have been involved in activities related to the production of bioorganic liquid fertilizers and have obtained various products within this framework. These have taken their place in the market after registration, and patent procedures have been completed. In addition, the use of bioorganic liquid fertilizers is increasing rapidly. However, due to the widespread use of chemical fertilizers, it constitutes the largest part among the existing sectors. However, especially under the leadership of the EU, the production of liquid bioorganic fertilizers is encouraged, and efforts are being made to replace chemical fertilizers in the market for these fertilizers in the future. As a result, the costs of chemical fertilizers imported by countries are increasing and therefore governments are trying to develop different policies. The value of organic liquid fertilizers and plant nutrition enhancers is expected to increase by 10–15% in 2022 compared to the previous year. As a consequence, there is a noticeable increase in the need for fertilizers applied to agricultural lands every year, both in Europe and in the world. However, it is aimed that the use of both bioorganic liquid fertilizers and regulators that increase plant growth can replace chemical fertilizers, and as a result, the use of chemical fertilizers in many agricultural lands will be reduced. As another outcome of the use of liquid fertilizer and plant nutrition enhancers, it is expected to contribute significantly to increasing food safety and reliability, which will increase as the global population is expected to increase in the future.

13.4 Conclusion and Future Perspective

In recent years, with the increasing population in the world, many innovative agricultural practices have been needed in order to ensure food safety and reliability. In this line, the effective use of fertilizers is one of the most important factors in increasing the yield and sustainability of plant production. There has been an increased interest in bioorganic liquid fertilizers and plant nutrition enhancers, which increase plant growth, as an alternative to chemical fertilizers, in order to both reduce costs and protect soil and plant health. For this reason, it is necessary to develop effective remedial measures in order to obtain the maximum yield from the products grown per unit area on arable soil. As a result, bioorganic liquid fertilizers developed as an alternative to chemical fertilizers and applications that promote plant growth can contribute to sustainable land management by increasing their trials in field and greenhouse conditions in terms of both low cost and increasing yield.

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Chapter 14
Production, Cost Analysis, and Marketing of Agricultural Effective Microorganisms

Amarchand Chordia Murugan, Vivekanandhan Perumal, and Swathy Kannan

Abstract By 2050, the planet must feed nearly 9 billion people, resulting in a 60% increase in food demand. Progress toward food security involves being available, accessible, and sufficient quantity and quality. In this dangerous scenario, new approaches to crop production are more crucial than ever. The global agricultural microbial market was worth USD 4.27 billion in 2019. During the 2020–2027 period, the market is expected to grow at a CAGR of 16.27%. Effective microorganisms (EM) have grown in a variety of industries. The global breakout of the COVID-19 pandemic has resulted in several global economy and agriculture sector issues. The market saw a mixed picture in terms of biostimulants and biofertilizers sales. Despite this, BASF SE and Syngenta saw an increase in their annual biological sales. The global agricultural biological market is expected to develop significantly due to the rising demand for organically derived productivity-enhancing products. The quest for new biocontrol microorganisms is ongoing and becoming more critical as challenges of pathogenic resistance rise.

Keywords Agriculture · Green revolution · Marketing · Effective microbes

14.1 Introduction

Agricultural technologies have been steadily improving since the Industrial Revolution and even more so since the mid-twentieth-century green revolution. Innovations in farming practices resulted in massive crop yields per area of arable land. This phenomenal increase in food production has supported a global population that has quadrupled over the last century. As the world's population expands, so does the amount of land devoted to feeding it. According to World Bank estimates, 47 million square kilometers of land is under agricultural production on the planet as of 2018.

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However, meeting the demand for increased agricultural productivity in the coming decades is likely to be far more complicated than it has been thus far. The reasons for this are ecological. Global climate change is threatening to destabilize a number of the natural processes that enable modern agriculture. However, modern agriculture is partially to blame for the sustainability crisis. Numerous techniques and modifications used by farmers to increase output also have negative environmental consequences. The universal food security challenge is straightforward: By 2050, the planet must feed nearly 9 billion people, resulting in a 60% increase in food demand. As a result, progress toward food security necessarily involves food that is available, accessible, and of sufficient quantity and quality to guarantee good nutritional outcomes, especially in long-term socioeconomic crises (FAO 2015). In this scary scenario, new approaches to crop production are more crucial than ever.

Effective microorganisms (EM), such as plant growth-promoting microbes, have been demonstrated to increase plant nutrient uptake, growth, and yield through several underlying mechanisms such as changes in soil structure, nutrient solubility, root growth and shape, plant physiology, and symbiotic relationships. Furthermore, they can boost plant tolerance to abiotic stressors and resistance to infections (Halpern et al. 2015; Brad Cova et al. 2019). EM are environmentally friendly technology. Effective microorganisms (EM) are a mixed culture of beneficial microorganisms that consist of a fermented mixed culture of coexisting and mutually compatible microorganisms in an acidic medium which can be used as bio-inoculants to augment the microbial diversity of soils and plants in ways that can improve soil quality as well as crop growth, yield, and quality. The microorganisms that make up EM are neither exotic nor engineered; instead, they are naturally occurring species isolated from natural environments around the world and selected for their specific beneficial effects and compatibility in mixed cultures.

14.2 Effective Microorganisms

The concept and application of EM began 30 years ago, in the late 1960s, and have been primarily aimed at resolving problems linked with soil degradation, diminishing productivity and crop yields, and the intensive use of agricultural pesticides in monoculture cropping systems. Interest in the notion grew steadily, and by 1982, EM had become a viable product with extensive on-farm testing to back it up. In Okinawa, Japan, Dr. Terugo Higa of the University of Ryukyus found a remarkable collection of naturally occurring helpful microbes with an incredible ability to revive, restore, and maintain in 1982. Effective microorganisms (EM) were the acronym he assigned to this group. Agricultural, environmental, and consumer organizations in various nations have indicated an interest in EM as a viable means of lowering chemical fertilizers and pesticides in their food production systems over the last decade. Many farmers have successfully moved from chemical-based conventional agriculture to non-chemical, organic agricultural systems, with substantially less risk. As a result, more than sixty countries are currently utilizing EM to

achieve more sustainable agriculture and environmental practices. EM technology has been incorporated into certain countries' national agricultural research and development programs and objectives (FAO 2011).

Agricultural effective microorganisms include up to 80 different species belonging to five major groups of microorganisms, including photosynthetic bacteria (*Rhodopseudomonas palustris*, *Rhodobacter sphaeroides*), lactic acid bacteria (*Lactobacillus Plantarum*, *L. casei*, *Streptococcus lactis*), yeasts (*Saccharomyces cerevisiae*, *Candida utilis*), actinomycetes (*Streptomyces Albus*, *S. griseus*), and fermenting fungi (*Aspergillus oryzae*, *Penicillium* spp., *Mucor hiemalis*) (Ndona et al. 2011). Effective microorganisms (EM), like other biostimulants, can positively affect plant nutrition, root morphology, and the structure of the rhizosphere–microbial community. Some authors have reported the use of EM as an amendment for various crops to improve soil fertility, increase crop yield, and control plant diseases (Ndona et al. 2011; Rezende et al. 2008; Javaid and Bajwa 2011; Hu and Qi 2013; Roberti et al. 2015; Talaat et al. 2015; Pierce et al. 2016; Shin et al. 2017). However, in cotton plants, application of EM alone did not result in a significant increase in yield over control, but when combined with organic matter, resulting in a 44% increase in yield over control (Khaliq et al. 2006). Similarly, in the green manure amendment, EM's application resulted in a significant decrease of 23% in mung bean grain yield, while it increased grain yield by 24% and 46% in farmyard manure and NPK fertilizer amendments, respectively (Javaid and Bajwa 2011). The effects of EM on photosynthesis have also been researched. EM treatment improved photosynthetic rate, transpiration rate, and intracellular CO2 concentration in cabbage plants (Chantal et al. 2010). Similarly, photosynthetic efficiency improved in periwinkle plants (Pierce et al. 2016).

14.3 Preparation of Effective Microorganisms

Effective microorganism concentration was developed by Higa and Parr (1994) and Higa (2001) in the 1970s from approximately 80 beneficial microbe species found in the environment. However, five of these bacteria are very important for the survival of EM. The study focused on utilizing safe and non-harmful microorganisms for agricultural uses. The potential of microorganisms in agriculture was realized after various trials were undertaken. EM have been demonstrated to be effective as an inoculant for increasing soil microbial diversity (Higa 1991; Higa and Parr 1994) and water. Sato et al. (2003) used EM1, EM3, and EMx to pioneer EM in concrete research. In recent years, research into using EM in concrete is increasing, albeit most studies have focused on comparisons with non-product microorganisms. Several investigations (Yatim et al. 2009; Andrew et al. 2012; Ali et al. 2017; Sam et al. 2019) have been undertaken since 2003 to investigate the performance and processes of EM-based concrete in fresh and hardened stages.

14.3.1 Types of Effective Microorganisms

There were four primary forms of EM at the time of its development. They contain varying percentages of cultures of various microorganism species in the solution. The following are the EM types:

1. EM1 is a mixed culture primarily composed of lactic acid bacteria and yeasts, with a minor percentage of photosynthetic bacteria.
2. EM2 is a photosynthetic bacterium, yeast, and fungal mixed solution culture.
3. EM3 is a mixed culture of bacteria that are primarily photosynthetic.
4. EM4 is a Lactobacillus-dominated culture with a few other bacteria.

Though Higa and Wididana (1991) developed four EM kinds, new types, such as EMC and EM5, were later developed. These EMs have primarily been used in agriculture (Khaliq et al. 2006; Ke et al. 2005; Gnanadesigan et al. 2014; Laskowska et al. 2017), cattle (Laskowska et al. 2017), waste treatment (Safwat 2018), and water treatment and remediation (Lananan et al. 2014; Ting et al. 2013; Muthaura et al. 2010).

14.3.2 Constituents of Effective Microorganisms (EM) for Crop Production

EM are a consortium of selected species of beneficial microorganisms. They include lactic acid bacteria, PSB, yeast, actinomycetes, and fungi (Ahn et al. 2014). Although EM consists of many microorganisms, its efficacy is dependent on the ratio of these dominant microorganisms. Therefore, adequate quantitative analysis and quantification of these microorganisms are necessary for optimum efficiency. The individual characteristics of EM's main constituents are discussed below.

14.3.2.1 Lactic Acid Bacteria

These facultative organisms can exist with or without oxygen since they do not rely on oxygen for energy (Ali et al. 2017). They get their energy from amino acids, sugars, and carbohydrates generated by photosynthetic bacteria and cane molasses in EM culture and other carbohydrates (Sun et al. 2014). The LAB bacteria are gram-positive bacterium that produces lactic acid or lactate as their primary product (metabolite). As a result, lactic acid has been discovered to increase the hydration of Portland cement (Singh et al. 1986). According to Kastiukas et al. (2015), the compressive strength of a concrete sample containing 1 % lactic acid increased from 39 MPa to 43 MPa after 7 days. *Lactobacillus plantarum*, *Lactobacillus casei* (Vidra et al. 2017; Sasaki et al. 2012), *Lactobacillus fermentum*, *Lactobacillus salivarius*,

and *Lactobacillus delbrueckii* (Bhatt and Srivastava 2018) are the most prevalent species of this kind of bacteria found in EM.

14.3.2.2 Photosynthetic Bacteria

These self-sustaining bacteria create amino acids and carbohydrates by using the sun as an energy source. The metabolites produced are occasionally used by other organisms in the culture. Depending on their roles and dynamics within an ecosystem, PSB can create and consume urea. *Rhodopseudomonas palustris* (Higa and Parr 1994), *Rhodobacter sphaeroides* (Sasaki et al. 2012), *Rhodobacter capsulatus*, and *Rhodospirillum rubrum* are common species of this class of bacteria.

14.3.3 Yeast

These organisms can consume sugars released by photosynthetic bacteria, extending their lives and precipitating $CaCO_3$. *Candida utilis* and *Saccharomyces cerevisiae* are the two most common yeast species. According to a review conducted by Bolobova and Kondrashchenko (2000), yeast fermentation waste (YFW) is a good biomodifier in concrete. According to their findings, YFW can affect water reduction capacity by up to 20%, compressive strength by up to 60%, and cement usage by roughly 15%.

14.3.3.1 Molasses

Molasses are an essential food source for bacteria in EM solutions, and it functions as a catalyst for latent spores in the fermentation process, activating the microorganisms. Molasses' efficiency in the fermentation process is determined by the amount of sugar present (Jumadurdiyev et al. 2005). Blackstrap molasses (type C) are recommended because of their lower sugar content; blackstrap molasses (type C) are recommended. Molasses' retarding effect on hydration has been confirmed by researchers (Ali and Qureshi 2019). This impact is caused by its adsorption on the surfaces of cement grains, which limits the interaction of cement grains and water molecules, particularly at lower dosages.

14.3.3.2 Microorganism Preparation

EM are a liquid concentrate of active microorganisms that are latent until activated. They become active due to the availability of a food source or nutrients. The microorganisms are activated by combining the concentrate with molasses and distilled water or rice rinse water. Molasses offers nutrients for microbial

proliferation (Higa and Parr 1994). EMA refers to activated EM. Activation is required to enhance the growth of effective microorganisms (Ismail and Saman 2014) and to rouse dormant spores by providing nutrition. For EM activation, chlorine-free or distilled water is needed. Tap water can be utilized; however, it must be kept open for 24 h for dichlorination because chlorine kills the germs. Because sulfur can kill microorganisms, pure unsulfured sugarcane molasses are preferable to beet molasses (Jumadurdiyev et al. 2005). EMA is made up of EM, molasses, and water. The most common mix percentage in concrete is 5% EM1, 5% molasses, and 90% distilled water (Yatim et al. 2009), while other research employed a subtly different mix ratio (Ismail and Saman 2014). After 7–10 days of incubation, the fermentation process begins. The acid generated by lactic acid bacteria reduces the pH of EMA suspension to around 3.5–4.0 (Yatim et al. 2009; Javadi et al. 2018). EMA should be stored at room temperature or in a calm environment to enable long-term storage and abstain from direct sunlight exposure.

14.4 Applications of Effective Microorganisms

According to Umi and Sariah (2006), using efficient microorganisms in agriculture has various advantages. By harmonizing and diversifying local microbes, effective microorganisms contribute to soil enrichment. Earthworms will feed on a diverse range of microorganisms, increasing the number of micropores. This improves soil health, promotes plant growth, reduces insect pressure, and allows for high-quality crops. Because it is derived from natural and organic materials, this category of bacteria is entirely safe for humans, plants, animals, and soil (Brown et al. 2000). Agriculture uses it extensively in various tasks such as planting, fertilizing, composting, and sanitation. The various organisms in effective microorganisms complement each other and have a mutually beneficial connection with plant roots in the soil environment. Plants would grow exceedingly well in soils populated and dominated by these powerful bacteria (Sun et al. 2014). Effective bacteria improve soil fertility and encourage crop growth, flowering, fruit development, and ripening. It can raise crop yields and crop quality while also hastening the breakdown of organic matter from agricultural leftovers (Cortez et al. 2000). The beneficial microorganisms community in the soil grows, which aids in the prevention of soil illnesses via competitive exclusion (Postma-Blaauw et al. 2006). The benefits of using efficient microorganisms in agriculture and gardening are discussed more below.

14.4.1 Improvement of Soil and Plant Nutrition Uptake

Effective microbes will boost the availability of nutrients in the soil for plants, reducing the need for continual replenishment and cultivation (Daniel and Anderson

1992). Foliar application of beneficial microorganisms results in abundant helpful microorganisms in the plant rhizosphere. Certain microorganisms in influential microorganism culture, including photosynthetic bacteria and N-fixing bacteria, are thought to improve the plant's photosynthetic rate, efficiency, and N-fixing ability (Pati and Chandra 1981). Vaid et al. (2017) stated that when bacterial isolates are used reciprocally, they perform better than when used individually, and these isolates substantially increase the mean Fe concentration in grain and straw by 34% and 52.4%, respectively, over the non-inoculated control. They also increase total Fe uptake in comparison to the non-inoculated control. The bacterial inoculation also improves the accessibility of Fe in the soil. When farmyard manures are sprayed during the blossoming and maturity stages, effective microorganisms improve plant nitrogen, phosphorus, and potassium nutrition. Effective microorganisms applied to FYM amended soil significantly improve plant nutrition at later mung bean crop growth (Javaid and Bajwa 2011). It is claimed that modern vegetables have substantially lower nutritional content than those grown in the past. EM vegetables have a great nutritional value and a higher sugar content, improving their flavor and tinning them for a more extended period (EMRO, Japan).

Muthaura et al. (2010) observed that inoculating the plants with efficient microorganisms boosted pigweed growth and yield, reducing the usage of fertilizers in the production of this food and boosting sustainable agriculture. In mung bean, EM improved grain production by 24% and 46%, respectively, in farmyard manure and NPK fertilizer supplements (Javaid and Bajwa 2011). According to Cheng and Yingchun (2013), long-term application of adequate microorganism compost may impact soil nematode community structure, wheat biomass, and grain yield. Some soil bacteria functioned as bio-inoculants, giving nutrients and encouraging plant growth, while rhizospheric microbes synthesized plant growth promoters, siderophores, and antibiotics and assisted with phosphorus uptake. We have seen rapid progress in understanding the diversity of environmental microorganisms and their potential benefits to sustainable agriculture and industrial systems. Soil is the primary substrate in which plants exist, absorbing nutrients and acting as a nutrient bin. One of the finest remedial strategies for soil contamination is "intrinsic bioremediation" (Vaxevanidou et al. 2015).

14.4.2 Suppressing Soil Pathogens

The incorporation of beneficial microorganisms into agricultural soil suppresses soil-borne pathogens and accelerates the decomposition of organic matter, increasing the bioavailability of mineral nutrients and essential organic compounds to plants (Singh et al. 2003). The addition of beneficial bacteria into the soil aids in reducing soil-associated microbiological illnesses. The inoculation of helpful microorganisms induces the "rotation effect," which occurs due to beneficial organism regeneration and harmful bacterium eradication. Disease suppression occurs due to the completion of available resources between disease-causing microbes in the soil and

beneficial microbes introduced into effective microorganisms. This results in an increased population of effective microorganisms through inoculation, depleting available resources in the soil and reducing pathogenic microorganisms due to starvation (Johan and Jesper 2005). Lactic acid sterilizes, and its presence in the soil inhibits nematode population expansion and protects against nematode-associated plant illnesses. Lactic acid bacteria found ineffective microorganisms also contribute to the decomposition of cellulolytic and lignified organic matter in the soil (Ouwehand 1998).

14.4.3 Enhancing Breakdown of Organic Wastes and Composting

Common and food-grade aerobic and anaerobic microorganisms make up effective microorganisms. When we introduce activated effective microorganisms into environments dominated by putrefactive bacteria, the microbiomes improve, and foul odors are reduced. Changing the microbial environment so that effective microorganisms thrive will result in faster organic matter fermentation and reduce the time required to produce high-quality compost. Using effective microorganism products, we can efficiently solve foul odors at waste treatment sites, such as incineration and sewage treatment facilities (EMRO, Japan). Hussain et al. (1999) discovered an increase in wheat and rice grain yield when effective microorganisms were applied in conjunction with farmyard manure and mineral NPK. Higher grain yields in the current and previous studies when effective microorganisms were combined with organic matter can be attributed mainly to the activity of the introduced beneficial microorganisms, which enhanced the decomposition of organic materials and the release of nutrients for plant uptake. According to Jusoh et al. (2013), compost treated with an effective microorganism has higher N, P, and K content than compost not treated with an effective microorganism. This study suggests that effective microorganisms are appropriate for increasing mineralization in the composting process. The finished compost indicated that it was in the matured range and could be used without restriction.

The management of agro-industrial wastes has become a big issue, and as a result, most farmers dispose of them by burning them. Open burning emits a massive amount of toxic pollutants in the atmosphere (particles and inorganic and organic gases), which have a negative influence on the environment and human health (Korenaga et al. 2001), as well as the risk of soil erosion and deterioration due to recurrent burning (Kahlon and Dass 1987). Furthermore, disposal in bodies of water (such as a river or lake) may contribute to a decline in water quality and exacerbate the problem of water bloom and habitat degradation. As a result of these problems, there is an urgent need to develop practical solutions for agro-industrial waste management. Composting is one of the most appealing because of its minimal environmental impact and low cost (Bustamante et al. 2008; Lu et al. 2009) and

its ability to produce a valuable product used to increase soil fertility (Weber et al. 2007). When complex organic molecules enter the soil, such as plants, animal dung, and organic fertilizers, IMO breaks these complex compounds into simpler forms or elements that can undertake ionic interactions (Anastasia et al. 2004). Compost residues are more beneficial than crop fertilization. Hanim et al. (2012) did a study to compost paddy husk and corn stalk residues and assessed the physicochemical parameters of different composts and humic acid extracted from the end product. This investigation revealed that IMO compost made from corn stalk had higher quality (chemical properties) than that made from rice husk. The methods for safe residue management are time-consuming, yet biocomposting is a reliable method for most farmers to recycle residue quickly.

14.4.4 Strengthening Native Microbial Activity

According to Bossuyta and Hendrix (2005), photosynthetic bacteria are self-sustaining microorganisms. They use sun and soil heat energy to transform exudates from root systems, soil organic fraction, and gases like ammonia into cell building resources, including amino acids, nucleic acids, and sugars. These can be absorbed directly into plants to enhance plant growth and stimulate and maintain the growth and establishment of other beneficial microbes in the soil system. For example, in the presence of amino acids released by beneficial bacteria, vesicular-arbuscular mycorrhiza fungi known to improve plant absorption of soil phosphates grow in the root zone. Furthermore, in the soil ecology, the VAM fungi coexist alongside Azotobacter and Rhizobium, boosting plant nitrogen fixation potential. Effective microorganisms boost the activity of beneficial indigenous microorganisms like mycorrhizae (both ectomycorrhizae and endomycorrhizae), which fix atmospheric nitrogen and accelerate phosphate and zinc uptake from the soil, augmenting the usage of chemical fertilizers and pesticides. Soil fertility improvement has a considerable favorable effect on crop plant growth, flowering, fruit development, and ripening (Lévai et al. 2006).

14.4.5 EM in Livestock Farming

Effective microorganisms will minimize disagreeable odors caused by ammonia and trimethylamine buildup, both alkaline. Feeding animals rations containing the efficient microbe Bokashi will improve their internal microbiota, reducing the offensiveness of dung odors (Higa 1991). Spraying effective microorganisms inside barns and adding effective microorganisms to animal feed and drinking water will improve the microbial ecology of the entire barn, including the intestinal bacteria flora of the

cattle, and keep the entire operation healthy. As a result, the fat quality will increase, there will be no unique meat odor, items will keep fresher for more extended periods, and the taste and quality of animal products will improve. Furthermore, it is possible to limit vaccines and antibiotics, thereby encouraging safer animal products produced by both producers and consumers (EMRO, Japan).

14.4.6 Reduction of the Number of Pollutants in the Environment

Valls and Lorenzo (2002) discovered that repeated application of efficient microbes reduces the demand for chemical fertilizers and pesticides in succeeding harvests, resulting in less pollution. Likewise, they detoxify contaminated water. The addition of efficient microorganisms to rivers increases the number of bacteria, which form the base of the ecological pyramid. When the bottom of the pyramid grows larger, so does the pyramid itself, resulting in greater diversity in the ecosystem. This will boost rivers' ability to self-purify, allowing them to become clean and attractive once more (Nikolopoulou and Kalogerakis 2010). *Mudball* is an effective microbe that activates and optimizes the inherent power of local and native microorganisms in the ground and water. These mudballs are said to have qualities that will purify water, improve water quality, and solve sanitary issues. Because there is little oxygen dissolved in the water, accumulating sludge in polluted rivers is oxygen deficient. By decomposing organic matter without oxygen, toxic fermentation bacteria inside the sludge produce dangerous gases such as methane, ammonia, and hydrogen sulfide. When mud balls are put to water under those conditions, they become lodged in the sludge's surface, and efficient fermentation bacteria contained in the mud balls begin to degrade the sludge (EMRO, Japan).

14.4.7 Effective Microorganisms in Bioremediation

Microorganisms' intrinsic capacities make them excellent for removing unwanted and heavy metals from solutions (Beveridge and Murray 1976; Langley and Beveridge 1999; Nies 1999). These capacities have been either passive or active in accumulation and biosorption (Brandl and Faramarzi 2006). Indigenous strains are better suited to overcoming obstacles such as high concentrations of heavy metals and acidic environments because they have adapted well to in situ circumstances. Bacillus strains have been widely employed in the removal of such metals (Pb, Cd, Cu, Ni, Co, Mn, Cr, and Zn) from wastewaters (Kim et al. 2007), as well as from soil solutions if administered to improve the intrinsic quality and fertility of the soil.

14.5 Production of Agricultural Effective Microorganisms

In line with the technology and objective of effective microorganism production, various facilities are required for the successful implementation of such projects, which are indicated below:

14.5.1 Laboratory Equipment, Glassware, and Other Necessities

(a) Compound biological microscope with low, high, and oil immersion lenses, measuring micrometers and hemocytometers for microscopic examination, counting vegetative and cysts/spore/buds
(b) Autoclave for sterilization of media
(c) Hot air oven for sterilization of glassware
(d) Incubator (25–40 °C range) for microbial growth
(e) Colony counter for CFU counts in agar plates
(f) Water bath-controlled temperature operations like melting of solid agar media
(g) Refrigerator for storing strains, etc.
(h) pH meter for measuring acidity/alkalinity of media
(i) Weighing balance for weighing ingredients of media
(j) Bunsen burner/sprit lamp.

Glassware and other necessary materials:

(a) Petri dishes for holding molten media
(b) Conical flasks, 50–1000 ml range size
(c) Test tubes or culture tubes without rim or culture tubes with caps (Biju bottles)
(d) Funnels for pouring media
(e) Pipette ranging from capillary size to all graduated size up to 10ml
(f) Measuring cylinders
(g) Beakers, 50–1000 ml range size
(h) Glass slides and coverslips
(i) Bent glass rods (spreaders)
(j) Inoculating loops and inoculating needles
(k) Dropping bottles for stains and reagents
(l) Saucepans for boiling media
(m) Wire baskets for holding tubes and Petri plates
(n) Filter papers
(o) Wax pencils/markers
(p) Metal containers for sterilized pipette and petri dishes
(q) Cotton wool
(r) Cheesecloth
(s) Scalpel and forceps

(t) Bottles containing disinfectants
(u) Detergent powder
(v) Necessary chemicals.

14.5.2 Effective Microorganisms' Production

The manufacturing facility setup needs a laboratory and other facilities and office. Space may also be required to install tube well/dug well and parking vehicles. A minimum of 1/2 acre of land is required for setting up a 150 TPA unit. Preferably, the entire site should be fenced with gates at suitable places. The boundary may be planted with thick and tall-growing species like Asoka to filter air and reduce dust. Given below are the financial plans for the production of biofertilizers in different scales (small, medium, and large) with technoeconomic viabilities, constraints, and marketing of biofertilizers.

14.5.2.1 Financially Outlay and Returns

1. Fixed assets: Fixed assets are the properties or equipment that the company owns and plans to generate income using it. Tables 14.1 and 14.2 are the land/property and laboratory equipment requirements for setting up an effective microorganism's production unit.
   a. Building: approximately 768 sg m at 2000.00/m^2 estimate of Rs. 15.36 lakhs with other charges.

Table 14.1 Land/property requirement for EM production unit start-up

S. no.	Components	Dimensions (ft.)	Area (sq. ft.)
1.	Media preparation room	20 × 18	360
2.	Sterilization room	20 × 18	360
3.	Fermenter hall	20 × 18	360
4.	Mixing room	20 × 18	360
5.	Packing room	20 × 18	360
6.	Store	20 × 18	360
7.	Saleroom and office	20 × 18	360
8.	Tin shade[a] (for storing and pulverization of carrier material)	20 × 100	2000
Total		2520 ft. (768 m^2)	

[a] Tin shade for lignite storage costs extra

Table 14.2 List of equipment required for EM production unit start-up

S. no.	Equipment	Quantity (no)	Total cost (Rs. in lakhs)	Justification for
1.	Balance	1	0.25	Weighing
2.	pH meter	1	0.15	Measuring pH of liquid media
3.	Laminar air flow chamber	1	1.50	Inoculation of microorganisms
4.	Autoclave (vertical)	2	1.00	Sterilization of liquid media and glassware
5.	Incubator (BOD)	2	1.50	Incubation of inoculated samples
6.	Refrigerator	2	0.25	Storage of microbial samples and chemicals
7.	Rotary shakers	2	1.50	For fermentation of cultures
Total			6.15	

Table 14.3 EM production unit operational cost

S. no.	Particular	Amount (Rs. in lakh)
1.	Glassware	2.00
2.	Chemicals and carrier materials	10.00
3.	Salaries (2 research assistants at 15,000/PM)	3.60
4.	Daily wages workers (5 persons @ Rs. 400/day)	7.20
5.	Miscellaneous (electricity, packing materials, etc.)	2.00
Total (Rs. in lakh)		24.80

Table 14.4 Total operational cost (for 3 years)

S. no.	Particular	I year (Rs. in lakh)	II year (Rs. in lakh)	III year (Rs. in lakh)	Total (Rs. in lakh)
1.	Building and equipment	21.51	NIL	NIL	21.51
2.	Operational cost for 3 years (working capital)	24.80	24.80	24.80	74.4
Total (Rs. in lakh)		46.31	24.80	24.80	95.91

b. List of equipment

2. Operational costs (per tear): Operating costs are incurred after the official project operations start. This group of expenses comprises everything required to keep the business going, including inventory, employee wages, technology, intellectual property, rent, and funds allocated to such vital activities as marketing, sale, and production. It means that both direct and indirect project expenses are considered operating costs if they take part in the daily maintenance and management of the project. Tables 14.3 and 14.4 summarize the EM production unit operating cost of up to 3 years.

14.5.2.2 Technoeconomic Viability/Cost–Benefit Analysis

Calculating the annual average selling price of 100 tons of biofertilizers (5 lakh packets per year, each containing 200 g of biofertilizer, subject to total production, supply, realization of money from sold packets, and lifting of packets from the production unit) (Table 14.5).

14.5.2.3 Tentative Production Target

This section summarizes the projected receipt of funds based on the total supply, the realization of funds from packets sold, and the lifting of packages from the production unit (Table 14.6).

14.5.2.4 Financials

The project financials center around the strategic planning of recurring and non-recurring expenses and give an idea of how much does the business is profitable (Table 14.7).

Table 14.5 Input cost, production cost, and selling price of EM

S. no.	Particular	Amount (Rs. in lakh)
I.	Input costs on the NRC+RC items	
1.	Fixed cost (building/equipment)	21.51
2.	Variable cost	24.80
II.	Production cost (for 5 lakh packets/annum)	
1.	Depreciation in building cost (15.36 lakh) at 5% on the total cost of the building	0.768
2.	Depreciation in equipment cost (6.15 lakh) at 10% on the total cost of equipment	0.615
3.	Operational cost	24.80
	Total (Rs. in lakh) of the production cost of 5.00 lakh packets	26.183
	The production cost of 100 pkts of biofertilizer (average)	523.66 (~Rs. 550)
III.	Selling price	
1.	Production cost/100 pkts	550
2.	Risk coverage for unsold pkts @25%	137.5
3.	Charges for transport and publicity	200
4.	Profit margin	100
	Total selling price per 100 pkts	987.50 (~Rs.1000)

Table 14.6 Net profit and anticipated receipt based on tentative production and supply of 100 ton EM per year

S. no.	Year	Tentative target	Production	Supply	Production cost	Anticipated receipt (Rs. in lakhs)	Net profit (Rs. in lakhs)
1.	1st	R & D and estimation of the production unit	NIL	NIL	NIL	NIL	NIL
2.	2nd	R & D and production	100 ton	99 tons (pkts 4.9 lakh each 200 g)	Rs. 27.22 lakh at Rs. 5.5/pkt	Rs. 49.5 lakh at Rs. 10/pkt	Rs. 22.27 lakh
3.	3rd	R & D and production	100 ton	99 tons (pkts 4.9 lakh each 200 g)	Rs. 27.22 lakh at Rs. 5.5/pkt	Rs. 49.5 lakh at Rs. 10/pkt	Rs. 22.27 lakh

Table 14.7 Cost of setting up a biofertilizer/biopesticide manufacturing plant with different capacities of 100–600 ton per annum (TPA)

Plant parameters		Cost	Small-scale production	Medium-scale production	Large-scale production
Land and site	–	Facilities	Rs. in lakh	Rs. in lakh	Rs. in lakh
No. of shifts	1	Non-recurring			
Working days/year	180	Basic infrastructures and facilities	–	10.00	20.00
Land required (sq. ft.)	3000–5000	Equipment/ machineries	25.00	40.00	60.00
Workforce		Total	25.00	50.00	80.00
Managerial	1	Recurring			
Skilled	1–2	Electrical power	4.00	6.00	11.00
Unskilled	3–10	Manpower	3.12 (1 + 1 + 3)	5.00 (1 + 2 + 6)	7.00 (1 + 2 + 10)
Utilities		Consumable, transport, trade margin, etc.	5.00	8.00	12.00
Electrical power (KVA)	10–50	Total	12.12	18	30
		Tech commercialization fee per product	1.5	1.5	1.5
		Total value of technology	38.62	69.5	111.5
		Economic analysis			
		Profit (net)	48.88	93	188.5
		Cost–benefit ratio after 1 year onwards	1:1.26	1:1.34	1:1.69

14.6 Market Opportunity for Agriculture Effective Microorganisms

Currently, there is a gap of 10 million tons of plant nutrients between the removal of crops and supply through chemical fertilizers. In the context of both the cost and environmental impact of chemical fertilizers, excessive dependence on agrochemicals is not a viable strategy in the long run because of the cost both in domestic resources and foreign exchange (Kumar et al. 2018). With the increasing demand daily for sale and residue-free foods, biofertilizers have an essential role in the organic farming system. The global biofertilizer market was worth more than $1.8 billion in 2018, increasing at a CAGR of around 14.3% between 2011 and 2018. Because biofertilizers are required in organic farming, the market is expanding rapidly worldwide (Kumawat et al. 2021). Even though India is the world's fourth largest user of chemical fertilizers (12.5 million tons of NPK nutrients), its soils are still being depleted of their inherent nutrient reserves due to a large gap between additions (12.5 million tons) and removals (18.5 million tons). One ton of produce removes 32 kg of nitrogen, 12 kg of phosphorus, and 58 kg of potash. Nutrient balance and efficient nutrient cycling are part of maintaining satisfactory yield levels. This can be accomplished by combining mineral fertilizer, bulky organic manures, compost, green manure, and biological inoculants, among other things. Currently, India's nutrient use is much lower than other countries. Compared to the global average consumption of 95 kg ha^{-1}, we use 74 kg ha^{-1} with a national productivity level of only 1.1 metric tons per hectare. Currently, India produces approximately 117 million metric tons of food grain for its expanding population of over 800 million people (Kumawat et al. 2021).

In June 2015, FAO/fertilizer organizations working group reported the prospects for inorganic fertilizer demand until 2015 to 2019 and supply and demand balances. It was evaluated that world demand for complete synthetic fertilizer had grown at 1.6% per annum from 2015 to 2019 (Panta 2018). Although biofertilizers were first commercialized in Europe and North America, their application is enhanced in South America and the Asia-Pacific. North America was the largest producer of biofertilizers, followed by Europe. Together these biofertilizers markets accounted for over 50–60% of the global returns. The Asia-Pacific is the third-largest biofertilizer market with increased demand from regional markets like India, China, and Taiwan. The Asia-Pacific is expected to witness a double-digit growth due to increased consumer preferences toward food and growing agricultural activities (Kumar and Chaudhary 2018). However, to other regions, an actual application of biofertilizers in the Asia-Pacific soil treatment compared to seed treatment.

South America is showing the fastest growth over the next seven years on account of growing agricultural activities in Brazil. Biological nitrogen-fixing biofertilizers are the largest bio-products and account for over 70% of global returns. International Plant Nutrition Institute (IPNI) has acknowledged that the application of biofertilizers in Brazil is about 60,000–70,000 tons/year. Each year, in the South

American regions of Uruguay, Bolivia, Argentina, and Paraguay, over 70% of 30 million hectares of soybean crops are inoculated with Bradyrhizobium sp., which favors a huge soybean market (Bianchi and Szpak 2017). A difference of 7–8.5% grain yield was recorded between inoculated and uninoculated control treatment, whereas Pseudomonas sp.

Furthermore, Azospirillum brasilense is approved for wheat and maize with an expected enhance in grain yield of 4–9% (Fukami et al. 2016; Oliveira et al. 2017; Zeffa et al. 2019). Although various nations with advanced research and demonstration and high biofertilizers technology like Japan, Taiwan, and Korea are moving forward toward biofertilizer production, small-scale farmers, due to literacy hindrance, need to be educated on the comparative applications of synthetic and organic constituents (Chien et al. 2007). Asian countries have opportunities to develop and commercialize bio-alternative products, contributing to food safety and eco-friendly concerns. In India, developing countries, more than one lakh hectares have been occupied by organic farming (Sekar et al. 2016) supported by nearly 100 biofertilizer manufacturers across the nation. They are receiving special attention in horticulture, vegetables, oilseed, cereals, and leguminous and medicinal plant cultivation. In China, 167 million hectares have been cultivated with microbial inoculants, and more than 300 manufacturers are found all over the nation.

14.6.1 Market Potential

The market is segmented for biofertilizers/biostimulants/biopesticides by form (liquid and carrier-based) and crop type. An overview of the cost–benefit ratio is given in Table 14.8 below to understand the potential of the effective microorganism current market value.

Table 14.8 Cost–benefit ratio for different EM production capacities

Sell of culture (pkts/year)	Revenue realized (Rs. in lakh)	Cost and profit statement		
		Total project cost	Profit (Rs. in lakh)	Benefit–cost ratio
Small-scale production (3.5 lakh pkts/year)	87.5	38.62	48.88	1:1.26
Medium-scale production (6.5 lakh pkts/year	162.5	69.5	93	1:1.34
Large-scale production (12 lakh pkts/year)	300.0	111.5	188.5	1:1.69

[a] Revenue realized = sell of culture/rate of culture sold (Rs. 25)
[b] Total project cost (only for 1st year) = NRC + RC + Tech. Comm fees

14.6.2 Effective Microorganisms' Market in India

The government of India is encouraging the use of biofertilizers through financial support, extension awareness programs, and subsides in sales with varying degrees of significance. At the same time, farmers are trained in new knowledge on biofertilizer technology focused on agricultural cultivation of crop quality in their regions. Thus, rural farmers are being encouraged to adopt the application of biofertilizers in sustainable agriculture. The government of India has been implementing the strategy to promote biofertilizers since the seventh Five Year Plan. The current annual manufacturing capacity of biofertilizers in India is 22,000 tons, and the end-user consumption is about 12,000 tons. The present level of biofertilizer use is still relatively low, and there is a substantial potential to increase it to 50,000–60,000 tons by 2020 (Survey 2018). Moreover, the government of India is focusing on generating additional demand through extension activities and promotion by regularly organizing seminars on the benefit of biofertilizer application in the legumes and cereal cropping system.

The government plays a dominant part in marketing biofertilizers in three possible channels:

1. State government via district-level officer's program and village-level workers to consumers
2. State marketing federation via cooperative organization to farmers
3. State agro-industries seven corporations via agro-extension service centers to farmers. However, the producers are free to sell through their own sales network or marketing channels. The government of India has provided the national biofertilizer development center at Ghaziabad with six regional centers in Jabalpur, Hisar, Bhubaneswar, Bangalore, Imphal, and Nagpur. In the absence of expressed information on the farm-level use of the agriculture inputs, this can help understand the progress of biofertilizer technology and its adaption in India.

14.7 COVID-19 Pandemic and Effective Microorganism's Market

In 2019, the global agricultural microbial market was worth USD 4.27 billion. The global impact of COVID-19 has been unimaginable and staggering, with agricultural microbials experiencing a drop in demand across all regions due to the pandemic. According to our analysis, the world market will grow at a slower rate of 8.7 % in 2020, compared to the average growth from 2016 to 2019. During the 2020–2027 period, the market is expected to grow at a CAGR of 16.27%, from USD 4.64 billion in 2020 to USD 11.81 billion in 2027. The increase in CAGR is due to this market's demand and growth returning to pre-pandemic levels once the pandemic has ended. Effective microorganisms (EM) have grown in various industries, including

agriculture, animal husbandry, wastewater treatment, and sanitization systems. Various trade exhibitions and expos have also assisted effective microorganism (EM) manufacturing businesses in expanding their client base, hence considerably contributing to the growth of the effective microorganisms (EM) market.

The sudden outbreak of the COVID-19 pandemic prompted the imposition of rigorous lockdown regulations in some countries, causing difficulties in the import and export of effective microorganisms (EM). COVID-19 impacted the global economy in three ways: directly impacting production and demand, causing supply chain and market disruption, and having a financial impact on enterprises and financial markets. The global breakout of the COVID-19 pandemic has resulted in several global economy and agriculture sector issues. Due to the lockdown imposition, border closures and restricted movement have caused supply chain disruptions. Market closures and disruptions in food services have negatively influenced demand. This has also resulted in a significant decrease and delay in the shipment of agricultural commodities. However, the market saw a mixed picture of biostimulant and biofertilizer sales, with some big manufacturers seeing a rise in product sales. Despite the adverse market conditions, BASF SE and Syngenta saw an increase in their annual sales of biological. Syngenta's total revenue was USD 13.6 billion in 2019.

Furthermore, industry players such as Novozymes and Valent Biosciences are launching an education website to help farmers and encourage sustainable agricultural production, which will improve sales in the following years. Fortune Business Insights identify major players that hold a significant portion of the global agricultural biological market share. These include Valent Biosciences, DowDuPont, Bayer, Arysta Lifescience, Syngenta, Marrone Bio Innovations, and Monsanto BioAg, Koppert.

The new Pesticide Registration Improvement Extension Act of 2018 (PRIA 4) is predicted to increase the growth of the worldwide agricultural biological market in North America. Similar efforts to effectively regulate biological products in the agricultural environment are envisaged in Europe, which will trail North America in market share and revenue. Currently, the two areas account for 60% of the worldwide agricultural biological market. The worldwide agricultural biological market is expected to develop significantly due to the rising demand for organically derived productivity-enhancing products. These biologicals have a slew of economic advantages, and agricultural yields increased significantly when biologicals were used. As a result, the economic feasibility of biologicals will increase the revenue of the global agricultural biological market in the coming years.

14.8 Future Perspectives

Through the performance of endophytic PGPR and its consortia has been promoting plant growth and enhanced productivity in the field conditions by several potential stains in different crops, the use of these EMs has not been popular among farmers

due to several reasons such as lack of information among farmers and availability and supply of quality bio-products. The industry value chain is critical in promoting and accepting biofertilizers (Srinivas and Bhalekar 2013). It consists of raw inputs for producers and suppliers, biofertilizer manufacturers, distribution channels, and end users. Most biofertilizer products are integrated across different stages of the values chain as demand and supply of bioprospecting are mainly dependent on the growth of end-user industries. 90% of the total biofertilizers manufactured are used in wheat, corn, soybean, chickpea, rice, mug bean, and maize.

With the promotion of alternate sources of integrated nutrition management, there is already knowledge among the consumers related to bio-inoculants and becoming popular gradually. Effective microorganisms can be easily positioned as eco-friendly growth enhancer manure with long-term benefits such as essential nutrient enrichments of soils; similarly, other benefits, for example, (a) save costs through the reduced dosage of agrochemicals, (b) improve resistance power against phytopathogens, and (c) enhance metabolites in crop plants. Now effective microorganisms/biofertilizers/biostimulators/bioenhancers of many brands are readily available through the regular distributor network. So, it is not very difficult to promote the appropriate crop-/region-specific effective microorganisms manufactured. Moreover, these effective microorganisms will have added benefits of lower transportation and marketing costs. Therefore, effective microorganisms can be marketed through the existing marketing channels. The point of sale (PoS) material giving details of proper technique for the application must be made available to all distributors/dealers. Similarly, tie-up with export-oriented crops like ginger, turmeric, fruits, and vegetable growers could be undertaken as this segment prefers the organic products due to the compulsion of importing nation policies of permissible limits of agrochemicals residues in the products.

14.9 Conclusion

Sustainable farming techniques can promote soil biodiversity, boost soil carbon sequestration, improve air quality, and avoid unnatural soil erosion. The quest for new biocontrol microorganisms is ongoing and becoming more critical as challenges of pathogenic resistance rise in the face of greater demand for crops to feed the world's growing population. It is acknowledged that the continual production of new efficient biofertilizers/biocontrol agents will be required to diversify the possible applications of bio-inoculants and to replace currently used biological agents in the event of resistance. As a result, cross-disciplinary partnerships and a better and more thorough understanding of soil–plant–microbe interaction are urgently needed.

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Chapter 15
Production, Cost Analysis, and Marketing of Biogas

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Abstract Biogas is an alternative to gaseous biofuels and is produced by the decomposition of biomass from substances such as animal waste, sewage sludge, and industrial effluents. Biogas is composed of methane, carbon dioxide, nitrogen, hydrogen, hydrogen sulfide, and oxygen. The anaerobic production of biogas can be made cheaper by designing a high throughput reactor and operating procedures. The parameters such as substrate type, particle size, temperature, pH, carbon/nitrogen (C/N) ratio, and inoculum concentration play a major role in the design of reactors to produce biogas. Multistage systems, batch, continuous one-stage systems, and continuous two-stage systems are the types of digesters used in the industry for biogas production. Biogas has a wide variety of applications across different industry sectors as mentioned previously. It can be directly used to produce heat and electricity, or it can be upgraded to remove water vapor, hydrogen sulfide, and CO_2 for use as a natural gas. It can also be used as an engine fuel in internal combustion engines or fuel cells for production of mechanical work and/or electricity generation. Biogas can also be used as a fuel for agricultural pumps depending on the requirements or can be directly upgraded to biofuels competing with biomass-based bioethanol and biodiesel production. This chapter concerns the production of biogas, setting up of small biogas plant, and provides the details about the expenditure and income generated in biogas production.

Keywords Biogas · Anaerobic digestion · Reactors · Cost · Economic investments

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Abbreviation

AD	Anaerobic digester
ABR	Anaerobic baffled reactors
ASBR	Anaerobic sequencing batch reactors
BOD	Biological oxygen demand
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
GHG	Greenhouse gases
GMI	Global methane initiative
HRT	Hydraulic reaction time
IEA	International Energy Agency
IRENA RES	International Renewable Energy Agency. Renewable Energy Target Setting
ORP	Oxidation–reduction potential
RDD	Research, design, development
THC	Total hydrocarbon
UNEP	United Nations Environment Programme
UASB	Upflow anaerobic sludge blanket digestion
VFA	Volatile fatty acids
VS	Volatile solids
WTE	Waste to energy

15.1 Introduction

In the current scenario, the irrational use of conventional fuels and the effect of greenhouse gases in the environment have led to the research efforts into alternative fuels production from bioresources. The use of energy has increased rapidly around the world, and about 88% of this demand is met by fossil fuels currently. Recent studies indicate that the energy demand will grow until 2050 up to 50%. In the meantime, the amount of greenhouse gas (GHG) emission in the atmosphere is also rising rapidly, with carbon dioxide (CO_2) being the main contributor (IEA 2015; UNEP 2014). Moreover, the security of energy supply is a crucial challenge, because most of the natural resources (oil and gas reserves) are found in politically unstable regions. In that context, biogas truncates one of the biggest challenges faced in this century which is to acquire a substitution for conventional fuels concerning the environmental issues. Besides the substitution of conventional fuels, it also aids in waste management, increasing agricultural productivity (Khanal 2008) and used as gaseous fuel in automotive applications.

Biogas is synthesized mainly for the production of heat, steam, and electricity because they do not require any pretreatment of gas (Baciocchi Renato et al. 2013). Biogas has been divulged as an alternative to petrol and diesel to expel the import

convolutions. Desulfurized and purified biogas can be utilized as natural gas. 1 m^3 of biogas can replace about 0.6 L of heating oil (Popli 2012). Biogas majorly constitutes methane and CO_2 with 40–75% and 15–60% volume, respectively (Baciocchi Renato et al. 2013). Developed countries such as Germany, Australia, Switzerland, and Italy use advanced large-scale plants for utilizing biogas. Biogas is regularly applied to generate heat, power, and electricity. Also, several industrial applications for its utilization in biogas plants as a substitute to natural gas are being progressed. Based on the analyzed data, a continuous increase in biogas production has been observed due to the global policies and programs.

A wide global market of biogas has been conspicuously promoted for the previous decades in various countries. Moreover, the advanced biogas production technologies have been supported by domestic or international supportive rules, such as research, design, and development (RD & D) financial funds, subsidization, and guaranteed electricity purchase contracts to make a competitive market against conventional energy suppliers (Teodorita et al. 2008). Biogas through AD provides significant advantages over other forms of bioenergy as AD is an energy-efficient and environmentally friendly technology for bioenergy production (Nishio and Nakashimada 2007). This AD technology can greatly eradicate GHG emissions in comparison to fossil fuels by employing locally available sources. Apart from that, the by-product called digestate is a high-value fertilizer for crops cultivation and can replace the common mineral fertilizers. There are various strategies around the world for producing biogas from agricultural products. In Germany, for example, the production of cheap agricultural products that require low processing (with no outcomes for consumers) provides feedstock for biogas plants. New policies recommend the use of crops and plant residents, life stocks remaining, and landfill use (IEA 2015). This chapter focuses on proposing a comprehensive analysis of the recent biogas technologies progress, cost requirements, and marketing of biogas.

15.2 Composition of Biogas

The demand for energy continues to rise with global population growth and rise in urbanization (Graham 2009). This has led to increased energy requirements and fossil energy utilization, increasing pollution across the globe (Kataki et al. 2017). With the limited supply of conventional fossil raw materials and their adverse environmental implications on air quality, research efforts on utilizing environmentally friendly renewable alternative sources have gained significant importance (Koberg and Gedanken 2012). In addition, continuous research coupled with technological developments in this field has helped the investment and deployment of clean energy technologies to surge across the world. As waste is an increasing issue worldwide, proper utilization of its energy potential via economically viable and technically feasible technologies can help promote sustainability and meet global renewable energy demand, while limiting risks pertaining to landfilling. One plausible and established waste-to-energy (WTE) option that has been widely adopted is

Table 15.1 Composition of biogas

Constituent	Chemical formula	Concentration	Property
Methane	CH_4	40–75%	Combustible
Carbon dioxide	CO_2	15–60%	Noncombustible
Moisture	H_2O	1–5%	Noncombustible
Nitrogen	N_2	0–5%	Noncombustible
Hydrogen	H_2	Traces	Combustible
Hydrogen sulfide	H_2S	0–5000 rpm	Combustible
Oxygen	O_2	<2%	Noncombustible
Trace gases	–	<2%	
Ammonia	NH_3	0–500 rpm	

the production of biogas from organic-rich waste streams via anaerobic digestion (AD) process or technology. Biogas production has a considerable role in waste management; it is not 100% greenhouse gas-free; nevertheless, it does not contribute to global warming. On the contrary, it helps to fight it. The methane can be combusted more cleanly than coal and can provide the desired energy with limited levels of carbon dioxide emission in the atmosphere. The carbon released from biogas can be absorbed by photosynthetic plants adding less total atmospheric carbon than the burning of fossil fuels. In preference to living species including humans, biomethane is one of the best ways to satisfy the increased need for energy without contributing to warming of the planet which threatens their living (Harrad 1996).

Biogas primarily consists of methane (CH_4) and carbon dioxide (CO_2) with small amounts of hydrogen (H_2), nitrogen (N_2), hydrogen sulfide (H_2S), oxygen (O_2), water (H_2O), and saturated hydrocarbons (i.e., ethane, propane). The detailed composition of biogas was given in the Table 15.1. The removal of the water and the toxic hydrogen sulfide is important in order to avoid detrimental effects. Biogas has composition that has to be taken into account during its use in spark ignition engines as the control system used is only for a single composition fuel. Barriers concerning the emission control during biogas combustion are directly connected to the methane oxidation and the appearance of low numbered alkanes in exhaust gases in case of a traditional three-way catalyst. The latter is because these hydrocarbons have strong carbon–hydrogen bonds and are nonpolar (Speight 2008). Liss and Thrasher reported that methane emissions from the combustion of all types of fuel make up one percent of the annual methane release. If emissions do not include a considerable amount of hydrocarbons, then the engine can be operated under rich conditions to control NoX emissions. Gaseous fuels unlikely to liquid fuels have to be stored and handled in a different way. For instance, in a vehicle, the natural gas is stored under high pressure, and when it is expanded, it will cause a significant pressure drop that may alter the fuel temperature and density and the same is applied for biogas (Liss and Thrasher 1991). Many experiments have been conducted in order to investigate the biogas combustion in spark ignition engines by the researchers (Juan Pablo Gomez Montoya, Andres A. Amell Arrieta and Jamie F. Zapta Lopez) of University

of Antioquia. By the experiments, it is reported that the removal of N_2 and CO_2 can achieve different biogas compositions and thereafter to be used as fuel. From the recent studies, it could be inferred that the increase of CO_2 concentration within the fuel results in an increase of total hydrocarbon (THC) and the CO emissions and in parallel causes a decrease in brake power because of the incomplete combustion due to slower burning rate (Porpatham et al. 2007). Biogas has various different negative characteristics as it suffers from low energy density, slow flame velocity, and incomplete combustion. However, biogas has beneficial properties as it requires less air to be combusted and has high auto-ignition resistance (Crookes 2006; Rounaud et al. 2002). In addition, the attainment of leaner operating conditions and heightened power generation can be based on hydrogen addition as hydrogen enhances the thermal efficiency by increasing the combustion rate and eliminating the cycle variation (Narayanan and Shrestha 2007).

15.3 Anaerobic Digestion for Biogas Production

A series of biological processing steps with the core conversion using the AD technology converts the complex organic matter in waste products to simple monomers by using a consortia of microorganisms which include *Methanobacterium bryantii* and acidogenic bacteria (Boone 2015). The AD technology facilitates organic decomposition and reduces inorganic matter in the absence of O_2 (GMI, USA). These organic materials are converted to final products, which are mainly biogas, digestate (a liquid form in most cases), and a combination of solid and liquid effluents derived from digestate treatments (Chen 2014). Biogas can be used to produce electricity and heat, while the effluent liquid rich in crop nutrients is used as agricultural fertilizer depending on the amount of nutrient loading, especially nitrogen (Chen 2014). The solid fraction from solid/liquid separation of the digestate can be used as dairy bedding or converted to potting soil mixes. Generally, the feed to the digester is pretreated using different physical operations to reduce maintenance issues. For example, the solids from wastewater primarily include primary and secondary sludge which are grinded, shredded, or screened for efficient operations. Additionally, the accumulation of grits inside the tanks reduces the working volume, which affects biogas production and increases maintenance and cleaning. Therefore, degritting is applied to prevent accumulation, which helps to improve process efficiency as well. Similarly, plastics, stones, and metals are removed from food waste during pretreatment depending on the collection methods (Water Environment Federation 2017).

15.4 Principle of Anaerobic Digestion

Biogas through AD, an energy-efficient and environmentally friendly technology, provides remarkable advantages over other forms of bioenergy (Nishio and Nakashimada 2007). AD technology greatly eradicates GHG emission compared to fossil fuels by employing locally available sources. Additionally, digestate, a by-product, is utilized as fertilizer has a high value in crops cultivation and can greatly replace the common mineral fertilizers. Biogas production through anaerobic digestion has witnessed an uncertainty of rural development in India. Initially in 1859, anaerobic digesters were installed in India to provide illumination. During 1910, anaerobic digestion of cellulosic materials was attempted to amplify the fall of nitrogen in Indian agriculture and to effectively dispose the animal wastes (Fowler and Joshie 1921). And in the year of 1935, biogas plants were developed to conserve nitrogen using animal dung. This was considered the ideal route for procuring nitrogen-rich manure before the dawn of nitrogenous fertilizers from fossil fuels. Concurrently, biogas plant for vehicle has seen a remarkable development in China and other developing countries. In 1970s due to the "fuel wood crisis," the community welcomed biogas plant. Later, a shift from fuel wood to biogas was witnessed in rural kitchen. It is certain to note that in terms of both environmental and economic virtues, AD is superior to composting, incineration, or the combination of digestion and composting due to its alleviated energy balance and reduced emission of volatile compounds such as ketones, aldehydes, ammonia, and methane. Therefore, AD provides both fuel and fertilizer in contrary to composting and direct burning (Deublein and Steinhauser 2008).

15.5 Steps Involved in Anaerobic Digestion

The AD process of organic biodegradable resources consists of four key stages: fermentation (hydrolysis), acidogenesis, acetogenesis/dehydrogenation, and methanogenesis (methanation). AD is a complex process involving several groups of bacteria and substrates and proceeds under strict anaerobic conditions (ORP under 200 mV) in order to transform organic material into mostly methane (CH_4) and carbon dioxide (CO_2). Each degradation step is carried out by different types of microorganisms, which partly act in syntrophic interrelation that requires different environmental conditions (Aquino and Stuckey 2008). During the hydrolysis step, the complex insoluble organic material such as lipids, carbohydrates, proteins, and nucleic acid is hydrolyzed into simple soluble compounds, amino acids, and fatty acids. Hydrolytic bacteria excrete enzymes like cellulase, cellobiase, xylanase, and lipase, most of them being strict anaerobes such *Clostridia* and *Bactericides* and some facultative anaerobes bacteria (e.g., *Streptococci*). However, the hydrolytic bacteria perform acidogenesis step by degrading the hydrolyzed products. Variety of products formed by acidogenesis comprises mostly of VFAs (volatile fatty acids),

alcohols, hydrogen, and carbon dioxide and their ratios can differ significantly related to process conditions. VFAs are also produced by acidogens (fermentative bacteria) along with ammonia (NH_3), carbon dioxide (CO_2), hydrogen sulfide (H_2S), and other by-products (Bharathiraja et al. 2016). Acidogenesis is followed by acetogenesis where organic acids and alcohols are converted by acetogens into acetate as well as carbon dioxide and hydrogen. The most common acetogenic bacteria are *Acetobacterium woodii* and *Clostridium aceticum*. The main controller of this step is the partial hydrogen pressure as the increasing of hydrogen amount can inhibit the metabolism of acetogens. It is compulsory to maintain a low partial pressure of hydrogen as hydrogen partial pressure influences the daily biogas production (Bharathiraja et al. 2016). The last step of anaerobic digestion includes the activity of methanogens producing methane. There are two groups of methanogenic bacteria: the acetotrophic bacteria degrade acetate into methane and carbon dioxide and the hydrogenotrophic methanogens consume hydrogen to produce methane. Although all methanogenic bacteria can consume hydrogen to produce methane, only few species can convert acetate to methane. The most typical acetotrophic bacteria include the *Methanosarcina barkeri*, *Methanococcus mazei*, and *Methanothrix soehngenii* (Bharathiraja et al. 2016). Many microbial details of metabolic networks in the bacteria consortium are not clear; however, recent studies refer that all the groups are interrelated. The anaerobic digestion is a sensitive process and requires the rates of degradation must be quite equal. For instance, if the first stage runs too fast, then acid augmentation occurs that results in lower pH and so misoperation of the reactor. The complex compounds like cellulose, proteins, or fats are hydrolyzed slowly into monomers within several days, whereas soluble carbohydrates are degraded within few hours. Therefore, to achieve complete degradation and avoid process failure the substrates properties have to be taken into account during the process design (Gavala et al. 2003).

Bioconversion of lignocellulosic waste provides the opportunity to create a biorefinery, producing fuel (biomethane), and high-value-added fertilizer (digestate). Processing of lignocellulosic waste to biomethane consists of three major unit operations: pretreatment, anaerobic digestion, and cleaning/conditioning. AD is a well-established process for renewable fuel production enhancing the energy economy and in parallel providing sustainable waste management. Codigestion with a carbohydrate-rich source and nitrogen-free/fewer raw materials can triumph the disadvantage of animal manure by significantly enhancing biogas production (Surendra et al. 2014). Biomass which contains carbohydrates, proteins, fats cellulose, and hemicellulose can be used as feedstocks for biogas production.

Nowadays, it is typical the addition of cosubstrates to increase the organics content and thus achieving higher gas yield. Typical cosubstrates are organic wastes from agriculture-related industries, food waste, and/or collected municipal biowaste from households. The composition and the yield of biogas depend on the feedstock and cosubstrate type. Codigestion of animal manures with food processing wastes in community digestion facilities is practiced in a number of European locations and could also be applicable in some dairy areas in California since it is the largest dairy

state in the USA with approximately 1.7 million cows generating 3.6 million tons of dry manure approximately (Krich et al. 2005)

Poultry dropping contains easily degradable organic materials than other agricultural waste products, but it is also known to have a high content of 40–50% of the TS of lignin and lignocelluloses which are one among the class of biofibers (Triolo et al. 2011). Another alternative for improving biogas yield is codigestion of poultry manure with other organic wastes which possess increased loading of readily biodegradable organic substances (e.g., such as whey, rice and wheat straws, municipal solid wastes, hog manure, buffalo manure, dairy manure and sewage sludge) which contains balance of nutrients and C/N ratio (Borowski and Weatherley 2013). Cattle dung is a widely used substrate which is mixed with hot water in the ratio of 1:1 and fed into the tank to give biogas and manure. This does not require any treatment before it is charged into the digester (Popli 2012). For avoidance of process failure, pretreatment of feedstocks is needed. The application of pretreatment methods enhances the degradation of substrates and therefore the process efficiency. Chemical, thermal, mechanical, or enzymatic processes can be applied in order to fast the decomposition process; it does not necessarily mean higher biogas yield (Mshandete et al. 2006) (Fig. 15.1).

15.6 Design of Reactors for Biogas Production

The pricing, building materials, and design complexity of digesters vary globally. To build any anaerobic digester, we must first address the three main requirements: producing a large volume of high-quality biogas, being able to manage a high organic loading rate continuously, and having a short hydraulic retention period to reduce reactor volume. Multistage systems, batch, continuous one-stage systems, and continuous two-stage systems are the most common types of digesters used in the industry (Abanades et al. 2021).

Plug-flow systems, anaerobic sequencing batch reactors (ASBR), tubular reactors, anaerobic baffled digesters (ABR), upflow anaerobic sludge blanket (UASB) reactors, and anaerobic filters are among the other types of reactors available. Typically, the main feedstock qualities, specifically total solid, are used to determine which digester type is appropriate. Continuous stirred tank reactors (CSTRs) are used to treat feedstocks with a large content of total solids and slurry, while soluble organic wastes are mostly digested in upflow anaerobic sludge blanket (UASB) reactors, anaerobic filters, and fluidized bed reactors. Codigestion is the most commonly used technique in single-step wet procedures (e.g., CSTR) (Tian and Trzcinski 2017).

Continuous systems are fed constantly, and the digestate residue is discharged at the same rate, resulting in a steady-state and a constant gas production rate. They are limited to substrates that can be pumped for continuous feeding. Alternatively, a semicontinuous method is used multiple times a day with a specific amount of feed. Continuous systems have several advantages, including ease of design, operation,

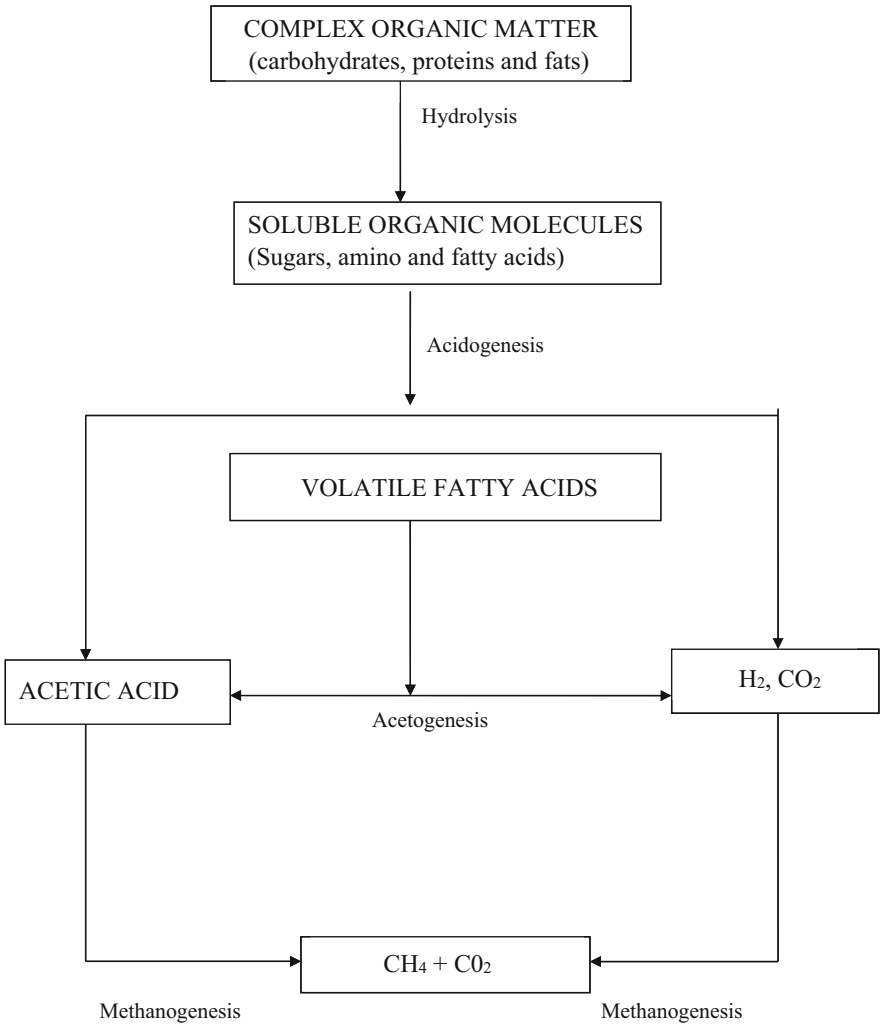

Fig. 15.1 Stages of biogas production

and low capital costs, but they also have drawbacks, such as quick acidification and high volatile fatty acid (VFA) production (Piechota 2021).

15.7 Factors Affecting AD Process

There are several operating factors affecting the production of biogas in the AD process. These mainly include temperature, hydraulic retention time (HRT), organic loading rate (OLR), and pH. Other factors affecting the gas production also include

tank volume, feedstock type, feeding pattern, and carbon to nitrogen (C/N) ratio. Temperature is a critical parameter for the AD process for survival of microbial consortia and to consistently produce biogas; as for each 6 °C decrease (20 °F), the biogas production falls by 50%. Two temperature ranges are most suitable for biogas production—thermophilic and mesophilic. Thermophilic bacteria operate at high-temperature conditions (48.9–60.0 °C or 120–140 °F), thus reducing the retention time to decompose more substrate and produce more biogas. However, these systems are costly to operate as energy is required to maintain higher operating temperature, and they are prone to acidification and are easily influenced by toxins (Mao et al. 2015). Alternatively, mesophilic bacteria functioning at lower temperatures (32.2–43.3 °C, or 90–110 °F) produce less biogas as compared to thermophilic but are easy to operate, low in investment costs, and more stable toward environmental changes. However, they have poor biodegradability and are susceptible to nutrient imbalance (Mtui 2007). In addition to temperature, HRT also affects biogas production. HRT is the average time (usually, a few days to 40 days, depending on the type of organic waste and digester) feedstocks reside in the digester to decompose based on chemical oxygen demand (COD)/biological oxygen demand. Longer retention times provide enough time for organic matter to degrade depending on the microbial consortia present in the digester at different rates and times. Shorter retention times would inhibit methanogenesis, while longer retention times lead to insufficient utilization of components (Singh and Sooch 2013). Similarly, the amount of volatile solids (VS) fed to the digester every day (OLR) is also an important parameter affecting biogas yield. Biogas production increases with higher OLR; however, it disrupts the bacterial population, leading to higher hydrolytic bacteria and acidogens. This would lead to lower methanogen population required for biogas production. The literature contains maximum OLRs for various organic feedstocks to avoid irreversible acidification and high biogas yields (9.2 kg VS/m^3/day for sludge, 10.5 kg VS/m^3/day for food waste), with an optirange between 1.5 and 6 kg VS/m^3/day for all waste (Chiumenti et al. 2009).

pH is another important factor affecting the bacterial activity and, thus, biogas production. Methanogens are highly sensitive to acidic environment (pH < 7), while acidogens are inhibited leading to a rapid increase in methanogens at higher pH levels. The optimal pH for acidogenesis is between pH 5.5 and 6.5 (Kim et al. 2003), while methanogenesis is most efficient between pH 6.5 and 8.2 (Lee et al. 2009). Thus, it is important to maintain pH between 6.5 and 7.5 to sustain an optimal concentration of acidogens and methanogens in the digester for higher biogas yields. Other factors affecting the AD process include type of feedstock for predicting the composition and rate of reaction, tank volume for determining the retention time, and C/N ratio, replicating the amount of nutrient levels in the digester required for AD steps affecting the biogas yield (Mao et al. 2015).

15.8 Cost Analysis

Cost analysis for a community biogas plant of 300 M^3/day capacity with biogas enrichment and bottling plant has been shown below.

15.9 Dung Requirement

For 300 q.m. biogas plant	6000 kg
Assuming cost of dung	Rs. 0.20/kg
Cost of dung for biogas plant	Rs. 1200/day

15.10 Capital Cost

Biogas plant (digester)	Rs. 3000/m^3 Rs. 900,000 for 300 m^3
Cost of scrubbing unit	Rs. 450,000.00
Cost of compressor	Rs. 750,000.00
C.N.G compressor	Rs. 100,000.00
Storage tank	Rs. 200,000.00
Installation exp.	Rs. 1,700,000.00

15.11 Operation Cost

15.11.1 Fixed Operational Cost

Interest on capital 10% per annum	0.1 × 1,400,000 = Rs. 140,000/annum
Depreciation 5% per annum	0.005 × 4,250,000 = Rs.70,000/annum
Total cost	Rs. 210,000/annum

15.11.2 Annual Running (Operational) Cost

Dung cost	Rs. 1200 × 350 = Rs. 420,000/annum
labor cost	Rs. 105,000/annum

(continued)

Electricity cost (10 kWh ×12 h)	Rs. 126,000/annum
Repair and maintenance cost	Rs. 28,000/annum
Total cost	Rs. 679,000/annum

Total cost of operation = sum of fixed operation cost + annual running cost = Rs. 210,000 + Rs. 679,000 = Rs. 889,000.00

15.12 Income

Total production of biogas per day is 300 × 80% (capacity utilized) = 240 m^3

In biogas, there is 60% pure methane gas, so we can say 240 × 60% = 144 m^3 methane

In terms of CNG cylinder, we get total 144 m^3 × 76% = 109 kg

Each cylinder has 12 kg = 9 cylinder

Cost of CBG Rs. 25/kg = 109 × 25 = Rs. 2725

Therefore, income generated by selling CBG in year = 9.54 lakhs

Total production of digested slurry 33% of daily feed = 2000 kg

Yearly production of digested slurry = 700 tons

Cost of slurry = Rs. 2000

Therefore, income generated by selling slurry in a year = 7.0 lakhs

Total income from biogas plant and slurry = Rs. 1,654,000.00 (yearly)

15.13 Conclusion

This paper summarizes the technical and economic perspectives of biogas production from wet wastes using detailed analysis of biogas energy yields to show the potential for waste utilization to satisfy growing energy demand**.** At this occasion, the current rate of biogas production specifies that these technologies are going to have major impact on the energy consumption in future. The impact includes reduced release of contaminants to the environment ensuring the battle against global warming. Uncomplicated handling of waste that are generated through agricultural activities will make economic feasibility and spent slurry from biogas production can be used as fertilizer for agricultural crops. In this review paper, the modern research associated with biogas production has been presented. Even though many researchers were contributed to biogas production, there are some gaps that need to be investigated. In future investigations, analyses of synchronized process of codigestion with substrates are needed to be executed and also researchers need to concentrate on multistage anaerobic codigestion with reduced cost through the selection of appropriate expertise. Purification of biogas is the major task behind the fixation of cost level, which should be analyzed in detail with methods like cryogenic upgradation and in situ methane enrichment. Further, the developing

methods for upgrading and refinement of the produced biogas will receive major attention due to rapid increment in the price of fossil fuels.

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Chapter 16
Mass Production and Marketing of Compost Caterpillar Fungus *Cordyceps sinensis*

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Abstract During COVID, global wide peoples realized the importance of natural food sources and their pharmacological values and now it has been used widely. It retained the previous decades of healthy human livelihood and attention created on

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conventional foods and medicines. From human evolution, mushrooms played a most unique role in food and medicine. Among them, *C. sinensis* is a temperate loving entomopathogenic fungus at altitudes of 3000 to 14,000 MSL. It is used as energy-sourced food and medicinal uses, via. Antioxidant, antihyperlipidemic, antiviral, antiaging, antidepressant, antifatigue, hypocholesterolemic, hypotensive, vasorelaxation, aphrodisiac, chronic urinal regulators, neuroregulators, anticancer, glycemic regulators, antimicrobial, immune modulators, and functional modifiers (FMs) to better live from their production of bioactive compounds. So, this fungal source drastically reduced overexploitation, climate change, mutations, and reduction of host population rate (ghost moth). These situations are pushed into artificial cultivation by complete and seminatural systems with extended times. Thus, simplification of cultivation progress will yield a better outcome in *C. sinensis* productions and new generation medicines in future when accompanied with scientific approaches with knowledgeable skilled persons in mushroom technology for human livelihood.

Keywords Aphrodisiac · Antifatigue · *C. sinensis* · Cordycepin · Hypocholesterolemic

16.1 Introduction

Consistently, studies about "medicinal mushrooms" have always become a very interesting and important one because of their potential uses in pharmacology and its impact in global markets (Schmit and Mueller 2007). Since a 1000 years ago, fungi have been used as food and medicine (folk) by humans. In particular, 2000 mushroom species are identified as edible among 14,000 recognized mushroom species (Vikineswary et al. 2013). Due to their presence of highly nutritive values, composition of mineral states, zero fat derivatives and productions of health pack medicinal properties for improving human beings' life from diseases (El-Ramady et al. 2022). Nowadays, fungal sources have been obtained from the two sources: natural (wild) or artificial culturing under in vitro (Chiu et al. 2016). Gathering of fungi from the wild resources causes serious difficulties due to identification, availability, nature, and environmental sustainability (Wei et al. 2021). Despite, researchers and pharma industrial companies mostly contributed in several mushroom species such as

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Agaricus, Antrodia, Albatrellus, Calvatia, Clitocybe, Cordyceps, Flammulina, *Fomes*, *Fungia*, *Ganoderma*, *Inocybe*, *Inonotus*, *Lactarius*, *Phellinus*, *Pleurotus*, *Russula*, *Schizophyllum*, *Suillus*, *Trametes, Tremella,* and *Xerocomus* (Patel and Goyal 2012). Above these mushroom species were given high attention due to their diversified production of novel anticancer, neuroregulatory, immunomodulators, anti-inflammatory, digestive, derma-care properties (pharmacological), health-improved properties (nutritional substances) derived ones (Hwang 2007). These properties are derived at very least quantities with highly input of cost and time oriented for production, standardization, and commercialization (Granato et al. 2010). Additionally, these mushroom (fungi)-yielded medicinal sources are highly valuable and biopotential with zero harm to humans than bacterial, algal, and plant species (Valverde et al. 2015). Globally, seven medicinal mushroom species play a vital role in pharmacological industries such as *Hericium erinaceus* (lion's mane mushroom), *Ganoderma lingzhi* (reishi mushroom), *Cordyceps* spp. (caterpillar mushroom), *Inonotus obliquus* (chaga mushroom), *Trametes versicolor* (turkey tail mushroom), *Lentinula edodes* (shiitake mushroom), and *Grifola frondosa* (maitake mushroom) which were most contributed than other species (Litwin et al. 2020; Lovett and Leger 2017). Among them, *Cordyceps* spp. were attributed a crucial role in drug discoveries in pre- and post-clinical studies of human's pharmacological activities like antioxidant (Shin et al. 2001), antihyperlipidemic (Wang et al. 2015a), antiviral (Yan et al. 2010), antiaging (Ji et al. 2009), antidepressant (Singh et al. 2014), antifatigue (Geng et al. 2017), hypocholesterolemic (Koh et al. 2003), hypotensive (Chiou et al. 2000), vasorelaxation (Wang et al. 2015b), aphrodisiac (Kashyap et al. 2016), chronic urinal regulators (Sun et al. 2019), neuroregulators (Wang et al. 2018), antioncology (Park et al. 2017), glycemic regulators (Yu et al. 2016), antimicrobial (Mamta et al. 2015), and immune modulators (Wang et al. 2011) ("*FMs*—functional modifiers") to better health (Fig. 16.1). Above these functional activities were rectified by several bioactive compounds (or) structural compounds, viz. nucleosides (Shaoping et al. 2001), sterols (Zhou et al. 2009), polysaccharides (Elkhateeb et al. 2019), proteins, and related nucleic acids (Sethy et al. 2016), fatty and organic acids (Das et al. 2021), metals (Bhetwal et al. 2021), and vitamins (Holliday and Cleaver 2008). Under this circumstance, identification, characterization, mass production isolation of biometabolites, standardization, and commercialization of *Cordyceps sinensis* or their species was highly oriented with scientific approaches and skillfully one in pharmacological science and industries (Abdul-Rehman et al. 2022). So, given this chapter thoroughly covered about origin, economic importance, nature, distribution, habitat, morphology, and lifecycle, ethnopharmacy (*eP*), structural bioactive compounds (SBCs), pharmacological uses, genealogical approaches, mass production, extraction and purification progress views, toxicology, commercialization, issues, and future perspectives of *Cordyceps sinensis* were discussed below.

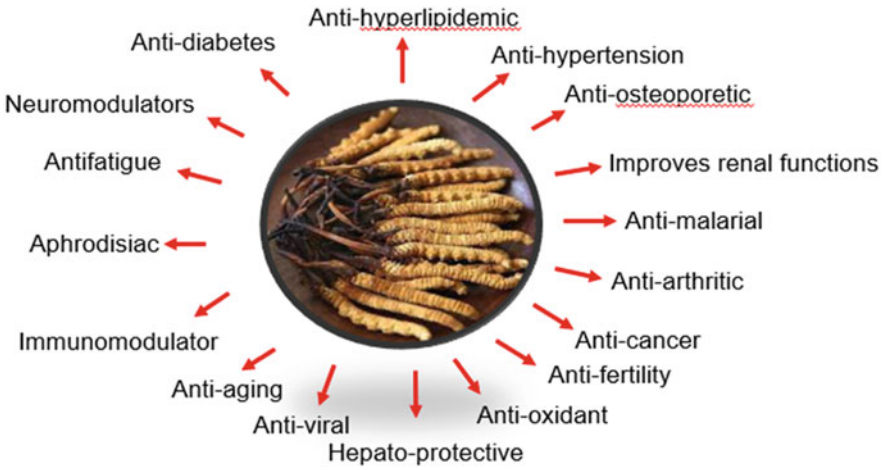

Fig. 16.1 Multiclinical beneficial approaches of *Cordyceps* spp. for human health. (Courtesy: Liu et al. (2015))

16.1.1 Origin

From 145.0 million years ago (Cretaceous period), this fungal species lived in wild hosted with entomopathogenic nature (Gibson et al. 2014). On time, they secreted a wide spectrum of secondary biometabolites during their infection and proliferation in insects (Asaia et al. 2012; Chakraborty et al. 2014a). In 1843, scientifically named as Sphaeria sinensis by Miles Berkeley, later it has renamed as Cordyceps spp. in 1878 by Andrea Saccardo (Nikoh 2000). Until 2007, it was called *Cordyceps sinensis*; now, it is also renamed *Ophiocordyceps sinensis* and otherwise commonly called "caterpillar fungus"/"yarsha gamboo"/"keera jhar"/"keera ghas" (Arora et al. 2008). It belongs to one of the 700 species on Ascomycota/Pyrenomycetes/Hypocreales/Clavicipitaceae. The word *Cordyceps* originated from the Greek word "Kordyle" (club) and Latin word of "ceps" (head) (Olatunji et al. 2018). Finally, the word *Cordyceps* denotes the fruiting body of the fungus coming out to form a mummified body of caterpillar structured one (Wu et al. 2015).

16.1.2 Economic Importance

Naturally, *Cordyceps* spp. are highly ecosystem-oriented growing mushroom species compared to other medicinal mushrooms. Therefore, collection of large-scale levels of this mushroom is a daunting task (Hardeep et al. 2014). However, an age group of 15–65-year-old men and women is contributed to collect this mushroom for domestic-level income in countries like Nepal, China, Bhutan, Tibet Himalayan regions, and northeastern states of India. The wild-collected mushroom (*Cordyceps*

sinensis) is sold at Indian market at Rs. 1,00,000/kg (Sharma 2004). Depending upon the demand, availability, and supply, the price level is also increased in the global market. Worldwide, *Cordyceps*-based pharmaceutical industries estimated global market value is 5–11 billion US$ (Shrestha 2012). In China, this is mushroom sold under the name "soft gold" at 35,000–60,000 $/kg. In Korea, Japan, and Thailand, several species of *Cordyceps* are used as functional foods with a price of $ 5.8/gram (Winkler 2008). Most of the North-Eastern states of Indian tribal (>3,00,000 families) livelihood and their income source depending by the collection of *C. sinensis* (Panda and Chandra Swain 2011).

16.1.3 Nature, Distribution, and Habitat

Cordyceps sinensis is a rare ubiquitous living entomopathogenic fungi, around 700 species of *Cordyceps*; 20 species were documented as parasitize to "Elaphomyces" genus (temperate, subarctic forest ecosystem living ones) (Baral et al. 2015). Interestingly, remaining some species such as *C. sinensis, C. ophioglossoides, C. militaries, C. gracilis, C. sobolifera, C. subsessilis, C. gunnii, C. cicadae, C. tuberculate, C. scarabaeicola, C. minuta, C. myrmecophila, C. canadensis, C. nutans, C. agriota, C. ishikariensis, C. konnoana C. nigrella, C. pruinose,* and *C. tricentric* were highly predominant pathogenic to insect orders of Arachnida, Coleoptera, Hemiptera, Hymenoptera, Isoptera, and Lepidoptera (Pal and Misra 2018). Among them, *C. sinensis* parasitizes the larval of "swift or ghost moths" (Hepialidae); this is specially found on the deep forests and meadows of Himalayan regions in India, Tibet, Nepal, and China at mean sea level of 3000–5000 m (Grehan and Ismavel 2017) and also is distributed in Bhutan, Japan, Korea, Thailand, Vietnam (Asia), Europe, and American countries (Fig. 16.2) (Elkhateeb and Daba 2022). In India, it is presented in highest altitude subalpine regions like Kumaun and Garhwal Himalaya (Pradhan et al. 2020). The constituents of biometabolites (quality and quantity) in *Cordyceps* spp. were changed due to the presence of geographical and climatic factors such as level of altitudes, low temperature, high precipitation, relative humidity, lack of O_2, and exposure of sunlight (Arora and Singh 2009).

16.1.3.1 Morphology and Life Cycle

During early spring, *C. sinensis* mycelium originates from an insect larva (*Hepialus armoricanus*) and pervaded on the whole body itself and ends to club-like cap consisted of two parts: a fungal endosclerotium (within caterpillar body) and stroma (Lo et al. 2013). The upper part (stroma) is dark brown or black, when the yellow color is fresh and longer (4–10 cm) than caterpillars (Fig. 16.3). It grows from the larval head and is clavate, sublanceolate (or) fusiform, unique from the stipe (slender, glabrous, and longitudinally ridged) (Toledo et al. 2013). Naturally,

Area *viz.*,

1. **Asia**
2. **Europe**
3. **USA & Canada**
4. **Australia**

Fig. 16.2 Global distribution of *Cordyceps sinensis* a diagrammatic view. Area, viz. (1) Asia; (2) Europe; (3) USA and Canada; (4) Australia

Fig. 16.3 Morphology of *Cordyceps sinensis*

C. sinensis life cycle comprises three phases, viz., infection, parasitism, and saprophytism (Guo et al. 2017). When the fungus grows on the immature larvae (host), usually it lies around 6 inches below the ground surface. As the fungus proceeds into maturity, they are heads up from underground to above soil. It consumes >90% of the infected insect larvae. The production of fungal sclerotia leads the host larvae from rigid and rapidly mummifying the hosts. Later, the sclerotial structure becomes dormant, after overwintering the fungus ruptures the

host body, a sexual fruiting body arises (perithecial stroma) from the larval head that rises upward to emerge from the earth and is joined to the sclerotia (dead larva) underground to complete the cycle and finally reaches the weight of *Cordyceps* about 300–500 mg (Zeng et al. 2006).

*16.1.4 Ethnopharmacy (*eP*)*

From human evolution, impacts of several diseases and disorders were cured by natural sources such as plants, animals, and algal and fungal derivatives. So, intake of mushrooms as pharmacological use has followed from the genesis of before civilic (Stamets and Zwickey 2014). *Cordyceps sinensis* has been utilized in traditional Chinese medicine (TCM) as tonic, syrup, powder, paste, pellets, and capsules mixed with milk (or) liquor (or) hot water in morning or evening for curing diseases like bronchitis, cough, cold, diarrhea, fatigue, headache, rheumatism, and enhancer of sexual potency of male and females. Mostly, these recommendations were based on the utilization of the "trial and error" method by local folk practitioners (Choda 2017). Additionally, it is also used as a highly nutritive food source due to the presence of essential amino acids and vitamins (B1, B2, B12, and K) (Zhu et al. 1998), slow-releasing glycemic (carbohydrates), dietary fibers, and minerals (Yalin et al. 2006). Additionally, several species like C. militaris (anticancer) (Shrestha and Sung 2005), C. pruinose (stomach diseases) (Ng and Wang 2005), C. bassiana (derma care & biopesticide) (Sung et al. 2006), C. cicadae (infantile convulsion) (Choda 2017), C. guangdongensis (inflammatory disorders, avian flu, regulation of menstrual cycle & antiaging) (Wang et al. 2021). Till now, C. sinensis is sold in capsule form for curing aphrodisiac by a commercially available name "Himalayan Viagra" (or) "Himalayan Gold" (Baral 2017).

16.1.5 Structural Bioactive Compounds (SBCs)

Due to the availability, pharmacological value, and demand, *C. sinensis* is a highly variable nature and it requires unique set of climatic conditions for their growth, development, and maturity including collection also (Shrestha 2011). The structural bioactive compounds were mostly obtained from the well-developed mycelium through extraction by using different solvents. The obtaining biochemical compounds differed from natural and cultured ones due to the exposure of ecosystem (Au et al. 2011). Until now, these biochemical compounds have been documented as a diversifiable natured one including nucleosides and nucleobases, nucleotides, polysaccharides, sterols and fatty acids, proteins, amino acids and polypeptides, cordymin, cordycedipeptide, cordyceamides A and B, and tryptophan (Fig. 16.4) (Zhang et al. 1991). Above these compounds, quality and quantity check were determined the varietal superiority among the cultured species (Chen et al. 2011).

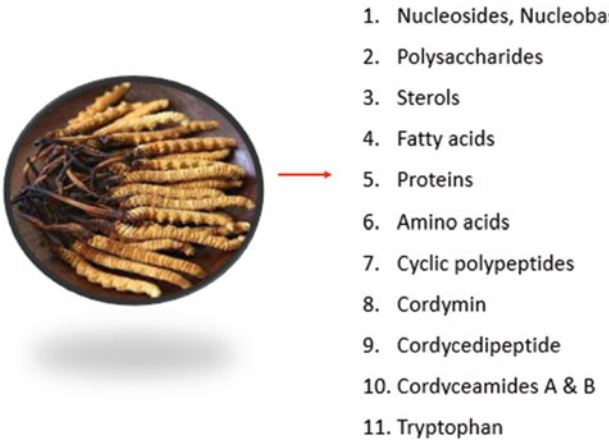

Fig. 16.4 Structural bioactive compounds of *Cordyceps sinensis*

16.1.6 Pharmacological Uses

Naturally, the obtained bioactive compounds are reported as diversified with unique characteristics of pharmacological nature. So, presenting bioactive compounds are different within species due to growing nature (Mizuno 1995). The bioactive compounds (structural) viz., nucleosides and nucleobases, nucleotides; polysaccharides, sterols and fatty acids, proteins, amino acids and cyclic polypeptides (large quantities compared to other molecules) are mostly predominant better against human pathological sciences (Table 16.1) (Chen et al. 2013).

16.1.6.1 Nucleosides and Nucleobases

It is one of the most important base compounds of *Cordyceps* spp., nearly more than 11 different nucleosides are extracted from mycelium of *C. sinensis* such as adenine, cytosine, guanine, hypoxanthine, thymine and uracil (6-nucleobases), adenosine, cytidine, guanosine, inosine, and thymidine (Yang et al. 2010); among them, guanosine has highest contribution ratio (quantity) to others, and these molecules are always present in nature of reciprocal linkages and mostly contributed through (purinergic/pyrimidine receptors and glial cells) regulators of CNS (central nervous system) in human physiology (Liu et al. 2015). Nucleobases of purine and pyrimidine play as a remarkable marker for nutrition values of *Cordyceps* spp. (Yang et al. 2009).

Table 16.1 Bioactive compounds and their pharmacological uses of *Cordyceps* spp

S. no.	Bioactive compounds	Pharmacological uses	References
1.	Adenosines and *N*-acetylgalactosamine	Anticonvulsant activity, antitumour, antidepressant, neurotransmitters, immunomodulators	Tescarollo et al. (2020) and Bockaert et al. (2010)
2.	Cordycepin	Immune enhancer, antimicrobial, antitumour, vasoregulation, antihypersensitive, antioxidant, hypoglycaemic	Phull et al. (2022)
3.	Polysaccharides	Antidiabetic, antiaging, internal organ protectant	Li et al. (2002) and Zheng et al. (2022)
4.	Cordycepic acid	Diuretic cures, liver fibrosis, WBC regulation in blood	Chatterjee et al. (1957) and Guo and Friedman (2007)
5.	Sterols and fatty acids	Hypercholesteraemic, aphrodisiac, vasorelaxation	Zhou et al. (2009)
6.	Proteins	Cardiovascular regulation, blood serum synthesis	Chiou et al. (2000) and Ye et al. (2004)
7.	Amino acids and cyclic polypeptides	Sedative hypnotic effect, blood glycemic regulation	Zhang et al. (1991), Ye et al. (2004), Vestergaard et al. (2009) and Qian et al. (2012)

Adenosines

Except abovementioned several nucleosides are found in specific deoxyuridine structures like adenosine, 2′-deoxyadenosine 2′ 3′-dideoxyadenosine, hydroxyethyl-adenosine, 3′-deoxyadenosine (cordycepin), pentostatin, N6-(2-hydroxy ethyl)-adenosine, tenellin, militarinones, fumosorinone, farinosones, oosporein, beauveriolides, beauvericin, cordycepin triphosphate, guanidine, and deoxyguanidine. During extraction, adenosine compounds most affected the time (extension) in natural *C. sinensis* (Shiao et al. 1994). Especially, adenosine act as an energy transfer and signal transductant molecules in intracells of cardiovascular tissues, anti-inflammatory properties, and antitumour (ROS-mediated mitochondrial membrane dysfunction) inducing and anticonvulsant activities through neuromodulators in human beings (Tescarollo et al. 2020). Specifically, these functions were mediated through activation of "GPCRs" (G-protein-coupled receptors), namely A_1, A_{2A}, A_{2B}, and A_3. By impulse of neurons and signal transduction to brain accompanied with nervous system and regulates psychological functions, viz. self-fear, immune modulation, anxiety, locomotion, and depression (Bockaert et al. 2010).

Cordycepin

Cordycepin is prominently found in the lowest amount in two species of *Cordyceps* such as *C. sinensis* and *C. militaris* and cannot be found in cultured ones (Tsai et al. 2010). Based on structure, cordycepin is 3′-deoxyadenosine and cordycepic acid (CA) is D-mannitol. The 3′-deoxyadenosine is derived from the absence of one O_2 molecule at third position carbon of ribose sugar with molecular weight of 251.24 Da. (228–231 °C—melting point) commonly extracted by the way of using acetonitrile and H_2O mixed ratio (5:95 v/v) at a flow rate of 1.0 mL/min (Hyun 2008). Under clinical diagnosis, cordycepin widely used as analgesic properties, immune booster, antitumour, antiglycemic, antiviral, antibacterial, anti-inflammatory, and neurostimulation stimulates steroidogenesis and pesticides (Phull et al. 2022).

Nucleotides

Prominently, three nucleotides are constituted commonly as adenosine-5′--monophosphate (AMP), guanosine-5′-monophosphate (GMP), and uridine 5′--monophosphate (UMP) with amphoteric molecules, base, and phosphoric acid (Lindequist et al. 2005). Reporting above these nucleotides were documented as enhancing the immune system of body, influence of fat metabolism, improve the gastrointestinal functions, inhibit urethral inflammation, regulates blood circulation and brain activeness (Lin and Li 2011).

16.1.6.2 Polysaccharides

Cordyceps spp. contains a diverse nature of polysaccharides; usually, fruiting bodies of *Cordyceps* consist of 3–8% of polysaccharides consisting of extracellular and intracellular at molecular weight of 16 kDa (Li et al. 2002). It is a compound nature of sugars (monosaccharides and polysaccharides) such as arabinose, fructose, glucose, galactose, mannose, mannitol, rhamnose, ribose, sorbose, and xylose. Monosaccharides mainly contributed to the organism's growth and maturity; remaining polysaccharides (APS, CPS-1 and CPS-2) others (cordycepic acid) exhibited toward pharmacological activities for human health (Guan et al. 2010). The acid polysaccharide (APS) at ratio of (3.3:2.3:1)—(mannose:glucose:galactose) triggering protective from internal cell injuries (Zheng et al. 2022); a water-soluble polysaccharide (CPS-1) was a glucomano-galactan sugars of glucose:mannose:galactose at 2.8:2.9:1 ratio in *C. sinensis*. This CPS-1 especially stimulates insulin secretion and maintains the level (insulin metabolism) in the pancreatic gland and cures diabetes (Li et al. 2006). Finally, CPS-2 (combo residues of mannose, glucose, and galactose with ratio 4:11:1, mostly contributed to internal wounding of blood vessels from regulation of PDGF-BB (platelet-derived growth factor—dimeric glycoprotein) (Chen et al. 2010).

Cordycepic Acid

In early days, CA (cordycepic acid) possessed an isomer of quinic acid, but later it is confirmed as D-mannitol by the variation presented in forming dextrorotatory lactones and usually 7–29% in *Cordyceps* spp. (Chatterjee et al. 1957). Cordycepic acid plays a viable role in treating diuretic, liver fibrosis, plasma osmoregulation, and synthesis of free radicals for better blood circulation and free of breathiness (Guo and Friedman 2007).

16.1.6.3 Sterols and Fatty Acids

Normally, sterols at low quantity level in all edible mushrooms. In *Cordyceps sinensis*, sterols have been identified in the form of ergosterol, ergosterol-3, ergosterol peroxide 3-sitosterol, daucosterol, and campesterol (Bok et al. 1999). In fungus, ergosterol contains a different molecular weight as 1.4 mg/g (vegetative stage) and 10.6 mg/g (fruiting stage) (Li et al. 2004). Additionally, two types of fatty acids (saturated and unsaturated) were found in *Cordyceps* spp. Usually, different fatty acids like docosanoic acid, linoleic acid, palmitic acid, pentadecanoic acid, oleic acid, and stearic acid were documented in *C. sinensis*. These act as osmoregulation and lipid metabolism in blood (Jerzy Jedrejko et al. 2021).

16.1.6.4 Proteins

Generally, proteins rate varies in different stages like dead larvae (29.1%), fruiting body (30.4%), and mycelial decomposition (14.8%). Mostly, proteins like spermidine, putrescine, flazin, cadaverine, perloyrine, and L-tryptophan were presented in the form with various nature and additionally CSDNase (deoxyribonuclease) and CSP (serine protease) at intracellular and extracellular (Yang et al. 2011). DNase mainly contributed in growth of mycelium by endogenous nature. CSP is an extracellular protease enzyme of single polypeptide chain with molecular weight of 31 kDa. It maintains blood serum albumin level and therapeutic effects on cardiovascular functions (Ye et al. 2004).

16.1.6.5 Amino Acids and Cyclic Polypeptides

C. sinensis consists of numerous amino acids like glutamic acid and aspartic acid in larval stage and cyclic polypeptides such as cyclo-(Gly-Pro), cyclo-(Leu-Pro), cyclo-(Phe-Pro), cyclo-(Val-Pro), and cyclo-(Thr-Leu) which were presented in all stages (Mishra and Upadhyay 2011). Among them, cyclo-(Leu-Pro) and (Phe-Pro) were confirmed as antimicrobial and antimutagenic properties against vancomycin-resistant *Enterococcus* and pathogenic yeasts. Including these, cyclodipeptides inhibit the synthesis of aflatoxins (Rhee 2004). A cordymin is a putative beneficial

peptide isolated from *C. sinensis* which is highly recommended for diabetic osteopenia and regulates blood serum glucose level (Vestergaard et al. 2009; Qian et al. 2012).

16.1.7 Genealogical Approaches

A fungus *Hirsutella sinensis* is an anamorph (asexual) stage of *C. sinensis*. Till now, molecular diagnosis is critical for this medicinal fungus. The fruiting body development is still unknown (Zhong et al. 2010). From internal transcribed spacer analysis, *Cordyceps* spp. yielded a fragment size of 539 bp (Zhang et al. 2010). Up to now, *Cordyceps* spp. characterized by genealogical approaches through functional protein genes, transcriptional factors such as Zn_2Cys6—type (fruiting body initiation), mitogen-activated protein kinase gene (MAPK gene) (Zheng et al. 2011), FKS 1 gene (induction of β-1,3 glucan synthase enzyme) for immunomodulator synthesis (Ujita et al. 2006), Cu, Zn SOD 1 gene (SOD 1) for stimulating anti-inflammatory properties (C. *militaris*) (Park et al. 2005), Two cuticle degrading serine protease genes, *csp 1* and *csp 2* essential role play in development of infection in host and pathogenesis of *C. sinensis* (Zhang et al. 2008). These kinds of several genes like glucanase, proteinase, and cyclic peptide synthase genes are involved in characterization of *Cordyceps* spp. (Liu et al. 2017).

16.1.8 Mass Production of C. sinensis

16.1.8.1 Technological Approaches

For the last 50 years, the *Cordyceps sinensis* has played a major role in global food and pharmaceutical industries due to their importance of beneficial bioconstituents. So, the demand and distributional availability have increased rapidly. It gives benefit to wild mushroom collectors and their socioeconomic status (Singha et al. 2020). Thus, global wide *C. sinensis* gets a great attention for mass production by artificial conditions. In early periods, it met several issues that were attained to low production with sustainability nature. During 1982, a successful attempt of cultivation of *Cordyceps* is carried out in Chinese academy of medical sciences. After several explorations of mass multiplication studies, two prominent methods were identified. It is complete artificial and semi-natural cultivation; both are different in their progresses viz., complete artificial: reared larvae are inoculated with cultured strains and the infected larvae are fed indoors. Later 1–2 years, *C. sinensis* has been harvested. In this pattern, all processes are conducted under artificial conditions only. It was more efficient than seminatural by survival rate of larvae and shortening growth period (early maturity) of *C. sinensis*, but cost is so much. In a seminatural method, the infected larvae were released to the natural ecosystem, liberally allowing

them to grow in free conditions. After 3–5 years, the fungus is harvested in those areas. It consumes natural resources but reduces the cost of input and takes much time. Both these methodologies are not familiar in *C. sinensis* mass production and not followed by a huge level of commercialization. Additionally, weather factorsinfluence the cultivation and yield from initial to harvest. *C. sinensis* cultivation consisted of three phases, viz. selection of host insects, preparation of culture, and inoculation of culture in to host and harvest (Qin et al. 2018).

16.1.8.2 Selection of Host Insects

In 1965, *Hepialus armoricanus* (ghost moth) was identified as the main host of the *C. sinensis*; till now, 50 species have been identified for host insects in *Hepialus*. The species have a wide distribution with 2500–5100 MSL with subalpine to alpine grasslands (Chu 1956). Natural vegetation and climatic factors decided the host population and occurrence, although nowadays the population diversity occurred due to deforestation and global warming (Outhwaite et al. 2022).

16.1.8.3 Artificial Rearing

Generally, sex ratio of (*Hepialus*) ghost moth female is always higher than male and mating is mostly at 6–9.30 pm. Female moths mated one time with their life span, but male attained 2–3 times. Normally, females lay 5–45 eggs each time and then die immediately. These eggs were collected, and they were used for further progress. *Hepialus* moth had stages of egg, larva, pupa, and adult. Eggs kept at a temperature of >10 °C with warm humid conditions (30–40 days for incubation), when hatching the egg shells turned from white to black in color. The larva (milk white, 2 mm length) mostly stayed in soil at a depth of 15 cm. During maturity, larvae are pale red in colour, beige body and length about 4–5 cm. Tender tuber roots or infantile buds are fed to larvae. In the month of June–July, the pupal stage is present. During rearing, abiotic factors such as temperature (15–20 °C/incubation; 10–18 °C/larval stage), humidity (36–45%/larval stage), and soil moisture maintained at 42–45%) are considered (Buenz et al. 2005).

16.1.8.4 Preparation of Culture

Isolation and Collection of Ascospores

The establishment of anamorph stage of *C. sinensis* (*Hirsutella sinensis*) plays a critical role in cultivation of *C. sinensis*. A viable strain from different climatic (temperate) zones was collected, and then, ascospores, stromata, and endosclerotia were collected which were used for culturing an anamorph stage of the fungus by using specific media, viz. 1% peptone potato dextrose agar, S31 agar, glycerol meal

peptone agar, milk agar, and confirmative by Sabouraud agar (SAB). Ninety percent of fungus confirmed on their conidial stage. The ascospores (fruiting bodies) by aseptic needle into sheathing sterile bags were collected and were kept under 0–4 °C (Liu et al. 2002).

Isolation of Culture and Mass Multiplication

Mostly, mass production of *C. sinensis* mycelia is obtained by the method of submerged cultivation. The collected ascospores are inoculated into liquid media (glucose 1.25%, sucrose 1.25%, peptone 0.02%, 0.06 g of 5% yeast powder, 0.025% KH_2PO_4, 0.012 g of $MgSO_4.7H_2O$, and 0.002 g of 5% vitamin B_1, and are kept in neutral pH with 24 °C for 8 days. Finally, it reached a biomass of 19.5 g/L (Liu et al. 2002). Compositional alteration of media by 20% potato, 0.08% beef extract, 0.2% peptone, 0.1% KH_2PO_4, 0.1% $MgSO_4.7H_2O$, 1.5% sucrose, and 2.5% glucose at 23 °C with 130 rpm/min on 4 days yielded better outcome in mycelial mass weight more than 2 times. After formation of fruiting bodies (ascospores, stroma and perithecium) by keeping under 8–17 °C for 7 months (Yue et al. 2013).

Inoculation of Culture to Host

The matured fruiting bodies, cultures are inoculated into cultured larvae of *H. armoricanus* under aseptic conditions in laboratory, after infected larvae keep under well-drained moist pits with favorite feeds under field conditions with monitoring for recorded mortality and survival rate. Later, it generates the full genesis of *C. sinensis* with fruiting bodies of stroma collected in respective 1–2 years later (Yuan et al. 2022).

Mechanisms of Infection

The fungal spores infected the larva in soil, and the following year, it died and turned to fruiting bodies. When made an infection by *C. sinensis*, it attacks the hemocelom of host larvae and makes small fragments of hyphae. After it proliferates as individual and entirely covers the hemocoel and later turned as cystid form of (exoskeleton body wall with hard) sclerotium and sprouts stroma from buccal cavity, all these established due to synthesis of lytic enzymes and mycotoxins (Shashidhar et al. 2013).

Extraction and Purification

Extraction, purification, and standardization of biometabolites or structural bioactive compounds (nucleosides and polysaccharides) from the fungus are final process of

commercial cultivation of *C. sinensis* and their pharma industries. Commonly, there are several methodologies available for above this process used by polarity, molecular weight-based like water extracts, ethanol, petroleum ether, acetone, methanol, ethyl acetate, and pressurized liquid extractions (PLE) (Xin Chen et al. 2013). Furthermore, several methodologies are involved for the determination of nucleosides (high performance liquid chromatography (HPLC), capillary electrophoresis mass spectroscopy (CEMS), liquid chromatography/electrospray ionization (LC/ESI-MS), ion-pairing reverse phase liquid chromatography (IR-RP-LC-MS) and polysaccharides (PMP-pre column derivation (monosaccharides), proteins (2D PAGEs, GCMS, periodate-oxidation and Smith degradation and nuclear magnetic resonance spectroscopy (NMRS) are followed (Nie et al. 2011; Feng et al. 2017).

16.1.9 Toxicology

Naturally, 1000 species of fungi act as an "insect pathogen" from families like Cordycipitaceae, Ophiocodycipitaceae, and Clavicipitatceae. Above these families consisted species of mushrooms have highly potential nature of pharmacological uses to humans (Chen et al. 2020). On the other hand, frequently taking these mushrooms or their metabolites in the way of food and medicines provide a slow release of toxic substances and are harmful to human physiology. There are numerous bioactive compounds transformed as toxic to later stages when we uptake regularly. These are listed in "Table 16.2" with their impacts including (Hatton et al. 2018).

Table 16.2 Bioactive compounds and their toxic nature to human health

S. No.	Bioactive compounds	Toxic nature	Producing *Cordyceps* spp	References
1.	Cordycepin	Gastrointestinal and bone marrow toxicity	*C. militaris, C. kyusyuensis*	Chen et al. (2020) and Hatton et al. (2018)
2.	Farinosones, oosporein, beauveriolides, beauvericin, cordycemadies, militarinones	Cytotoxicity	*C. militaris, C. bassiana, C. sinensis, C. farinosa, C. cicadae*	
3.	Pentostatin	Gastrointestinal, nausea, diarrhea, renal and neurocells, pulmonary toxicity	*C. militaris*	
4.	N6-(2-hydroxyethyl)-Adenosine	Induction of oxidative stress	*C. militaris, C. cicadae*	
5.	Tenellin	Toxic toward erythrocytes	*C. bassiana*	

16.2 Production Cost Analysis

In each and every investment is mostly for the output/benefit/net return for every all industries in global wide thought and need. Mushroom production acts as secondary-based sector for farmers during crop failure or additional returns (Singh et al. 2001). It applicable in tropical and subtropical natured mushroom cultivation, but *Cordyceps* spp. are highly depended on the climatic favored ones, and it influenced the production enhancement (Rawat et al. 2020). Furthermore, several reports recorded the production cost analysis, and its outcome is beneficial to artificial cultivators of Cordyceps spp. (Table 16.3), (Singh et al. 2010).

16.2.1 Commercialization

Till now, the wild mushroom collectors of *Cordyceps* spp. have increased day by day due to the market value and demand (Chakraborty et al. 2014b). Additionally, several multinational pharma industries took this collection and cultivation progressed due to their pharmacological nature and also available in "online mart" in different names like raw fungus, dry powder, organic capsules, and liquid drinks from the manufacturer like "Himalayan Herbs" (Paterson 2008). Globally, it costs approximately 6.7 US$ and Indian market rate at Rs.100/− per piece and 1000/− for 10 grams (Gupta and Karkala 2017). This wild collection trade is a big setup of a network from local gatherers (uncountable)—broker or agent (local market)—regional broker/agent (district)—wholesaler (state/national—market)—international

Table 16.3 Production cost analysis (PCA) of *Cordyceps* spp. under artificial cultivation

Particulars	Categories of cultivation farms			Average (Rs.)
	Small (Rs.)	Medium (Rs.)	Large (Rs.)	
Land sources(Rs. 2,00,000/cents)	1–2 acres	5–10 acres	>100 acres	1,50,000
Investment for buildings	57,230	1,56,230	2,12,606	1,42,022
Equipment (generator, trays, forks, tubs, sprayers, exhaust fans, coolers, heaters, weighing balances, chemicals, LAC)	62,672	1,66,160	2,36,147	1,55,187
Production	10–20 (kg)/2 times/year	25–50 (kg)/2 times/year	70–100 (kg) /2 times/year	10/25/70 (kg)/year
Net income (kg/Rs. 1,00,000)—Grade I (kg/Rs. 30,000–35,000)—Grade II (kg/Rs. 12,000–20,000)—Grade III	Grade III	Grade II	Grade II and grade III	1,20,000/ 7,50,000/ 70,00,000
Benefit–cost ratio	1:10	4:10	42:100	

Singh et al. (2010)

Fig. 16.5 Systematic diagram of *Cordyceps sinensis* commercialization from local to global

(multinational pharma industries) (Fig. 16.5) (Sharma et al. 2017). Globally, *Cordyceps* spp. products are high priced in nature, so mostly it has been used by celebrities, players, and physicians and not by local peoples (Anita 2019).

16.2.2 Issues in Cordyceps *Spp.*

Cordyceps sinensis is a highly temperate lovable entomopathogenic Ascomycetes fungus. So, it attained several problems (or) issues that are categorized in multiple ways through (collection, cultivation, extraction and standardization, commercialization, and intake) (Raethong et al. 2020).

16.2.2.1 During Collection

From collection to *Cordyceps sinensis* met several issues like, fungus attained mutation, diversity, continuous exploitation of natural sources, global warming and climate change provides a great destruction of wild species gradual to rapidly. So, gatherers met unexpected disappointments during collection by identification and poor availability (Gupta and Karkala 2017).

16.2.2.2 During Cultivation

Under cultivation process, selection of viable (host–insect) and their availability in nature to keep that ecosystem (under artificial) and maintaining the population rate (survival: mortality), parasitic to saprophytic elongation period, are daunting tasks in practically (Gupta and Karkala 2017).

16.2.2.3 During Extraction and Standardization

From the early period to now, the fungus turned as mutant and expected bioactive compounds changed in their quality and quantity. Additionally, these processes were highly cost-effective, and they consumed time also (Gupta and Karkala 2017).

16.2.2.4 During Commercialization

During commercialized conditions, *C. sinensis* price is not a stable one, and it depends upon changing circumstances of availability, demand, and supply. So, it is always in the "pendulum" position (Gupta and Karkala 2017).

16.2.2.5 During Intake

Anything that is excess in human life is always poisonous, and it includes physiological and psychological also. So, nowadays, frequent intake of mushrooms as functional foods and medicines gave negative outcomes in human health of social and physical life (Gupta and Karkala 2017).

16.3 Future Perspectives

Nature gives a better source for human's life when we protect it. During disturbance, it also gives an unwanted way of impact in all stages. So, *C. sinensis* plays a vital role in previous to current decades also. Thus, preserving the natural ecosystem to the safest way avoided the destruction of human essential mushrooms. Additionally, continuous research on *C. sinensis* in the future gives a better way in human life through multidimensional scientific approaches of new species identification, and simplification of cultivation methodologies will provide better outcomes in soon (Nguyen et al. 2020).

16.4 Conclusion

During the Stone Age to now, mushrooms are used as conventional food and medicines in rural villages to global streets. In the last two decades, new diseases have emerged and created great pandemics. In these situations, pharma industries highly focused their views on available wild natural sources and their importance in human life. During that time, *C. sinensis* was attempting a huge familiar view on multibenefits from temperate to tropical. Currently, the species evolved several

mutations due to global climate change, continuous exploitations, commercial collectors from multinational pharma-industries with unofficial thefts has created a great impact in the next step of artificial cultivation. Till now, it does not give better fulfillment in outcomes due to lack of scientific approaches, skilled persons, and unfavored changing ecosystems. Definitely, when we carry out these fields without lacking, we will gain better outcomes in *C. sinensis* by better nutraceutical (nutrition + pharmaceutical) ways to human livelihood with the safest thing.

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Chapter 17
Mass Production Methods, Markets, and Applications of Chitosan and Chitin Oligomer as a Biostimulant

Prasant Kumar and Hitakshi Korat

Abstract Global organic food demand is increasing as consumers understand about the problems of the food grown under conventional farming. In the current situation, farmers are looking for alternatives to boost its productivity with minimal use of chemical fertilizers and pesticides. In this transition period, biostimulant plays a key role to increase nutrient uptake efficacy and boost plant yield. This chapter focuses on a specific biostimulant, i.e., chitosan and chito-oligomers, and their mass production methods and their pros and cons. Various applications of chitosan and chito-oligomers as biostimulant and crop protectant are also discussed. At the end, this chapter focuses on market demand of chitosan and chito-oligomers.

Keywords Biostimulant · Chitosan · Fertilizer · Organic · Yield

17.1 Introduction

Organic farming is in demand of twenty-first century due to consumer's awareness of quality food. Global organic food demand is increasing as consumers understand about the problems of the food grown under conventional farming. In conventional farming, the major agriculture inputs are chemical driven from seed coating to harvesting. Productivity in conventional farming truly depends on chemical fertilizer and pesticides. The chemical fertilizer and pesticides enter directly or indirectly in our food system and environments. In the current scenario, food quality and nutritional fits for consumption are on priority, but population growth is also in alarming conditions.

The current world population of 7.9 billion is expected to reach 8.5 billion by 2030 and 9.7 billion in 2050 as per UN reports. There is a need to increase the food production by 70% by 2050.

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As a consequence of population growth, food consumption is also increasing. On the other hand, cultivable agricultural land is significantly reduced due to global industrialization, drought, salinity, and global warming (Zandalinas et al. 2021).

The demand of quality food is day by day increasing, so there is need to find the pathway to feed the alarming population. Increasing crop productivity and maintaining quality food are on priorities.

In this contest, a new group called biostimulant is introduced in the farming system to maintained eco-friendly environment with positive effects on plant growth and crop productivity. Many biostimulants have proven to be effective in improving crop health and resilience to pests.

In this chapter, the initial part gives the overview of biostimulant, its benefits, and market size. In rest of the chapter, our main focus is on chitin, chitosan, and derivatives as a plant biostimulant, their production methods, and market demand.

17.2 Biostimulant, Its Benefits, and Market Size

17.2.1 Biostimulants

The word biostimulant was coined by horticulture specialists for describing substances promoting plant growth without being nutrients, soil improvers, or pesticides. In 1997, Zhang and Schmidt from the Department of Crop and Soil Environmental Sciences of the Virginia Polytechnic Institute and State University defined biostimulant as "materials that, in minute quantities, promote plant growth." In scientific literature, the word biostimulant was first defined by Kauffman et al. (2007) in a peer-reviewed paper, with modifications: "Biostimulants are materials, other than fertilizers, that promote plant growth when applied in low quantities." The term "biostimulant" is defined in the 2018 Farm Bill as "A substance or microorganism that, when applied to seeds, plants, or on the rhizosphere, stimulates natural processes to enhance or benefit nutrient uptake, crop quality and yield." In 2012, the European Commission has assigned an ad hoc study on plant biostimulant to evaluate the substances and materials involved, which was published by du Jardin (2012) as: "The science of plant Biostimulant—A bibliographic analysis." Based on scientific literature, the following definition was proposed: "Plant biostimulants are substances and materials, with the exception of nutrients and pesticides, which, when applied to plants, seeds, or growing substrates in specific formulations, have the capacity to modify physiological processes of plants in a way that provides potential benefits to growth, development, and/or stress responses" (du Jardin 2012).

In modern agriculture, biostimulant is a group of substances from natural origin that contributes to boosting plant yield and nutrient uptake, while reducing the dependency on chemical fertilizers. Developing biostimulants from byproducts pave the path to waste recycling, generating benefits for growers, food industry, registration and distribution companies, as well as consumers (Lin Xu and Danny Geelen).

Biostimulants have been defined by different authors, based on source materials, mode of action, and other parameters.

The active ingredients present in different types of biomass with potential biostimulant activity fall into a diverse range of molecules that include phytohormones (cytokinin, auxins, gibberellins, brassinosteroids, ethylene, abscisic acid) and polyamines (Yakhin et al. 2017).

17.2.2 Biostimulant Is Categorized into Seven Different Classes by du Jardin (2015)

- *Humic and Fulvic Acids:* Humic substances (HSs) arise from the decomposition of plant and animal tissues yet are more stable than their precursors (Gaffney et al. 1996).
  HSs include humic acids (HAs), fulvic acids (FAs), and humin and are known to stimulate the root system and plant growth and to mitigate stress damage; their effects extend to soil properties and microbial community structure. The use of these substances in promoting plant growth generates physiological, transcriptional, and metabolic changes (da Silva et al. 2021). These acids occur naturally in soils as a result of the breakdown of organic matter. HSs effects extend to soil properties and microbial community structure (Puglisi et al. 2013; Canellas and Olivares 2014). The effect on plant growth of these substances depends on the mode of application of HSs to the plant, content of bioactive molecules, source, dose and molecular weight of the humic fraction, and plant species (Canellas et al. 2010; Nardi et al. 2021; Calvo et al. 2014). The role of HSs in plant nutrition goes beyond the increase in plant root morphology, and these substances can form a complex with cations present in the soil, improving the uptake of nutrients such as P, Zn, and Fe by plants (Olaetxea et al. 2018; Nardi et al. 2021; Van Oosten et al. 2017).
- *Protein Hydrolysates:* Protein hydrolysates (PHs) are an important group of plant biostimulants defined as mixtures of polypeptides, oligopeptides, and amino acids which are manufactured from protein sources using partial hydrolysis. PHs can be derived from various sources such as milk, casein, animal-derived (meat, collage), and vegetable origin (wheat, gluten, rice, protein, pea protein, soy protein) (Vijai et al. 2010). PHs are use to increase the germination, productivity and quality of a wide range of horticulture and agronomic crops. Plants are colonized by an abundant and diverse assortment of microbial taxa that can help plants acquire nutrients and water and withstand biotic and abiotic stress. Indeed, recent studies have provided evidence that plant microbiomes are modified by the application of PHs, supporting the hypothesis that PHs might be acting, at least in part, via changes in the composition and microbial activity to enhance culture (Colla et al. 2017).

- *Seaweed and Plant Extracts:* Seaweed extracts contain major and minor nutrients, amino acids, vitamins, cytokinins, auxin, and abscisic acid like growth promoting substances (Mooney and Van Staden 1986), and it stimulates the growth and yield of plants (Rama Rao 1991), develops tolerance to environment stress (Zhang and Schmidt 2000; Zhang et al. 2003), increases nutrient uptake from soil (Verkleij 1992; Turan and Köse 2004), and enhances antioxidant properties (Verkleij 1992). Soybean (Glycine max (L.) Merr.) is one of the richest sources of protein.

  Plant-based extracts are new, natural, and multicompound products which possess antifungal, antimicrobial, antiparasitic, antiprotozoal, antioxidant, medicinal, and anti-inflammatory properties.
- *Inorganic Compounds:* It includes Al, Co, Na, Se, and Si and presents in soils and in plants as different inorganic salts and as insoluble forms like amorphous silica present. Beneficial functions are strengthening of cell walls by silica deposits, like pathogen attack for selenium and osmotic stress for sodium. Inorganic salts of beneficial and essential elements—chlorides, phosphates, phosphites, silicates, and carbonates—have been used as fungicides (Deliopoulos et al. 2010). Beneficial elements which promote plant growth, the quality of plant product, and tolerance to abiotic stress include cell wall rigidification, osmoregulation, thermal regulation, enzyme activity by cofactors, interactions with symbionts, protection against heavy metal toxicity, plant hormone synthesis, and signaling (Pilon-Smits et al. 2009).
- *Beneficial Microbes:* Beneficial microbes include fungi and bacteria.
  - *Fungi*: Fungi interact with plant roots in different ways, from mutualistic symbioses to parasitism (Behie and Bidochka 2014). Mycorrhizal fungi promote sustainable agriculture, considering the widely accepted benefits of the symbioses to nutrition efficiency, water balance, and biotic and abiotic stress protection of plants (Augé 2001; Gianinazzi et al. 2010; Hamel and Plenchette 2007; Harrier and Watson 2004; Siddiqui et al. 2008; Van der Heijden et al. 2004).
  - *Bacteria:* Bacteria interact with plants in all possible ways (Ahmad et al. 2008): For fungi, there is a continuum between mutualism and parasitism, bacterial niches extend from soil to interior of cells, and some bacteria are transmitted via seed, increase in nutrient use efficiency, induction of disease resistance, and modulation of morphogenesis by plant growth regulators.
- *Chitosan and Other Biopolymers:* Chitosan is commercially important compound and offers a range of applications from presowing seed treatment to postharvesting treatment in the fields of agriculture (Vaghela et al. 2020). Chitosan is a deacetylated form of the biopolymer chitin produced naturally and industrially. Chitosan elicited much interest as biostimulant and crop protectant due to its effect against biotic and abiotic stress, besides being environmentally friendly and inexpensive.

17.2.3 Benefits of a Biostimulant

1. Biostimulant promotes germination and root development, leading to increased vigor and growth.
2. Biostimulant increases plant tolerance under abiotic stress such as extreme temperature, drought, water logging condition, and high winds.
3. Biostimulant reduces the doses of chemical fertilizers and hence eco-friendly.
4. An enhanced root system promotes more efficient nutrient, water uptake, and translocation throughout the growing season.
5. Biostimulants generally operate through different mechanism than standard fertilizers and provide essential nutrient and minerals for plant metabolism and plant growth. These metabolic pathways upregulate gene expression that can have an effect on cell division and sizing, root and shoot growth, reproductive development, and timing.
6. Biostimulant improves the quality in produce such as the sugar content, color, fruit seeding, and good palatability.
7. Biostimulant enhances soil fertility by positively supporting the development of soil friendly microorganisms.
8. They help in remove heavy metals from contaminated soils.
9. Biostimulants help to amplify antioxidants in plants.

17.2.4 Market of Biostimulant

The global biostimulant market size was estimated at USD 1.74 billion in 2016, projected to expand at a CAGR of 10.2% from 2017 to 2025 (Fig. 17.1) (Grand

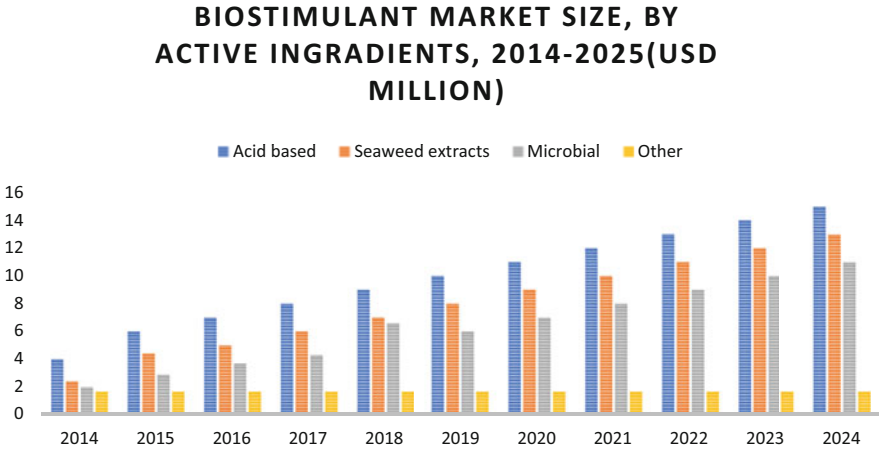


Fig. 17.1 Size of biostimulant market, by active ingredients

View Research 2018). Rising focus on enhancing productivity, coupled with rapid soil degradation, is likely to drive the market over the forecast period.

Biostimulants are emerging as an essential component in sustainable agricultural practices. Instances of environmental hazards and soil contamination from injudicious and excessive application of chemical-based products on crops have been a key issue for the industry in recent times. Biostimulants are an effective substitute for chemical-based agro-products and are replacing these at a higher rate on account of their environment-friendly nature and performance efficiency.

Companies are adopting backward and forward integrations to increase their profit margins and gain higher bargaining power. This is bound to intensify the competition over the forecast period. Research and development activities, along with expanding product lines, are predicted to majorly impact the overall competitive scenario through 2025. Global agriculture professionals are increasingly adopting biostimulants for the cultivation of row crops and cereals.

17.3 Mass Production Methods of Chitin, Chitosan, and Its Derivatives

17.3.1 Chitin, Chitosan, and Derivatives

The chitin isolation and characterization began in 1811 by the studies of the French chemist Henri Braconnot. The first reports of chitosan production appeared in 1970 in Japan and the United States. Chitin is a biopolymer from crustacean shells, and chitosan, the deacetylated form of chitin, has potential applications in food, cosmetics, and industrial processes.

17.3.1.1 Structure of Chitin

Chitin is a beta (1, 4)-linked photopolymer of *N*-acetyl glucosamine derivatives of glucose (Renuka et al. 2019) (Fig. 17.2), and it shares close structure similar to cellulose in that it is also indigestible by vertebrate animals due to lack of enzyme system required for its degradation. In chitin, alcoholic OH group of the second

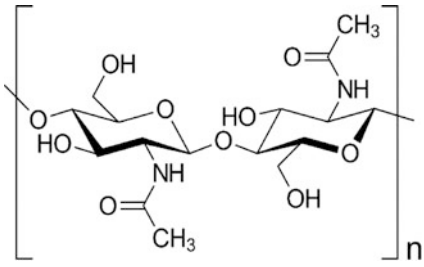


Fig. 17.2 Chitin

carbon atom of beta-D-glucose unit is replaced by an *N*-acetyl amino group (Antaonio et al. 2017).

17.3.1.2 Structure of Chitosan

Chitosan is a polysaccharide derived from chitin. Its molecular weight is typically between 300 and 1000 kDa depending on the source of chitin. A deacetylated form of chitin, at least 50% of the free amine form, has a heterogeneous chemical structure made up of both 1–4-linked 2-acetamido-2-deoxy-B-D-glucopyranose as well as 2-amino-2-deoxy-B-D-glucopyranose (Novikov et al. 2020) (Fig. 17.3).

Chitosan is differing from chitin by the presence of amino group which appears in its solubility in dilute acids (pH < 6) and forming complexes with metal ions so that it can be used for purification.

17.3.1.3 Glucosamine

Monosaccharides.

Structure of Glucosamine: D-glucosamine is made naturally in the form of glucosamine-6-phosphate (Fig. 17.4) and is the biochemical precursor of all *N*-containing sugar specially in humans.

The binding of chitin and chitosan to cell receptors induces physiological changes, triggering an oxidative burst reaction with H_2O_2 accumulation and Ca^{+2} leakage into cell. The cellular response to chitosan involves also NO, and the

Fig. 17.3 Chitosan

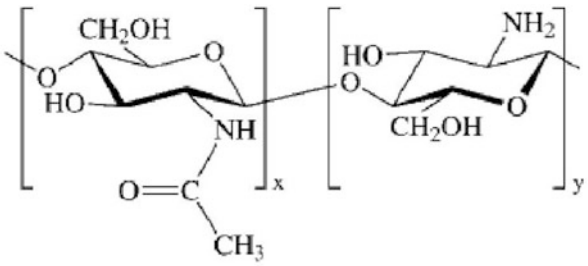
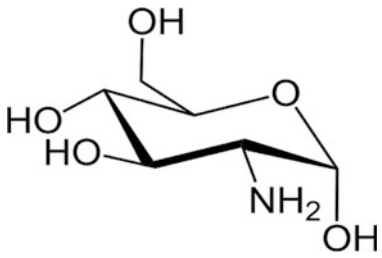


Fig. 17.4 Glucosamine



phytohormone regulators jasmonic acid, abscisic acid, phosphatidic acid all relate to abiotic stress gene regulation.

Due to its potency to induce defense mechanisms and stress response pathways, chitin and chitosan are used to improve crop resilience to pathogen attack and abiotic stress conditions (Riofrio et al. 2021; Sharma et al. 2016).

Chitin and chitosan can be used in cosmetics, agriculture (act as plant vaccine) (Rahman et al. 2018), food and nutrition, antimicrobial agent, water engineering, chromatography, paper industry, textile industry, tissue engineering, batteries, and drug delivery (as a carriers) (Reshad et al. 2021).

17.3.2 Commercial Production Methods of Chitin, Chitosan, and Its Derivatives

Seafood is a major source of animal protein in many countries; however, besides the edible part, these raw materials have an inedible one (Santos et al. 2020). The waste produce from the seafood contains a large proportion of chitin. Chitin produced annually by sea food organisms as wastes is about 10^{12}–10^{14} tons (Yadav et al. 2019). This huge quantity of chitin would provide enough raw material waste and a potential source of raw material for production of chitin and its conversion into value added products such as chitin, chitosan, chito-oligomers, and glucosamine.

There are different commercial methods adopted by industries for production of chitin, chitosan, and chito-oligomers. Different combinations of methods are currently on trails by industries, and also experimentation is done in laboratory to overcome the disadvantage of previous commercial methods.

Here, our focus is to list out the major methods adopted by industries and laboratory. In this chapter we discuss the advantage and disadvantage of different existing methods.

17.3.2.1 Chemical Method of Extraction of Chitin, Chitosan, and Chito-Oligomers

Chemical extraction method of chitin is composed of 3 basic steps: (a) demineralization, (b) deproteinization, and (c) discoloration. Further, conversion into chitosan and chito-oligomers adds an additional fourth steps: (d) deacetylation process to convert chitin into chitosan.

To start the chemical extraction of chitin or any other methods, there is a need to clean the materials as chitin obtained from diverse sources as wastes are subjected to washing and drying followed by crushing into powders.

(a) *Demineralization Process*: Demineralization of raw materials is based on acid treatment (0.25 N or 2 N HCl) to remove minerals like calcium carbonate and calcium phosphate. The most common acids used in this treatment process are

sulfuric acid, hydrochloric acid, acetic acid, nitric acid, and formic acid (Pighinelli et al. 2019; Hamdan et al. 2020; Pachapur et al. 2016; Pal et al. 2014). Demineralized raw materials are washed with distilled water for 15–20 mins until the pH become neutral. After that, they are dried in an oven around 60 °C for 24 h.

Demineralization occurs through the decomposition of calcium carbonate in calcium chloride, with the release of carbon dioxide. Similar minerals also react with acid and produce salts, which are removed by filtration. This process differs with different types of raw materials, time extraction, size, concentration of acids, temperature, and solute/solvent ratio.

(b) *Deproteinization Process*: Deproteinization of demineralized raw materials is carried out by using an alkali treatment. This method involves the disruption of chemical bonds between protein and chitin, requiring chemical depolymerize and biopolymer. The most commonly used reagents are NaOH, Na_2CO_3, $NaHCO_3$, KOH, $NaHSO_3$, Na_3PO_4, $Ca(OH)_2$, and Na_2S (Hahn et al. 2020). The protein is removed from raw materials using 40% KOH/NaOH with constant stirring for approx. 2–2.5 h at around 90 degree Celsius. Partial Deacetylation process leads to low molecular weight chitosan.

(c) *Decoloration Process*: This is final step of chitin preparation. Decoloration usually done when colorless chitosan is required. An organic solvent mixture or acetone is employed to eliminate the pigments like carotenoids and astaxanthin (Yadav et al. 2019).

(d) *Deacetylation Process to Convert Chitin into Chitosan*: Chitin is processed homogeneously or heterogeneously. In the homogenous method, chitin is treated with conc. Alkaline condition—NaOH (40–50%) at 25 ^{0}Celsius for 3 h and allowed to disperse in compressed ice at around 0 degree Celsius. This method gives a soluble chitosan. In the heterogeneous process, the chitin is treated with hot high concentrated solution of NaOH/KOH and high temperature (100–120 °C) for few hours, and chitosan is produced as an insoluble residue, it's deacetylated form upto 85% to 99%. After this above process filtration and drying of the soluble chitosan, as it's appropriate condition.

Another process is solubilizing chitin freezing at −193 °C and drying at 25 °C so that the chitosan obtained with acid salts.

Advantages and Disadvantages of Chemical Process

Advantages of the chemical extraction methods are short processing time, used at industrial scale, remove the all-organic salt, and fully developed process.

Disadvantages of the chemical steps are generally performed under high temperature conditions, higher energy input, harsh conditions which reduce quality of chitin, environmental unfriendly (Rao et al. 2000), solubilized minerals and proteins that cannot be used for human and animal nutrients, uncontrollable hydrolysis of product, consume large amount of acids and alkali, and difficulty in recovering waste products such as pigments and proteins.

17.3.2.2 Chemical and Enzymatic Deacetylation Process

Demineralization, deproteinization, and decolorization processes are same as above mention steps in chemical process to reach the chitin purification.

Deacetylation process to convert chitin into chitosan and chito-oligomers: The mechanism of enzymatic process is based on catalyzed hydrolysis of *N*-acetamide bonds of chitin by using enzyme chitin deacetylase. These enzymes are obtained from *Mucor rouxii, Absidia Coerulea, Aspergillus nidulans, Pencillium oxalicium,* and *Colletotrichum lindemuthianum* (Pareek et al. 2013; Younes and Rinaudo 2015). This enzyme is thermally stable and has a binding affinity toward beta-(1, 4)-linked *N*-acetyl-D-glucosamine polymers.

Advantages of this combining process are relatively short period time and highly deacetylated products.

Disadvantage of this process are (a) Chemical treatment leads to pollution, (b) Concentrate NaOH inhalation leads to health issues like severe burns to the eye, skin, digestive system & lungs, resulting in permanent damage of organs and some time death.

17.3.2.3 Mechanical and Chemical Process

This method requires a highly pure grade and ultrapure water used for preparation of all solution (Samar et al. 2013). The raw materials shrimp's wastes are rinsed several times with boiling water to eliminate all other impurities in the material and then were washed with distilled water, and dried at 80 °C overnight in an oven. Finally, they were grounded to pass through a sieve 200 micrometer and then subjected to demineralization and deproteinization.

Demineralization Process: The demineralization reaction was performed in a beaker/flask placed in a domestic microwave oven, the shrimp shell wasted is heated for 8 mins at a power 500 W, and then, it was filtered and washed with distilled water using vacuum distillation pump and then overnight dried at 80 °C overnight.

Deproteinization Process: Deproteinization reaction was performed in a flask placed in a domestic microwave oven at a different power, from the range 160–350 W. During this process, the washed sample is heated for 5 mins at a power 160 W and then 3 mins at power 350 W; after that, the sample was filtered and washed with distilled water using vacuum distillation pump and then oven-dried at 80 °C overnight (Oh et al. 2007).

Deacetylation Process: The preparation of chitosan was carried out by deacetylation of extracted chitin and 45% NaOH was placed in a conical flask, covered tightly with cotton, and then heated in a domestic microwave oven at a power 350 W for 8 mins. The mixture was then cooled with cold water, and after filtration, chitosan was washed to neutral pH and freeze-dried using VIRTIS freeze mobile 5EL with sentry microprocessor control freeze dryer or washed with distilled

water using vacuum distillation pump and then oven-dried at 80 °C overnight (Reys et al. 2017).

Advantages of the process are rapid compared to chemical process, speed up the reaction rate by order of magnitude over conventional heating, and efficient for several reaction and eco-friendly.

Disadvantages of this process are energy consumption, solubilized minerals, and proteins that cannot be used as human and animal nutrients, and uncontrolled hydrolysis of product.

17.3.2.4 Biological Process

The biological process involves the use of microbes that produce enzymes and organic acids at a relatively low cost, favoring high reproducibility in shorter time, simpler manipulation, smaller solvent consumption and lower energy input (Younes and Rinaudo 2015).

Chitin extraction from shrimps is other sea waste which involves two steps: (a) demineralization and (b) deproteinization. Deacetylation process involves to biotransform the chitin into chitosan and chito-oligomers.

(a) Demineralization Process: Shrimps and other sea waste contain calcium carbonates to protect their external shells. In biological methods, microbes are capable to dissolve this external shell by secreting organic acid. Microbes are capable to secrete organic acid such as lactic acid producing bacteria. Organic acid secreted from microbes reacts with calcium carbonate and calcium salts and precipitate it. These precipitated salts are removed from culture medium, and precipitated organic salts are removed by washing.

(b) Deproteinization Process: Deproteinization of the waste from the industry to obtain hydrolyzed protein is a method based on the addition of enzymes or microbes for protein fragmentation. In this process, some proteases (alcalase, pepsin, papain, pancreatin, devolvase, and trypsin) from bacteria can eliminate proteins from waste material and minimize the deacetylation and depolymerization during purification of chitin (Khorrami et al. 2012).

Fermentation: The cost of using enzymes can be decreased by performing deproteinization by fermentation process, which can be achieved by endogenous microorganisms (called auto-fermentation) or by adding selected strains of microorganisms (Younes and Rinaudo 2015). Fermentation can be achieved by single-stage fermentation, two-stage fermentation, co-fermentation, or successive fermentation. Fermentation methods are optimized based on the microbes use for organic acid, and proteases secrete extracellularly (Prameela et al. 2010).

(c) Deacetylation Process: Microbes and their enzymes are used to convert chitin into chitosan. Due to the eco-friendly nature, the deacetylation process attracts significant interest (Ghorbrl-Bellaaj et al. 2012). The use of microbial enzymes prevents irregular deacetylation and molecular weight reduction caused due to

acid and alkali treatment. In deacetylation process, the degree of deacetylation is a major concerns, and it depend on proportion of glucosamine monomer residues in the chitosan chain, and it can affect the solubility and performance of chitosan in many of its applications. Enzymatic deacetylation of chitin with deacetylase is isolated from various organisms such as fungi (*A. niger, F. velutipes, C. lindemuthianum, M. racemosus*, etc.) (Teng et al. 2001) and bacteria (*V. cholera and other bacteria of Vibrionaceae family*) (Mahmoud et al. 2007). However, chitin deacetylase was less effective for natural chitin which is insoluble and crystalline in nature (Yadav et al. 2019). To enhance the accessibility of chitin deacetylase to acetyl groups of natural crystalline chitin, pretreatment is needed to carry out with physical or chemical methods such as sonication, grinding, heating, and derivatization. Biological process of deacetylation are still limited due to long processing time and currently limited to laboratory scale (Kaczmarek et al. 2019).

The advantage of biological methods includes the production of chitin with higher reproducibility. Moreover, the solubility of chitin is limited and the biological approach is limited (Gadgey and Bahekar 2017).

17.4 Applications of Chitosan and Chito-Oligomers as Biostimulant and Protectant in Plants

Application of Chitosan biopolymer in a different category like Agriculture, Cosmetics, Food processing, Biomedical engineering, Waste water treatment, Chemical Industry and others (Bakiyalakshmi et al. 2016). In India, agriculture is a powerhouse of the world. In recent years, environmental-friendly measures have been developed for managing crop disease as alternative to chemical pesticides, fertilizers, biostimulant, and plant disease control, including the use of natural compounds such as chitosan. Currently chitosan is use an an alternative to chemical pesticides in seed coating material, plant elecitors, soil conditioners and crop protectant (Malerba and Cerana 2016). Chitosan has been reported to possess antifungal and antibacterial activity, and it showed to be effective against seed-borne pathogens when applied as seed treatment.

17.4.1 Antimicrobial Activity

Chitosan acts as an antimicrobial agents, it's activity as an antimicrobial were observed in various microbes such as fungi, bacteria, viruses (Casadidio et al. 2019). Antimicrobial substance kills or inhibits the growth of microorganisms. The inhibition was observed at different pathogen development stages such as mycelial growth, sporulation, spore viability, germination, and the production of fungal virulence factors. Antimicrobial effectiveness of chitosan seems to be higher

against fungi than bacteria. Antimicrobial activity of chitosan depends not only on external conditions (target microorganism, nature of the medium, pH temperature, etc.), but also on different intrinsic factors such as its molecular weight, and degree of polymerization and deacetylation. Two theories have been proposed for explaining the antimicrobial mechanism of chitosan. Theory one states the polycationic nature of chitosan is the reason for its antimicrobial nature to interact with the negatively charged microbial cell membrane which results in the disruption of the cytoplasmic membrane and, ultimately, leakage of intracellular constituents. By the other theory, chitosan oligosaccharides easily permeate into the nucleus of eukaryotic cell and interfere with the transcription of RNA and the synthesis of proteins (Kumar et al. 2018). Tran Minh Quynh reported in his study that the irradiation of chitosan through gamma radiation modifies the intrinsic viscosity and the number average molecular weight which in turn improves its antibacterial and antifungal activities. This irradiated chitosan can be applied as antimicrobial coating films for the preservation of fresh fruits. Chitinolytic microbes produce extracellular chitinase enzyme to degrade chitin-rich tissue of other organisms. Other studies showed the chitinase enzyme produced from *Streptomyces rubiginosus* shows maximum activity after 72 h of incubation period after which the activity decreases gradually, and it will act against plant pathogenic fungi. The addition of chitin to the soil promotes the growth of antagonistic microbes.

17.4.2 Plant Resistance Elicitation

All plants, whether they are resistant or susceptible, respond to pathogen attack by the induction of a coordinate signaling system, which results in the accumulation of different gene products. The response to pathogen attack is effective at different levels. The most studied plant responses to chitosan treatment are the formation of chemical and mechanical barriers and the synthesis of new molecule and enzymes involved in defense response. In some cases, chitosan causes the induction of hypersensitive response, mainly around infection site, that leads to programmed cell death. The signals able to triggers the defense mechanisms in plants called elicitors; they can be produced in the site of infection both by infected plant cells and by pathogen itself.

17.4.3 Chitosan Seed Treatment

In agriculture, "seed treatments are biological, physical, and chemical agents and techniques applied to seed to provide protection and improve the establishment and improve the establishment of healthy crops." It represents the first line of defense for seeds and seedling against pests infecting the seed teguments or living in soil (seed-borne and soilborne pathogens). It provides protection during the critical stage of

germination and the very first seedling development, when seeds and seedling are unable to protect themselves from invasive pathogens. The substance applied to the seeds can be various kinds such as chemical pesticides, biochemical substance, and natural compounds, and there are many difference techniques that can be used for this purpose. Among them, seed coating and dressing represent a common procedure of seed treatment applied for preventing disease and pests other than improving the seedling performances, i.e., the seedling emergence time, synchronized emergence, improved germination percentage, emergence rate, and yield in many field crops. Chitosan represents an interesting prospective like they can be used as film, forming physical barriers around the seeds preventing the pathogen infection.

For example, different kinds of chitosan seed coating with or without essential oils like thyme (*Thymus vulgaris*) and tea tree (*Melaleuca alternifolia*) essential oils, incorporated at different concentrations and applied with different thickness, have been studied for controlling disease and reduce the risk of pathogen attack.

Another main application of chitosan as seed treatment concerns the elicitation of systemic resistance in plants. Basing on recent evidence, chitosan, when applied as seed treatment, behaves as a resistance elicitor, inducing a physiologically enhanced defensive ability in seedling and plants, whereby the plant's innate defenses are potentiated. Chitosan seed treatment can also be effective in insect control because it stimulates plants to produce systemic antibodies with repellent effects on insect pests, as reported in soybean against *Agrotis ipsilon*, soybean pod borer, and soybean aphid.

17.4.4 Chitosan as Soil Amendment

As previously described, chitosan can be used in several ways to reduce plant disease levels and prevent the development and spread of disease, thus preventing crop yield and quality. Chitosan as soil amendment was found to successfully decrease *Fusarium* wilt in several plant species. Part of the observed effect of chitosan in the reduction in these pathogens comes from the fact that it enhances plant defense responses. It has been demonstrated that chitosan acts as an elicitor of plant systemic immunity by the accumulation of defense-related antimicrobial compounds and plays an important role in the activation of induced resistance.

In field conditions, chitosan alters the equilibrium of the rhizosphere, disadvantaging microbial pathogens and promoting the activity of beneficial microorganisms. For instance, soil treatment with chitin and/or chitosan from shrimp waste has been shown to decrease the rate of infection of plant roots by nematodes and enhance the suppressiveness against soilborne disease.

Recently, an innovative bioremediation strategy uses the ability of chitosan to chelate minerals and other nutrients, making them more available for the uptake by the plants. Thus, if chitosan can increase absorption of essential minerals, enhancing the plant's nutritional value (biofortification), it is possible that it can also help plants to take up higher concentrations of toxic elements.

17.4.5 Nematode Control

Nematodes in the genus Meloidogyne, such as M. incognita, cause economically significant, damaging root galls on a variety of plants. In potted plant experiments, Radwan et.al found that chitin and chitosan both significantly reduced root galls on tomato plants. Under laboratory conditions, chitosan increases sporulation and the mycelial growth of beneficial *P. chlamydosporia* and causes an increase in the production of a protease used by the fungus to parasitize plant-damaging root-knot nematodes. These effects are not observed in agricultural soils. Instead, chitosan appears to promote the colonization *P. chlamydosporia* in plant roots, which in turn makes the fungus a more effective biocontrol (Escudero et al. 2017).

17.4.6 Postharvest Protection of Crops

Chitin derivatives and particularly chitosan protect fruits from postharvest disease, being used as soluble additives to provide antimicrobial property and capable of forming gas semipermeable films. Soft rot damage is significantly reduced in tomato by coating with chitosan films. Preharvest treatment in strawberry decreases infection levels and improves fruit quality. In carrots, chitosan application 3 days prior to Sclerotina sclerotium inoculation decreases pathogen incidence, resulting in smaller lesions. Studies in chitosan-treated pepper at storage conditions showed that the gray mold appeared 7 days after than untreated fruits. Chitin derivatives were not only used to coat fruits but also used to increase quality of sliced fruits as shown in studies of sliced red pitayas and mango. In general, chitosan has shown a behavior similar to that of chemical fungicides, so it can be used instead of them, with the advantage of being a biodegradable product. The use of these alternatives in agriculture is due to the lower production costs of chitin derivatives and its advantages over the currently applied phytosanitary products.

17.5 Market Demand of Chitin, Chitosan, and Its Derivatives

Chitin, chitosan, and derivatives' market has witnessed significant growth over the last few years and is projected to reach by USD 8531.7 million by 2027 at a CAGR of 8.7% from 2020 to 2027. Derivatives of chitin such as chitosan and glucosamine find application in various industries. Chitin is found in crustacean shells, insect skin, and the cell wall of fungal species. The global chitin, chitosan, and derivatives market is highly competitive with key industry players focusing on product, regional presence, strategic alliances, and industry experience. Chitosan's antioxidant and antimicrobial activity allow it to be used to extend the shelf life of foods, and its

excellent emulsifying properties allow it to replace synthetic surfactants in food technologies. Easy availably of fungi waste in agriculture and artificial production of fungi through cheap nutrients by simple chemical procedures may create lucrative opportunities for the chitosan market growth.

17.5.1 Market Dynamics

Naturally obtained chitin and its derivatives are widely used in various application industries such as food and beverages, personal care, pharmaceuticals, nutraceuticals, textile, waste water treatment, paper manufacturing, hemostatic agent (coagulation), dietary supplement, cholesterol lowering agent, and agriculture. Chitosan can also be used as a functional ingredient against hypercholesterolemia, hypertension, and inflammations. Thus, the wide application scope of chitin and its derivatives is fueling the growth of the global chitosan and derivatives' market. The abundant availability of raw material is another factor driving the growth of the market as it ensures the continuous and economical supply of raw material.

The global chitin, chitosan, and derivatives' market has been segmented on the basis of type, application and region. The dominance of the segment can be attributed to the high demand for chitosan in various application industries such as food and beverages, agriculture, aquaculture, food packaging, water treatment, pharmaceuticals, and animal feed (Sastry et al. 2015) (Figs. 17.5 and 17.6).

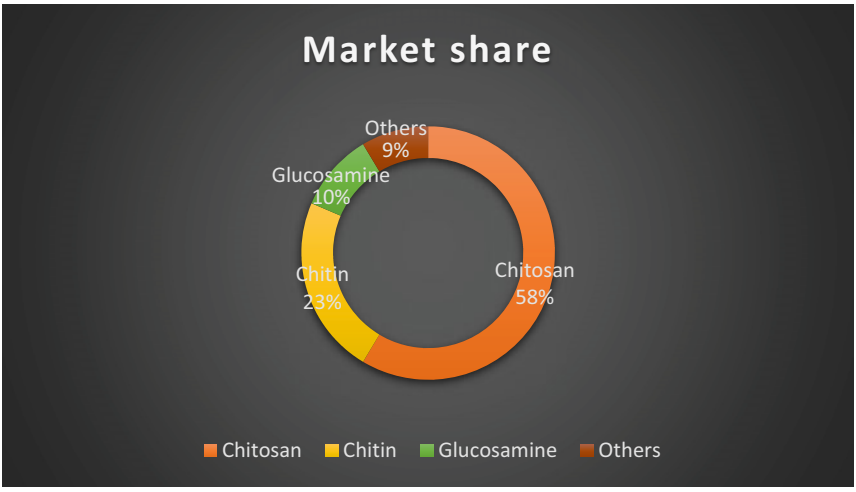


Fig. 17.5 Global chitin, chitosan, and derivatives' market share, by type, 2018 (%)

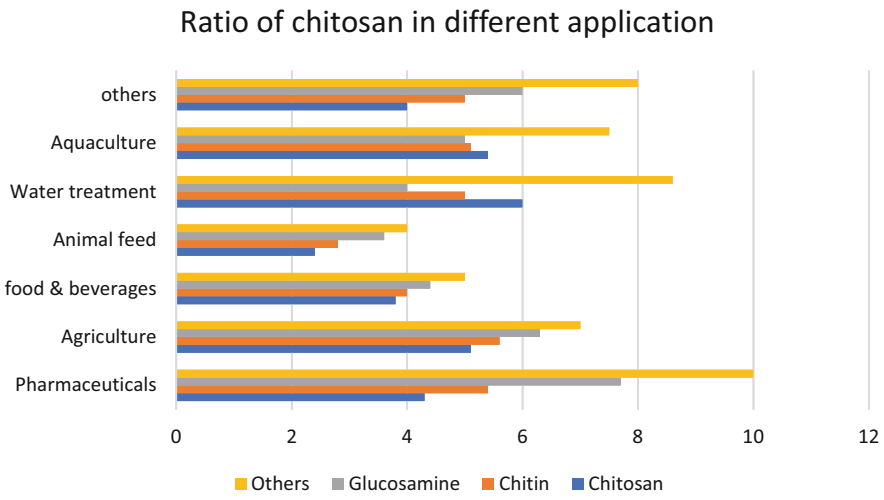


Fig. 17.6 Different (%) of chitosan in various industrial application

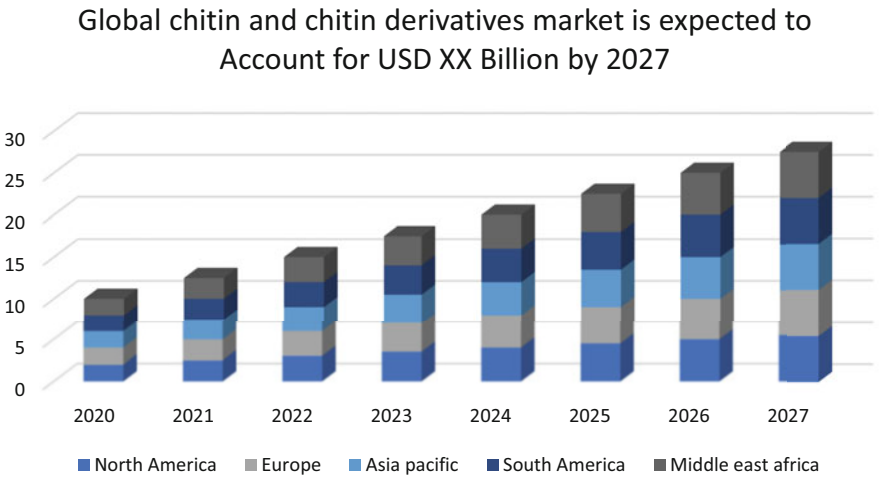


Fig. 17.7 Global chitin and chitin derivatives' market share expected in 2027

17.5.2 Regional Analysis

On the basis of region, the global chitin, chitosan, and derivatives' market has been categorized as North America, Europe, Asia-pacific, South America, and the Middle East and Africa (Fig. 17.7).

North America accounted for the largest market share in 2018, while the market in Asia-Pacific is expected to register the highest consciousness, and increasing awareness regarding the benefits of chitin, chitosan, and their derivatives is the key

factors driving the growth of the North American market. The presence of numerous pharmaceutical and nutraceutical manufacturers in North America is another factor boosting the consumption of chitin, chitosan, and derivatives in the region, with the USA being the largest country-level chitin market.

In addition, in India 2019, the government of India with BARC planned to install water filtration plants in village. The government targets to provide clean drinking water to everyone by approximately 2024–2026.

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Chapter 18
Mass Multiplication and Production Cost Analysis of Phosphate Solubilizing Microorganisms

P. Maheshwari, G. Gayathry, P. Murali Sankar, P. Sangeetha, and P. Anandaraj

Abstract The use of redundant quantity of phosphorus (P) fertilizers to develop the production and productivity of food, to meet out the global food demand, depletion of soil fertility, groundwater contamination, or eutrophication and accumulation of high concentration of toxic elements such as arsenic (As) and selenium (Se) in the soil. Several groups of soil microorganisms have the ability to mineralize or solubilize the insoluble form of soil P into soluble form of P. These groups of microorganisms are highly involved in the growth and development of many crops. Inoculation (nursery/seed/seedling dipping/soil application) of PSM (phosphate-solubilizing microorganisms) is promising technology for enhancing the world food production scenario without any deterioration of environmental impact. Several groups of bacterial and fungal species have the ability to solubilize the P under in vitro condition, and some of the microbial species are able to mobilize P into plants. PSM have urged to force the attraction of agriculturists which has used as a biofertilizer or soil inoculums for improving the plant growth and yield. PSM can be enumerated through the series of processes, namely isolation of organisms in the respective medium, purification, and characterization. After that characterization, process the identified microbes may be mass multiplied in the concerned medium. In this chapter, we discussed the mass multiplication of phosphate-solubilizing microorganisms, production cost, and their marketing condition.

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Keywords Marketing · Phosphorus · Phosphate-solubilizing microorganism · Production cost

18.1 Introduction

Phosphorus (P) is one of the essential and vital macronutrients for development of plant growth and constitutes nearly around 0.2–0.8% of the dry weight of the biomass (Sonam et al. 2011). Phosphorus nutrition is absolutely essential for the activities of food synthesis, cell reproduction, growth of shoot and root, flower formation, fruits, and seed setting. The inorganic forms of the element in soil are compounds of calcium, iron, and aluminum. The organic forms are compounds of myoinositol phytins, phytate phospholipids, and nucleic acids, and these compounds may be hydrolyzed by phytase and phosphatase enzymes, respectively (Neal et al. 2017). A large portion of phosphatic fertilizer applied to soil is fixed in soil by conversion in the form of insoluble phosphates such as calcium, magnesium, aluminum, and iron phosphates, which are not available to plants. P content in soil is approximately 0.5%, and this can be available to plants only up to 0.1%. Only 20–25% of phosphorus applied in the form of fertilizers is available to plants. The remaining insoluble and unavailable form of phosphorus is present in soil which is unutilized by plants.

Rhizosphere soil is rich source of beneficial microflora and fauna because of root exudates and enhancing the availability of plant nutrients. They are widely used in the biological processes as well as transformation of insoluble form into soluble form of nutrients. They include different groups of microorganisms such as bacteria and fungi, which convert insoluble inorganic phosphate compounds into soluble form, e.g., *Pseudomonas striata*, *Bacillus megaterium var. phosphaticum, Bacillus subtilis*, *Penicillium digitatum,* and *Aspergillus awamori*. They are capable of solubilizing calcium, magnesium, aluminum, and iron phosphates as well as rock phosphate. In case of soils low in phosphates, phosphatic fertilizers can be added with low-grade rock phosphate, basic slag, or bone meal. Apart from the addition of phosphatic fertilizers, phosphate-solubilizing microorganisms play an important role in the phosphorus nutrition solubilization and mineralization and thereby make it available to the plants (Bhattacharyya and Jha 2012). Hence, these groups of microbes are called as phosphorus-solubilizing microorganisms (PSM) and presented in Fig. 18.1. They solubilize insoluble inorganic (mineral) phosphorus and mineralize insoluble organic phosphorus (Sharma et al. 2013).

Many species of soil fungi and bacteria are able to solubilize phosphorus in vitro, and some of them can mobilize phosphorus in plants. PSM increase the bioavailability of soil insoluble phosphorus for plant use. The salt-tolerant or halophilic soil microorganisms that also exhibit the ability to solubilize insoluble phosphorus facilitate the development of saline-alkali soil-based agriculture (Zhu et al. 2011).

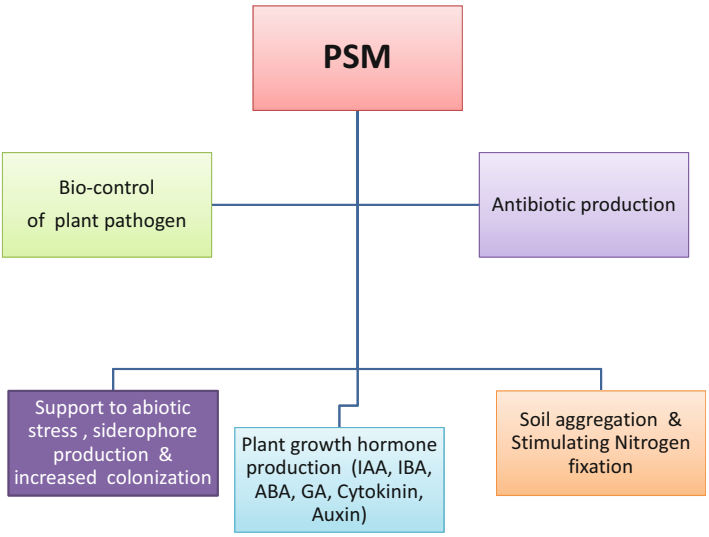

Fig. 18.1 Growth enhancement by phosphate-solubilizing bacteria

18.2 Mechanisms of Phosphorus Solubilization

P is solubilized by the production of metabolites like organic acids. This organic acids convert Ca_3 $(PO_4)_2$ to di and monobasic phosphates and releases P to plants. Solubilization of phosphates by plant roots and microorganism is dependent on soil pH, moisture, and air along with their carbonyl and hydroxyl groups which may chelate the cation-bound form of phosphate. Microorganisms including *Bacillus polymyxa, Pseudomonas striata*, *Micrococcus, Bacillus megaterium*, *Penicillium awamori*, *Fusarium, Aspergillus,* and Mycorrhizae secrete high amount of phosphatase enzymes which also solubilize bound form of phosphates. In neutrals and alkaline soils having a content of calcium, precipitation of $CaPO_4$ takes place. Microorganism and plant root readily dissolve such PO_4 and make them available to plants. PSM utilize lecithin in the medium or soil and increase the soil acidity. This acidic condition may stimulate the enzymes to act upon lecithin and releases the choline (Zhu et al. 2011).

Mycorrhiza is one of the best known symbiotic interactions which exist between the roots of higher plants and fungi. This mycorrhizal association has been found to improve plant growth through better uptake of phosphorus (Browne et al. 2009) and zinc from soil and suppression of root pathogenic fungi and nematodes. Vesicular arbuscular mycorrhizal fungi (AMF) have extended plant root system which may aid to increase the phosphate availability to the plants by PSM, namely *Glomus mosseae, Glomus aggregatum*, (Zai et al. 2017), and *Rhizophagus irregularis* (Mackay et al. 2017). Cyanobacteria and Actinobacteria possess the ability to secrete organic acids that could solubilize phosphorus (Jog et al. 2014; Chaiharn et al. 2018). Hamdali et al. (2008) reported that *Micromonospora* sp. and *Streptomyces* sp. produce siderophores

or calcium ion chelators that are able to solubilize phosphorus. In addition to the secretion of organic acids, production of chelators such as Al, Ca and Fe by phosphate solubilizing bacteria (PSB), phosphate solubilizing Actinomycetes (PSA) and Blue green algae [Yandigeri et al. (2011); Whitton et al. (1991); Zhao et al. (2002)].

The PSM act as biofertilizer for agriculture for increasing the rate of production and productivity (Babalola and Glick 2012). Biofertilizers or bioinoculants are carrier-based inoculum preparations consisting of efficient strain of N_2-fixing or PO_4-solubilizing microorganisms (PSM). Biofertilizers or bio-inoculants are formulated mainly based on the carrier-based inoculants or liquid inoculants. Carrier-based bacterial bioinoculants are more cost-effective than liquid biofertilizers, since they contain more bacterial cells which allow the cells to survive for an extended period of time.

18.2.1 Media Composition for Isolation of PSM

Sperber's Hydroxy Apatite Agar (Sperber 1957).

Glucose	10.00 g
Yeast extract	0.20 g
$MgSO_4.7H_2O$	0.10 g
$(NH_4)_2\ SO_4$	0.10 g
KCl	0.20 g
Soil extract	200 mL
Tap water	1000 mL
pH	6.8
Agar	20.0 g
K_2HPO_4 (10%)	40 mL
$CaCl_2$ (10%)	60 mL

The soil extract was prepared by autoclaving 1 kg of field soil suspended in 1 L of water for 1 h at 15 psi. Then, a pinch of calcium carbonate was added, allowed to stand for a while and then filtered. The filtrate was collected sterilized and stored for further use. Calcium chloride (10%) and dipotassium hydrogen phosphate (10%) were sterilized separately and added to the molten medium before plating. The pH was adjusted to 7 at the time of plating with sterile 0.1 N NaOH till a white precipitate appeared in the medium.

18.2.2 Collection of Soil Sample and Serial Dilution Plating Technique

Soil samples were collected from the paddy crop rhizosphere region. Ten grams of the rhizosphere soil sample was transferred to a 90-mL sterile water blank containing 250-mL Erlenmeyer flask. This solution was kept in a rotary shaker for 5–10 min for obtaining uniform sampling. This will give a dilution of 1:10 (10^{-1}). Aliquots of 1 mL were transferred from 10^{-1} dilution to another 9 mL sterile water containing test tube which will give a dilution of 10^{-2}. Likewise, the serial dilution was performed up to 10^{-4} dilution. Aliquots of 1 mL were plated from 10^{-1} and 10^{-2} dilutions in the appropriate media and incubated at room temperature (30 ± 2 °C). For each dilution, at least three replications are required for obtaining greater accuracy of the result. Pour approximately 10–15 mL of the molten and cooled (45 °C) Sperber's hydroxyapatite agar medium (Sperber 1957) to each petri dish and mix the inoculum by swirl or gently rotating (clockwise and anticlockwise) the petri dish for isolation and enumeration of phosphobacteria. When the agar gets solidified, plates are inverted at room temperature for 3–5 days. This serial dilution and plating work were performed in the laminar airflow chamber.

18.2.3 Isolation and Screening of PSM

The clear halo zone was observed around bacterial colonies surrounded by turbid white background after 3–5 days of incubation. Such cultures are isolated and purified for further use. The diameter of the clearing zone in and around the colonies and the solubilization efficiency was calculated. The potentiality of the P solubilization ability or solubilization index (SI) can be measured by using the following formula (Nguyen et al. 1992; Buddhi and Min-Ho 2013).

$$\mathrm{SI} = \frac{\text{Colony diameter} + \text{halo zone diameter}}{\text{Colony diameter}}. \tag{18.1}$$

18.2.4 Purification of Phosphate-Solubilizing Bacteria and Fungi

Microorganisms are present in nature a mixed cultures only. If more than one type of microorganisms is present, it is called as a mixed culture. A culture that is grown from a single cell is called as pure culture. The three most commonly used methods for obtaining pure cultures of bacteria are as follows: (a) streak plate, (b) pour plate, and (c) spread plate method. General principle involved in these techniques is to

dilute or divide the mixed population to the maximum so that single cells are separated and develop into single colonies. The individual colonies could be picked out and grown as a pure culture of the organism.

The fungi can be purified by the single spore isolation and single hyphal tip methods, when certain fungi are found mixed with other species of fungus or other microbes. The single spore isolation method can be conveniently employed when the spores of fungi are dark-colored and large-sized. The fungi producing colorless small spores can also be purified by this method, but such fungi can be purified with single hyphal tip method which is a more appropriate technique for the purification of all fungi from mixed cultures.

18.3 Characterization of the Phosphate-Solubilizing Microorganisms

The purified bacterial and fungal cultures may be preserved for further use. The pure cultures are further processed for identification and classification of microorganisms. Morphological characterization can be done for the pure colony based on its size, shape, arrangement, color, elevation, margins, surface form, and texture (Lal 2002).

18.3.1 Gram Staining

The Gram stain has been used as a taxonomic tool for many years, aiding in the classification and identification of bacterial cells (Gram 1884). The identified test isolates were characterized based on the cell wall morphology by using Gram-staining characteristics as per the below-mentioned standard protocol. The most commonly used differential stain is the Gram stain, first described in 1884 by Christian Gram. The staining procedure occurs in four parts: The first step is the addition of the primary stain, crystal violet, which stains the contents of the slide purple. After a rinse, Gram's iodine (mordant) is added to chemically bond the alkaline dye to the bacterial cell wall. The third step is the decolorizing step where the slide is exposed to an alcohol wash. The last step is the application of a counter stain. The most common counter stain is safranin, which colours decolorized cells pink. The gram positive bacterium appears purple in colour and gram negative bacterium appears pink in colour.

18.3.2 Identification of PSM by Molecular and Biochemical Methods

The purified form of test cultures is further processed for identification and classification of microorganisms by molecular (DNA extraction and DNA sequencing) and biochemical methods (methyl red and Voges–Proskauer test, starch hydrolysis, catalase test, H_2S production test) of characterization (Claus and Berkeley 1986).

18.4 Mass Multiplication of Phosphate-Solubilizing Microorganisms (PSM)

The mass multiplication of phosphate-solubilizing microorganism can be produced by two kinds of methods.

1. Mass production of phosphate-solubilizing bacteria (PSB).
2. Mass production of phosphate-solubilizing fungi (PSF)-AM (arbuscular mycorrhizal) inoculant.

18.4.1 Mass Production of Phosphate-Solubilizing Bacteria (PSB)

The mass multiplication of bacterial inoculants by carrier-based biofertilizers involves three steps:

1. Mass multiplication of microorganisms.
2. Preparation of carrier material.
3. Mixing of carrier material with liquid inoculum.

18.4.1.1 Mass Culturing of Phosphate-Solubilizing Bacteria

The media used for mass multiplication of PSB is phosphobacteria or *Bacillus* (nutrient broth), *Pseudomonas* (Kings B broth), and *Rhizobium* (yeast extract mannitol agar medium). Mass production of phosphobacteria is standardized for obtaining higher cell load Fig. 18.2. The nutrient broth is prepared in the small conical flasks, and the inoculum from pure culture or mother culture is transferred to flasks. The culture is grown under shaking conditions at 30 ± 2 °C as submerged culture. The culture is incubated until maximum cell population of 10^{10}–10^{11} cfu/mL is produced. Under optimum conditions, this population level could be attained within 2–3 days for phosphobacteria. The microbial culture obtained from the flask is called as **mother culture or starter culture**. For commercial production

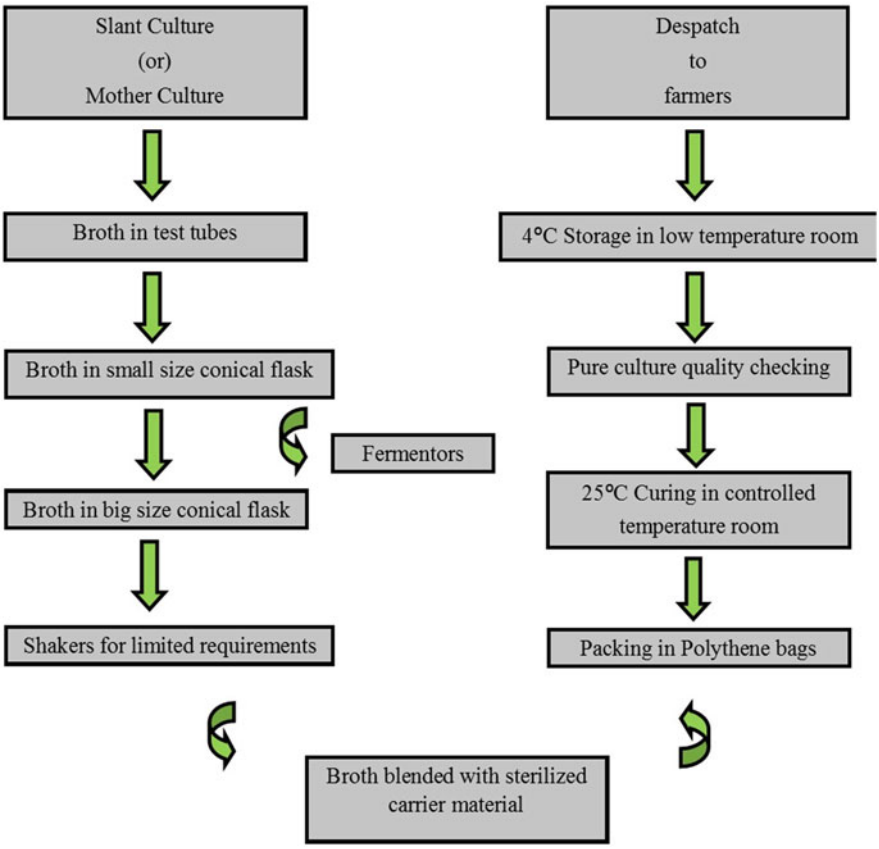

Fig. 18.2 Schematic representation of mass production of phosphobacterial biofertilizers

of microbial inoculant, inoculum from starter culture is transferred to large flasks or seed tank fermenter and grown until required level of cell count is reached.

Inoculum Preparation

- Prepare appropriate media for specific to the bacterial inoculant in 250-mL, 500-mL, 3-L, and 5-L conical flasks and sterilize it.
- The media in 250 mL flask is inoculated with efficient bacterial strain under aseptic condition.
- Keep the flask under room temperature in rotary shaker (200 rpm up to 5–7 days) for obtaining uniform microbial growth.
- Observe the flask for growth of the culture and estimate the population, which serves as the starter culture.

- Using the starter culture (at log phase), inoculate the larger flasks (500 mL, 3 L and 5 L) containing the media, after obtaining growth in each flask.
- The above media is prepared in large quantities in fermentor.

Fermentor

Mass culturing of biofertilizer requires large-capacity fermentors. Fermentor is the vessel which maintains the controlled environmental conditions for the growth and multiplication of microorganisms in order to obtain a desired product. Fermentor provides access for inoculation, sampling, aeration, and cleaning. It should be made of stainless steel to withstand high pressure and also to resist corrosion. High-quality fermentor will have smooth surfaces inside.

Basic Functions of a Fermentor

- Fermentor vessel should be capable of being operated aseptically for a number of days. It should withstand high pressure. The power consumption should be as low as possible. The vessel should be designed to require minimal use of labor for operation.
- The vessel should be constructed to ensure smooth internal surfaces without cracks and crevices.

Description of a Fermentor

The vessel in which industrial fermentation process carried out is called as fermentor. The volume of the fermentor varies from 5–10 L to 500,000 L. Similarly, the fermentor model will vary for aerobic process and for anaerobic process. It is domed at the top and bottom and held upright by a stainless steel skirt. The top has accessories like a steam pressure gauge, a safety pressure release valve, aeration system with filters, and inoculation port. Inlet and outlet ports are provided for water passage through stainless steel cooling coil. The sampling port is provided at the bottom to withdraw samples or to harvest the cultures. The basic structure and function of the fermentor are furnished in the Table 18.1.

However, the actual operation volume in a fermentor is always less than that of the total volume (2/3), because it requires head space for splashing, foaming, and aeration of the liquid inside.

Sterilization of Growth Medium in the Fermentor

- Prepare required quantity of growth medium and adjust to the required pH.
- Pour the medium into the fermentor vessel after closing the sampling valve.

Table 18.1 Components of the fermentor

1. Inlet	To feed the substrates, inoculum, microorganism, etc.
2. Outlet	To collect the products, to take samples
3. Stirrer	To mix the contents so as to get contact between nutrients and microbes
4. Air sparger	To provide oxygen to the microbes by small air bubbles
5. pH meter or controller	To check the pH and if needed to add acid and alkali to maintain optimum pH
6. Thermometer	To check the temperature
7. Outer jacket	By which cool water will be circulated to cool the fermentor (because during fermentation, lot of heat will be generated)
8. Sterilization unit	By which steam will be supplied to the fermentor to sterilize the medium and container

- Keep the air outlet valve open.
- Bring the growth medium to boiling under maximum heat by using steam generator.
- Close the air outlet valve and allow the pressure to build up inside the vessel.
- Maintain a pressure of 15 1b/in^2 at 121 °C for 20 mins.
- Switch off the fermentor and cool the medium by circulating cool water.

Mass Culturing in Fermentor

- Spray the inoculation port with alcohol and flame thoroughly.
- Allow the port to cool, and inoculate the media in the fermentor vessel with the log phase culture grown in 5-L flask. Usually 1–2% inoculum is sufficient; however, inoculation is done up to 5% depending on the growth of the culture in the larger flasks.
- Turn on the air pump, and open the air outlet valve.
- Regulate the airflow to 3–10 L of air per hour per liter of the medium. The sterile air provides aeration and agitation for the growth of culture.
- Draw samples and analyze for growth, periodically if necessary.
- Once the culture reaches full growth, turn off the air supply and the cells are harvested with the population load of 10^9 cells/mL after incubation period through the sampling port.
- There should not be any fungal or any other bacterial contamination at 10^{-6} dilution level.
- It is not advisable to store the broth after fermentation for periods longer than 24 h. Even at 4° C, the number of viable cells begins to decrease.

18.4.1.2 Processing of Carrier Material

The use of ideal carrier material is necessary in the production of good-quality biofertilizer. Peat soil, lignite, black ash, vermiculite, charcoal, press mud, farmyard manure, and soil mixture can be used as carrier materials. The neutralized peat soil/lignite is found to be better carrier materials for biofertilizer production. The following points are to be considered in the selection of ideal carrier material (Table 18.2).

Preparation of Carrier Material

- The carrier material (peat or lignite) is powdered to a fine powder so as to pass through 212 micron IS sieve.
- The pH of the carrier material is neutralized with the help of calcium carbonate (1: 10 ratio), since the peat soil/lignite is acidic in nature (pH of 4–5).
- The neutralized carrier material is sterilized in an autoclave to eliminate the contaminants.

18.4.1.3 Mixing of Broth Culture with the Carrier and Packing

Inoculant packets are prepared by mixing the broth culture obtained from fermenter with sterile carrier material as described below:

Preparation of Inoculants Packet

- The neutralized, sterilized carrier material is spread in a clean, dry, sterile metallic, or plastic tray.
- The bacterial culture drawn from the fermentor is added to the sterilized carrier and mixed well by manual (by wearing sterile gloves) or by mechanical mixer. The culture suspension is to be added to a level of 40–50% water-holding capacity depending upon the population.
- The inoculant packet of 200 g quantities in polythene bags, sealed with electric sealer and allowed for curing for 2–3 days at room temperature (curing can be done by spreading the inoculant on a clean floor/polythene sheet/by keeping in

Table 18.2 Ideal method of carrier material selection process

1. Cheaper in cost	5. Water-holding capacity of more than 50%
2. Should be locally available	6. Easy to process, friability, and vulnerability
3. High organic matter content	7. Amenable for mixing
4. Nontoxic chemicals	

open shallow tubs/trays with polythene covering for 2–3 days at room temperature before packaging).

Specification of the Polythene Bags

1. The polythene bags should be of low-density grade.
2. The thickness of the bag should be around 50–75 micron.
3. Each packet should be marked with the name of the manufacturer.

   (a) Name of the product,
   (b) Strain number,
   (c) The crop to which recommended,
   (d) Method of inoculation or application,
   (e) Date of manufacture,
   (f) Batch number,
   (g) Date of expiry,
   (h) Price,
   (i) Full address of the manufacturer, and.
   (j) Storage instructions, etc.

18.4.1.4 Quality Control of Biofertilizers for Phosphobacterial Inoculant

Quality control must begin with the pure culture maintenance of mother culture and broth culture before addition to the carrier and finished product. The population of bacteria should be maintained and contaminants should be checked periodically using serial dilution plating technique. Quality assurance or specification of the inoculants packets may be furnished in the Table 18.3.

Storage of Biofertilizer Packet

- The packet should be stored in a cool place away from the heat or direct sunlight.
- The packets may be stored at room temperature or in cold storage conditions in lots in plastic crates or polythene/gunny bags.
- The population of inoculant in the carrier inoculant packet may be determined at 15-day interval. There should be more than 10^9 cells/g of inoculant at the time of preparation and10^7 cells/g on dry weight basis before expiry date.

Table 18.3 Gazette notification, 2009-GOI—Indian Standard Specifications for PSM

Parameters	PSM
Base	Carrier-based in the form of moist/dry powder or granules or liquid-based
Cell no. at the time of manufacture	CFU minimum 5×10^7 cell/g of powder, granules, or carrier material or 1×10^8 cells/mL of liquid
Contamination level	No contamination at 10^5 dilution
PH	6.5–7.5 for moist/dry powder and 5.0–7.5 for liquid-based
Moisture percent by weight maximum in case of carrier-based	30–40%
Particle size in case of carrier-based material	All material should pass through 0.15–0.212 mm IS sieve
Efficiency character	The strain should have phosphate-solubilizing capacity in the range of minimum 30% when tested spectrophotometrically. In items of zone formation, minimum 5 mm solubilization zone in prescribed media having at least 3

18.4.2 Mass Production of Phosphate-Solubilizing Fungi (PSF)-AM (Arbuscular Mycorrhizal) Inoculants

AM fungi can be mass-produced only in the presence of living roots due to their inability to grow independently without plant host. For inoculum production, VAM fungi require a host plant and a growth medium. Soil is often used as growth medium as it provides optimum conditions for the growth and reproduction of fungus. Inert materials like vermiculite, perlite, and sand are used commonly as media for VAM inoculum production.

18.4.2.1 The Procedure for Mass Production of VAM Fungi Is as Follows

- Form the trench with the size of 1 m x 1 m x 0.3 m lbh and line with black polythene sheet to be used as a plant growth tub.
- Mix 50 kg of vermiculite and 5 kg of sterilized soil and pack in the trench up to a height of 20 cm.
- Spread 1 kg of AM inoculum (mother culture) 2–5 cm below the surface of vermiculite.
- Surface-sterilize maize seeds with 5% sodium hypochlorite for 2 mins before sowing.
- Apply 1 g urea, 1 g superphosphate, and 1 g muriate of potash for each trench at the time of sowing seeds. Further, apply 10 g of urea twice on 30 and 45 days after sowing for each trench.
- Quality test on AM colonization in root samples is to be done on 30th and 45th day.

- Grow the stock plants for 60 days (8 weeks). The inoculum is obtained by cutting all the roots of stock plants. The inoculum produced contains a mixture of vermiculite, spores, pieces of hyphae, and infected root pieces. Thus, within 60 days, 55 kg of VAM inoculum could be produced from 1 sq. meter area. This inoculum will be sufficient to treat 550 m^2 nursery area having 11,000 seedlings.

Storage of Inoculum

VAM bulk inoculum can be stored for a period of 6 months under room temperature.

Economics of Liquid Phosphobacteria Mass Production

The approximate production cost of the liquid phosphobacteria was summarized in Table 18.4.

18.5 Conclusion

Phosphorous is one of the important nutrients required for plant growth and development. Phosphate-solubilizing microorganisms have gained a wider acceptability rate because of its eco-friendly in nature, improve the soil fertility status and crop growth, and involve in biogeochemical cycle. The applied form of chemical fertilizer may be quickly fixed into the soil which cannot be available to plants. Application of PSM microbial inoculants can contribute minimal cost of input, easy to handle, and cleaner environment which may lead to urge the production of PSM. However, phosphate-solubilizing microorganisms require a specific technology to develop the microbial inoculants, and this should be useful to farmers in a short time without affecting the soil fertility.

Table 18.4 Economics of liquid phosphobacteria mass production

Sl. no.	Particulars	Numbers	Amount (Rs. in lakhs)
I.	**Fixed cost (nonrecurring cost)**		
a.	Building (including the land) 600 sq.ft.	1 no	25.00
b.	*Equipment and apparatus*		
	1. Fermentor (200 lit capacity)	2 nos	20.00
	2. Shaker	1 no	0.50
	3. Laminar flow chamber	1 no	1.00
	4. Autoclave	2 nos	1.50
	5. Hot air oven	2 nos	0.50
	6. Incubator	1 no	0.25
	7. Refrigerator	1 no	0.60
	8. Microscope	2 nos	0.60
	9. pH meter	1 no	0.10
	10. Balance	1 no	0.10
	11. Automated bottling unit	1 no	3.00
	12. Working benches	4 nos	3.00
	13. Stainless steel or plastic trays	10 nos	0.20
	14. Trolley	2 nos	0.20
	15. Storage cabinets	2 nos	0.75
	Subtotal (b)		**32.30**
II	**Variable cost**		
c	*Recurring contingencies*		
	1. Salary for staff (technical staff) @Rs.25,000/–	1 year (1 no.)	3.00
	2. Salary for laborers	1 year (2 nos.)	2.00
	3. Travelling expenses	1 year	0.50
	Subtotal (c)		**5.50**
d.	*Working capital*		
	1. Cost of mother culture (production)		0.10
	2. Purchase of glasswares		2.00
	3. Purchase of chemicals for media preparation, culture protectants (glycerol/Piko media)		1.00
	4. Other consumables		0.25
	5. HDPE bottle/container (liquid PPFM)		2.00
	6. Carton boxes/labels		1.00
	7. Miscellaneous expenditure (electrical and transport of materials)		2.00
	Subtotal (d)		**8.35**
e	Administrative expenses		1.00
f	Interest on loan/depreciation (5%)		1.25
	Grand total (b + c + d + e + f)		**48.40**

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Chapter 19
Large-Scale Production and Business Plan for Novel Corona Vaccine

Amrita Kumari and Seema Rani

Abstract Frontline healthcare workers will play a central role in encouraging COVID-19 vaccination. Many studies have found that physicians are the most important influencers of vaccine decision making. To substantially reduce morbidity and mortality from COVID-19, an efficacious and safe vaccine must be delivered swiftly and broadly to the public as soon as it is available. However, the mere availability of a vaccine is insufficient to guarantee broad immunological protection; the vaccine must also be acceptable to both the health community and general public. Vaccine hesitancy is a major barrier to vaccine uptake and the achievement of herd immunity, which is required to protect the most vulnerable populations. Depending on varying biological, environmental, and sociobehavioral factors, the threshold for COVID-19 herd immunity may be between 55% and 82% of the population. This chapter outlines the structure of coronavirus and vaccine development, different approaches of COVID-19 vaccines, whole virus vaccines, protein subunit vaccines, DNA vaccines, and composition of novel corona vaccine. It also illustrates the steps for corona vaccine development with preclinical, clinical evaluation, large-scale production of vaccine, quality control, packaging, storage shipping, and marketing. It describes the key challenges to scale up vaccine production, overcoming key challenges related to vaccine scale-up with business plan for novel corona vaccine.

Keywords Coronavirus · Acute respiratory syndrome · Vaccine development · Herd immunity · Death rate · Clinical evaluation

19.1 Introduction

Coronavirus is a zoonotic virus, a new positive-strand RNA virus which belongs to the family Coronaviridae of the order Nidovirales (Padron-Regalado 2020). The types of coronavirus known to date are as follows: the alpha coronaviruses

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HCoV-229E and HCoV-NL63; the beta coronaviruses HCoV-OC43 and HCoV-HKU1; SARS-CoV, which causes severe acute respiratory syndrome (SARS); MERS-CoV, which causes Middle East respiratory syndrome (MERS); and SARS-CoV-2, a new coronavirus which was first reported in December 2019 in China (Padron-Regalado 2020), which causes the disease known as coronavirus disease 2019 (COVID-19). The full genome sequence of coronavirus was first shared by China on January 12, 2020, for countries to use in developing specific diagnostic kits (Kyriakidis et al. 2021). By September 2020, GISAID (Global Initiative on Sharing All Influenza Data), a large international repositories of SARS-CoV-2 uploaded and shared almost 100,000 full SARS-CoV-2 genomic sequences on GSAID SARS-CoV-2 Genomic Epidemiology platform (Peera 2020). According to WHO Report October 2021, globally over 23.7 crore people were affected from COVID-19 with 48.5 lakh confirmed deaths that were solely attributable to this disease (Peera 2020). Most people infected with COVID-19 reported respiratory tract illness which can range from very mild rhinorrhea to severe acute respiratory distress syndrome and death (Guan et al. 2020a; Chen et al. 2020; Bhatraju et al. 2020). However, other nonrespiratory symptoms have also been associated with COVID-19 such as anosmia, diarrhea, rash, thromboembolic disorders, myocarditis, and vasculitis (Chen et al. 2020; Bhatraju et al. 2020; Wang et al. 2020; Pan et al. 2020; Huang et al. 2020; Giacomelli et al. 2020; Zhou et al. 2020; Song et al. 2020a; Recalcati 2020). After exposure to the virus in 2–14 days, one will get persistent fever, on an average of 5–6 days. The mean incubation period is estimated to be 5 days (2–7), with a majority developing symptoms by 11.5 days (Lauer et al. 2020; Zou et al. 2020). Prolonged exposure for at least 15 minutes within 6 ft to an infected person and briefer exposures to individuals who are symptomatic (e.g., coughing) are associated with higher risk for transmission, while brief exposures to asymptomatic individuals are less likely to result in transmission of COVID-19. The death rate of COVID-19 is consistently reported to be age dependent, with a higher percentage in elderly (aged >70 years) cases dying, although other factors are also associated with intensive care admission and mortality (Global Polio Eradication Initiative 2020; Guan et al. 2020b) including sex (male > female), hypertension, obesity, and diabetes.

According to WHO, on October 12, 2021, there are currently 194 candidate vaccines that are in preclinical development worldwide. There are 40 vaccines which are in Phase I trial undergoing safety tests in healthy young individuals, 35 vaccines are in Phase II trial which are being tested in broader groups of people, 34 vaccines are in Phase III trial which are in large international trials to test their impact on COVID-19, and 8 vaccines are being monitored in the wider population after being approved (WHO 2020). Most of these trials are enrolling healthy adults (from age 18 years) only, with the upper age limit of inclusion ranging from 50 to 60 years.

19.2 Structure of Coronavirus and Vaccine Development

Coronaviruses have a large (30 + kb) single-stranded positive sense RNA genome which belongs to *Coronaviridae* family. The genome encodes several structural proteins like nucleocapsid phosphoprotein (N) which forms the helical capsid which is further surrounded by an outer envelope comprised of matrix protein (M), envelope protein (E), and spike proteins (S) (Boopathi et al. 2020). Other transcribed nonstructural proteins include orf1ab, ORF3a, ORF6, ORF7a, ORF10, and ORF8. The S protein, which naturally occurs in a trimetric form, contains the receptor-binding domain (RBD) responsible for binding onto the angiotensin converting enzyme 2 (ACE2) and entry primarily into the lung cells (Fig. 19.1).

With the availability of genome sequence of SARS-CoV-2 on January, 2020, different research groups across the world were employed to develop a vaccine against the coronavirus by using a variety of platform technologies that could stimulate the immune system against several viral antigens. Traditionally, vaccine development strategies such as live attenuated viruses (e.g., measles, mumps, rubella), inactivated viruses (e.g., inactivated polio vaccine), protein or polysaccharide conjugated subunit vaccines (protein: acellular pertussis, hepatitis B; polysaccharide conjugated: pneumococcus, meningococcus), and virus-like particles have been used in vaccines licensed in humans, but over the last decade, new sophisticated technology platforms to expedite vaccine development have been explored which include vaccines composed of nucleic acid (DNA and RNA) and viral vectors and recombinant proteins (Rauch et al. 2018). At present, the majority of the candidate vaccines for SARS COVID-19 that employ administration of viral antigens or viral gene sequences aim to elicit neutralizing antibodies against the viral spike protein (S), preventing uptake through the human ACE2 receptor and, therefore, blocking infection (Buchholz et al. 2004; Walls et al. 2020). Currently, there are four approaches of COVID-19 vaccines according to the platform technology used in their development: whole virus, protein subunit, viral vector, and nucleic acid (RNA and DNA). Some of them try to smuggle the antigen into the body; others use the body's own cells to make the viral antigen.

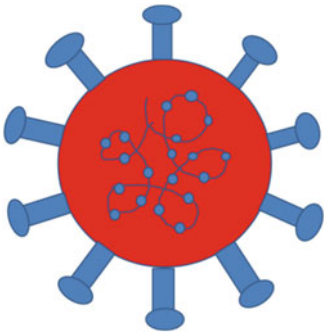

Fig. 19.1 Structure of coronavirus

19.3 Different Approaches of COVID-19 Vaccines (Fig. 19.2)

19.3.1 Whole Virus Vaccines

Whole virus vaccines use a weakened (attenuated) or deactivated form of the pathogen that causes a disease to trigger protective immunity to it. There are two types of whole virus vaccines, i.e., live attenuated and inactivated vaccines.

Live attenuated vaccines use a weakened form of the virus, which can still grow and replicate, but cause no or only very mild disease. These vaccines trigger the immune system by mobilizing a range of defenses against it, including killer T cells (which identify and destroy infected cells), helper T cells (which support antibody production), and antibody-producing B cells (which target pathogens lurking elsewhere in the body, e.g., the blood). This immune response continues until the virus is cleared from the body, meaning there is plenty of time for memory cells against the virus to develop (Koirala et al. 2020) (Fig. 19.2).

However, they may be unsuitable for people with compromised immune systems (e.g., those with HIV) and pregnant women though, because even a weakened virus may trigger disease in these individuals.

They are used for rubella virus, varicella zoster virus, and influenza virus and measles virus. Live attenuated SARS-CoV-2 vaccine candidates include: Cold-adapted mutants, temperature-sensitive mutants, and codon deoptimized mutants.

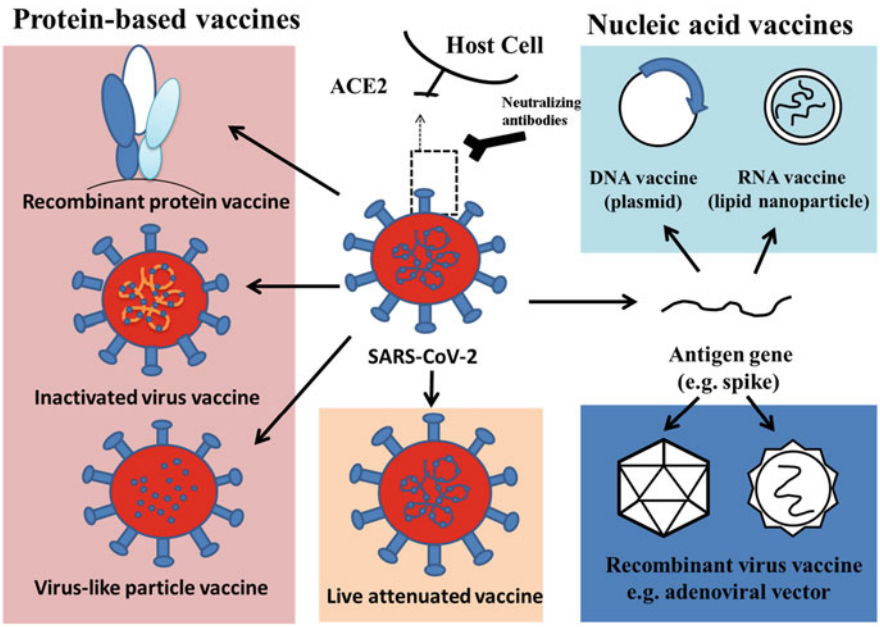

Fig. 19.2 SARS-CoV-2 vaccine candidates under development

According to Okamura S et al. 2021, cold-adapted mutants used for SARS-CoV-2 virus replicate more slowly in the lower respiratory tract and lungs, compared to the wild-type strain, whereas temperature-sensitive (TS) mutants could replicate not replicate at these locations. However, Mueller Steffen and his coworkers reported that codon deoptimized mutants of virus show lower proliferation rates than the wild-type strain, at any location (Okamura and Ebina 2021).

Although live attenuated vaccines are considered as one of the most powerful vaccine modalities, there are several problems associated to their use. The most serious problem is that viruses may reacquire their toxicity due to mutations after vaccination. A possible solution to this problem is to construct strains with various mutations by using reverse genetic methods such as BAC DNA and CPER methods (Ye et al. 2020; Torii et al. 2021) and thus maintain live attenuation, even when one responsible mutation is replaced by the wild-type sequence. In addition, adverse reactions like coagulopathy in elderly COVID-19 patients and induction of cytokine storm due to the proliferation of the live attenuated vaccine strains in the nasal cavity have been reported due to SARS-CoV-2 infection (Yuan et al. 2020; Song et al. 2020b). The temperature-sensitive strains that we isolated were able to proliferate in the nasal cavity as the wild-type strain, thereby potentially disrupting the epithelial tissues. Therefore, its effects need to be evaluated in detail.

Inactivated vaccines contain viruses whose genetic material has been destroyed by heat, chemicals, or radiation to stop disease-producing capacity, but they can replicate and still trigger an immune response. For this reason, they are considered safer and more stable than live attenuated vaccines, and they can be given to people with compromised immune systems. The disadvantage of inactivated vaccines is that they only stimulate antibody-mediated responses, and this response may be weaker and less long-lived. To overcome this problem, inactivated vaccines are often given alongside adjuvants (agents that stimulate the immune system), and booster doses may be required. Moreover, production of attenuated or inactivated vaccines requires large amounts of virus or bacteria to grown, which must then be isolated and purified, depending on the vaccine. Each of these steps requires specific equipment, reagents, and stringent procedures to avoid, and check for, contamination, which can further increase costs.

An inactivated SARS-CoV-2 vaccine was first reported in July 2020 (Song et al. 2020b). Gao et al. inactivated the virus with betapropiolactone, and the vaccine candidate was evaluated in mouse, rat, and nonhuman primate models. This vaccine-induced anti-S and anti-RBD antibodies, and the serum from immunized animals showed neutralizing activity. Kaabi et al. investigated the effect of 2 inactivated SARS-CoV-2 vaccines (SARS-CoV-2 WIV04 and HB02) on symptomatic COVID-19 infection in adults. Xia et al. reported that β-propiolactone-inactivated whole virus vaccine provides important interim safety, tolerability, and immune response against COVID-19. To date, four SARS-CoV-2 inactivated vaccines (Sinopharm, Sinovac, Sinopharm-Wuhan, and Bharat Biotech) have been approved and are being used in China, U.A.E, and India (Kaabi et al. 2021).

19.3.2 Recombinant Vaccines/Viral Vectors

Viral vector-based vaccines differ from most conventional vaccines in that they do not actually contain antigens, but rather use the body's own cells to produce them. They do this by using recombinant viruses which are constructed by inserting the coding sequence of an antigen into a viral genome or by replacing a part of it. In the case of COVID-19, spike proteins found on the surface of the virus are used as antigens (Feng et al. 2020). Upon infection, the antigen's coding sequence can be delivered to the cytoplasm or the nucleus of the infected host cell and instructing them to make large amounts of antigen, which then trigger an immune response; the vaccine mimics what happens during natural infection with certain pathogens, especially viruses. This has the advantage of triggering a strong cellular immune response by T cells as well the production of antibodies by B cells. There are two main types of viral vector-based vaccines. Nonreplicating vector vaccines are unable to make new viral particles; they only produce the vaccine antigen. Replicating vector vaccines also produce new viral particles in the cells they infect, which then go on to infect new cells that will also make the vaccine antigen. The COVID-19 viral vector vaccines under development use nonreplicating viral vectors.

Adenoviruses are commonly considered backbone vectors for the development of SARS-CoV-2 vaccines. They are nonenveloped double-stranded DNA (dsDNA) viruses with a packaging capacity of up to 7.5 kb of foreign genes (Schiedner et al. 1998). In almost all cases, the deletion of genes such as E1 and E3 genes has been engineered for the expression of the SARS-CoV-2 spike (S) protein or epitopes of it such as the receptor-binding domain (RBD) (Feng et al. 2020) gene therapy. An example of a viral vector vaccine is the rVSV-ZEBOV vaccine against Ebola. The recombinant vesicular stomatitis virus–Zaire Ebola virus (rVSV-ZEBOV) Ebola vaccine is currently the only vector vaccine that has been licensed and available for human use and only produced and used to a limited extent (Regules et al. 2017; Kennedy et al. 2017; World Health Organization 2019). A MERS-CoV vaccine (MVA-MERS-S_DF1) using modified vaccinia virus Ankara and expressing the spike (S) protein of MERS-CoV was evaluated in an open label, Phase 1 trial on 26 individuals aged 18–55. It showed a favorable safety profile without any severe adverse effects but induced only a relatively limited humoral and T-cell response to the MERS CoV (Koch et al. 2020). Reassuringly, the study showed that although vector specific neutralization antibody was elicited, the vaccine still elicited antibody responses against the transgene following booster immunization (Koch et al. 2020). Results of the Phase 1 clinical trial for an alternate vaccine, ChAdOx1 MERS vaccine that uses a replication-deficient simian adenoviral vector expressing the spike (S) protein in 24 individuals aged 18–50 years, showed that a single dose was able to elicit both humoral and cellular responses against MERS CoV. The majority of solicited and unsolicited adverse events (AEs) reported by participants were mild or moderate, and all were self-limiting, and there were no serious AEs related to vaccine administration, which supports progression into Phase 1b and Phase 2 trials (Folegatti et al. 2020).

In the context of viral vector-based vaccine candidates against SARS-CoV-2, a number of preclinical studies have been conducted (Lundstrom and Aljabali 2021). Among the 162 ongoing COVID-19 vaccine candidates, studies listed by the WHO (Folegatti et al. 2020), utilize viral vectors or virus like particles (VLPs). Adenovirus-based vectors have dominated the field including human Ad5 and Ad26 and the simian AdChOx1. For instance, in a preclinical study, the intramuscularly administered Ad5 vector was utilized for the expression of the codon-optimized SARS-CoV-2 spike protein (S) in rhesus macaques (Feng et al. 2020). The immunization with Ad5-S-nb2 elicited systemic S-specific antibody and cell-mediated immune (CMI) responses. Currently, four adenovirus vector vaccines are in widespread use. These are the products (in alphabetical order) of CanSino Biological Inc./Beijing Institute of Biotechnology, Janssen—Johnson & Johnson, Oxford–AstraZeneca, and The Gamaleya Institute Moscow.

19.3.3 Nucleic Acid Vaccines

This is a relatively new technology, which utilizes antigen/genetic material from a disease-causing virus or bacterium-encoding plasmid DNA or RNA, messenger RNA (mRNA), or viral replicons. The nucleic acid, once taken up by a cell, provides the instructions for making a specific protein from the pathogen, to which a humoral and cell-mediated immune response is expected to occur, similar to natural infection. Nucleic acid vaccines use (a pathogen) to stimulate an immune response against it.

So far, DNA and RNA vaccines are being developed against various diseases, including HIV, Zika virus, and COVID-19, but none of them have yet been approved for human use. Beside this, several DNA vaccines are licensed for animal use, including a horse vaccine against West Nile virus. Such vaccines have been trialed for veterinary infectious diseases and demonstrated immunogenicity, for example, for foot and mouth disease, deer Powassan virus, and rabies virus (Pulido et al. 2010; VanBlargan et al. 2018; Saxena et al. 2009). However, Phase I trials in humans are underway for nucleic acid vaccines against Ebola, influenza, and Zika virus (Rauch et al. 2018).

Currently, there are 4 DNA vaccines under clinical trials (World-Health-Organization 2020). Among these, Phase I clinical trials have been conducted on SARS-CoV and MERS-CoV DNA vaccine candidates. Phase I trial on SARS-CoV DNA vaccine INO-4800 was investigated in 10 adults which includes the use of a recombinant SARS DNA coding for the SARS-CoV N protein genome, developed by the National Institute of Allergy and Infectious Diseases (NIAID) (Martin et al. 2008), and MERS-CoV DNA vaccine (GLS-5300), developed by GeneOne Life Science/Inovio and coding for the full length S protein genome, which had a higher number of participants ($n = 75$) (Modjarrad et al. 2019). Both vaccines showed acceptable safety profiles and induced humoral and cellular responses; the MERS-CoV DNA vaccine has advanced into a Phase 2 clinical trials (ClinicalTrials.gov 2020). The only other SARS vaccine produced by Sinovac Biotech that has entered a

Phase I trial is an inactivated vaccine (ISCV) (Lin et al. 2007). There were no reports of human studies in which vaccinated subjects were challenged by the natural virus. The advantage of nucleic acid vaccines is the ease with which it allows antigen manipulation and the speed of production, as manufacturing can be synthetic and entirely cell-free so circumventing the need for BSL2 laboratories. The disadvantages are that nucleic acid, especially mRNA, is so feeble than DNA and needs a continuous cold-chain process for transport and storage (Zhang et al. 2019). mRNA-1273 (Moderna/US NIAID) is the first mRNA-based vaccine developed by Boston-based Moderna Therapeutics partnered up with the National Institute of Allergy and Infectious Diseases (NIAID) that entered clinical trials in 63 days after the genome sequencing of SARS-CoV-2 (Kyriakidis et al. 2021).

19.3.4 Protein Subunit Vaccines

Rather than injecting a whole pathogen to trigger an immune response, subunit vaccines (sometimes called acellular vaccines) contain purified pieces of viral proteins which are generated through recombinant synthesis and purification methods after cultivating large amounts of the pathogen, such as bacteria and yeast. Beside this, strict hygiene should be maintained to avoid contamination with other organisms. The main advantages of subunit vaccines are that these fragments are incapable of causing disease, thus considered very safe. Moreover, these are suitable for people with compromised immune systems. The disadvantage is that these vaccines explicit weaker immune response than with other types of vaccines. To overcome this problem, subunit vaccines are sometimes delivered alongside adjuvants (agents that stimulate the immune system) and booster doses may be required. Further, these vaccines are relatively complex and more expensive to produce than chemically synthesized vaccines, such as RNA vaccines. Yang et al. constructed a subunit vaccine composed of residues 319–545 of the SARS-CoV-2 RBD and produced it through the baculovirus expression system. The preclinical study reported that the vaccine could protect the nonhuman primates from SARS-CoV-2 infection with little toxicity (Yang et al. 2020). Several teams across the world are currently working on engineering protein-based vaccines; however, the clinical results have not been published to date.

19.4 Composition of Novel Corona Vaccine

A novel corona vaccine comprises tiny fragments of the disease-causing organism or antigen which may be a whole virus in attenuated or inactivated form, recombinant virus, nucleic acid (DNA/RNA), or protein subunit and other ingredients like adjuvants, preservatives, stabilizers, surfactants, and residual which keep the vaccine safe and effective. Each component of vaccine serves a specific purpose, and each

ingredient is tested for safety in the manufacturing process. Preservatives prevent the vaccine from becoming contaminated once the vial has been opened. The most commonly used preservative is 2-phenoxyethanol. Stabilizers prevent chemical reactions from occurring within the vaccine and keep the vaccine components from sticking to the vaccine vial. Stabilizers can be sugars (lactose, sucrose), amino acids (glycine), gelatin, and proteins (recombinant human albumin, derived from yeast). Surfactants keep all the ingredients in the vaccine blended together. Residuals are tiny amounts of various substances used during manufacturing or production of vaccines that are not active ingredients in the completed vaccine. Substances will vary depending on the manufacturing process used and may include egg proteins, yeast, or antibiotics.

19.5 Steps for Corona Vaccine Development

Vaccine development is a complex and lengthy process that has evolved and expanded considerably over the last few decades. Early on, the focus of the vaccine development process was the immunogenicity and efficacy of the vaccines, which were generally developed for diseases with significant burdens of morbidity, often with high mortality as well. Nowadays, greater emphasis is shifted on benefit-risk profiles of the vaccines under development. Moreover, economic evaluation of a new vaccine is also considered before its implementation (Roels et al. 2011). A viral pandemic requires rapid manufacture of effective treatments and vaccines. Billions of vaccine doses are required across the globe and the manufacturing of vaccines needs to occur at scale and pace while maintaining consistent high standards in quality. The first initial step in vaccine development is determining the burden of disease and defining the target population for a new vaccine. Disease burden is the impact of a health problem in a region or population, dependent upon the frequencies of the disease, the impact on quality of life (mortality and morbidity), healthcare resource use, and other indicators such as financial cost to society. A health problem or disease can have a relatively low incidence, but have a high case-fatality or case-disability incidence and treatment costs, resulting in a high burden of disease (Roels et al. 2011). The disease burden also differs greatly between the developed countries and developing world, due to differences in sanitation, healthcare, and other contributing factors, such as socioeconomic factors, access to preventive measures of communicable diseases, supportive care, and antibiotics. Funding is most important part in production of vaccine. Fund for Vaccine Procurement was developed by the Pan American Health Organization in 1979 for the purchase of vaccines, syringes/needles, and cold-chain equipment for countries in Latin America and the Caribbean. A major benefit of the fund's role has been to ensure access to vaccines and thereby significantly improve population health. This gives vaccine manufacturers a return on their development costs, followed by availability of the vaccine in the market at an affordable price. Governments of developing countries are able to budget and plan for immunization programs, knowing that vaccines will be available in

sufficient quantity, at a price they can afford, for the long term. Hence, for the production of a vaccine on large scale and in short time period requires a collaborative approach across pharmaceutical companies, governments and international organisations, medical professionals, policy makers, public or private health maintenance organisations.

Further, the World Health Organization (WHO) CHOICE (CHOosing Interventions that are Cost-Effective) project has the objective of providing policymakers with the evidence for deciding on the interventions and programs which maximize health for the available resources (Roels et al. 2011).

Vaccines have many challenges to overcome before they become licensed products. Each vaccine under development has gone through many steps before it can be introduced in a country's vaccine program. Generally, vaccine development is a long and complex process often lasting for 10–15 years. The process of vaccine production starts with exploratory work on design of vaccine and evaluation in animal models. This process is followed further in different phages in which more preclinical experiments are performed. The process of vaccine production is designed, and proper toxicology studies are carried out to check any toxic effect of vaccine; further, this stage can also last for several years. But, during the development of COVID-19 vaccines, the clinical trial phases are overlapped to speed up the process, so the vaccines could be used as quickly as possible to control the pandemic.

These steps for the production of new vaccine are broadly categorized into three stages: preclinical, clinical, and postlicensure development, which ensure the safety and immunogenicity/efficacy of the final licensed vaccine.

19.5.1 Preclinical Evaluation

Each vaccine under development must first undergo screenings and evaluations to determine which antigen should be used to invoke an immune response. Before a vaccine can be tested in humans, it is first tested in animals to assess reactogenicity and/or characterize further the action of the antigen and any adjuvant. At this point, the vaccine manufacturing process is also defined. Compulsory initial submissions are made to regulatory authorities, such as an Investigational New Drug (IND) application to the Food and Drug Administration (FDA) in the USA, in order to begin clinical development. Before initiation of human trials, pharmacological and toxicological effects of new vaccines must be assessed which include single-dose toxicity, primary pharmacodynamics, secondary pharmacodynamics, pharmacokinetics, and local tolerance of the experimental vaccine.

19.5.2 Clinical Evaluation

After initial development, vaccines go through three phases of clinical trials to make sure they are safe and effective. Prior to regulatory approval, a vaccine usually undergoes three phases of clinical trials in humans. But, during the development of COVID-19 vaccines, the clinical trial phases are overlapped to speed up the process, so the vaccines could be used as quickly as possible to control the pandemic. The clinical trials for COVID-19 vaccines have involved tens of thousands of volunteers of different ages, races, and ethnicities. Clinical trials for vaccines compare outcomes (such as how many people get sick) between people who are vaccinated and people who are not. Because COVID-19 continues to be widespread, the vaccine clinical trials have been conducted more quickly than if the disease was less common. Results from these trials have shown that COVID-19 vaccines are effective, especially against severe illness, hospitalization, and death.

19.5.2.1 Phase I

Phase I trials are short-term studies in which the vaccine is given to a small number of volunteers typically 30–100, to evaluate its safety, confirm its ability to induce an immune response, and determine the right dosage and preferred route of administration to achieve the effective immune response. Generally, in this phase, vaccines are tested in young, healthy adult volunteers who are at low risk of acquiring natural infection (WHO Technical Report 2004; European Medicines Agency, Committee for Medicinal Products for Human Use 2005; Hudgens et al. 2004).

19.5.2.2 Phase II

After the successful completion of Phase I trials, the vaccine is then given to several hundred volunteers to further evaluate its safety and ability to generate an immune response. Participants in this phase have the same characteristics (such as age, sex) and include the responses of such individuals who are at risk of acquiring the infection. There are usually multiple trials in this phase to assess immune response in various age groups, gender, and different formulations of the vaccine. Phase II trials usually include a comparator group that did not get the vaccine to determine whether the changes in the vaccinated group are attributed to the vaccine or have happened by chance (WHO Technical Report 2004; Hudgens et al. 2004; Farrington and Miller 2001).

19.5.2.3 Phase III

In Phase III trials, the vaccine is given to thousands of volunteers. These trials should be randomized, double-blinded, and placebo-controlled (the placebo may be a saline solution, a vaccine for another disease or some other substance). Phase III trials are typically designed to evaluate vaccine efficacy and its safety in large number of people (WHO Technical Report 2004; European Medicines Agency, Committee for Medicinal Products for Human Use 2005; Farrington and Miller 2001).

After the successful completion of Phase III trials, a biologics license application is filed with regulatory agencies (e.g., the United States Food and Drug Administration (FDA) or the European Medicines Agency). The licensing process can take another 1–2 years, especially if additional data are requested. Importantly, because it is very expensive, the overall process of vaccine development is slowed by economic risk assessment at every step. Vaccine development progresses through these stages only if the developer is convinced that the data are promising that the risk of failure is relatively low and that there is (still) a market for the vaccine.

In various cases, the production process of vaccines was simply adapted from the preexisting vaccines or vaccine applicants, and in certain cases, preclinical and toxicology data from related vaccines could be used. As a result, the first clinical trial of a vaccine candidate for SARS-CoV-2 began in March 2020 (NCT04283461). Trials were designed such that clinical phases are overlapping, and trial starts are staggered, with initial Phase I/II trials followed by rapid progression to Phase III trials after interim analysis of the Phase I/II data. Currently, several manufacturers have already started the commercial production of vaccines at risk without any results from Phase III trials. Although the licensure pathways are not yet completely clear, it is possible that reviews could be expedited and that vaccines could even be approved through an emergency use authorization. The FDA has released a guidance document for the development and license of SARS-CoV-2 vaccines, which as well as providing additional details states that an efficacy of at least 50% will be required (FDA 2020). It is very important to point out that moving forward at financial risk is the main factor that has enabled the accelerated development of SARS-CoV-2 vaccine candidates, and no corners have been or should be cut in terms of safety evaluation. Although vaccine development is moving forward at an unparalleled speed, there are still many open questions. It is likely that two doses of a vaccine will be required, with booster doses potentially necessary at later time points. In this case, at least 16 billion doses will be needed to meet the global demand. Many of the vaccines that are described below are being developed by entities that have never brought a vaccine to market or use technologies that have never resulted in a licensed vaccine. Therefore, unforeseen issues with scaling could cause delays. It is also not yet clear whether bottlenecks will occur in the availability of syringes or glass vials. Finally, for certain vaccine candidates against SARS-CoV and MERS-CoV, vaccine-enhanced disease was reported in some animal models. For SARS-CoV-2 vaccine candidates, there have so far been no signals of enhanced disease in animal models or in humans; however, such a safety signal would certainly derail the

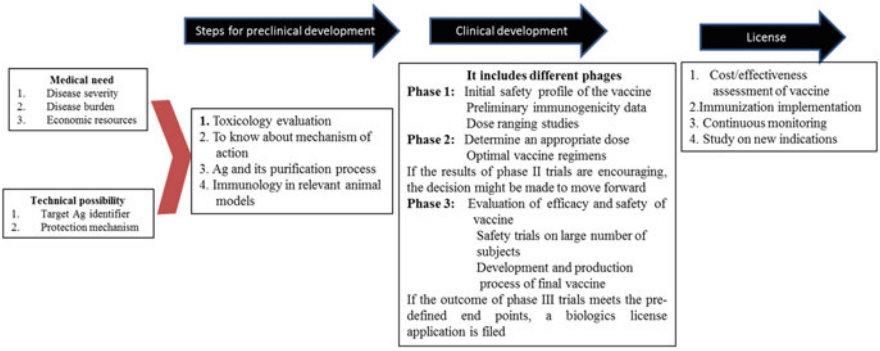

Fig. 19.3 Steps for vaccine development

development of a vaccine candidate and would negatively affect vaccine development in general (Fig. 19.3) (Krammer 2020).

19.6 Large-Scale Production of Vaccine

19.6.1 Manufacturing Steps

The most difficult, time-consuming, and resource-intensive aspect of vaccine development is to scale up the production of a vaccine from small amounts to commercial levels. For scientists, it is easy to work readily with vaccine in 1–10 L bioreactors in a bench-level laboratory. But, transferring the technology from laboratory to the pilot scale of 50–100 L volumes is not simple. It requires a close check on the behavior of the microorganisms, biochemical and physiological interaction, and the rate of yield to ensure that the product is equivalent to that developed in the laboratory. Hence, production of vaccine on large scale (550 liters or more) by well-established pharmaceutical firms is a quite challenging process. For example, the recent scale-up of a *Haemophilus influenzae* type b conjugate vaccine (Hib-CV) and a Hib-CV-diphtheria and tetanus toxoids and pertussis vaccine (DTP) combination was more difficult than anticipated (Siber et al. 1992). Siber et al. reported that many manufacturers of single-component Hib-CV noted reductions in the immunogenicities of their vaccines that appeared to coincide with the scale-up process itself (Siber et al. 1992). In these recent cases, sophisticated physical and biochemical characterizations of the vaccines and animal testing did not predict the reduced immunogenicity.

Considering viral vector vaccines as an example, once the required concentration of vaccine has been grown and extracted from the cells, to achieve a large volume of pure vaccine a series of key steps take place: filtration, membrane chromatography, and ultrafiltration.

Filtration—like sieving—removes unwanted residual products. This is achieved using special membranes with pores.

Membrane chromatography allows the vaccine to bind to a surface to ensure only the product that is needed is left.

Ultrafiltration "buffers" the vaccine to control how acidic or alkaline it is.

To ensure that the vaccine is readily acceptable and serves the purpose, there should be zero chances of errors happening. There are three main factors taken into consideration, while mass-producing any vaccine includes production of antigens and other biochemical compounds, adding adjuvants and enzymes for support, using DNA and mRNA technologies to develop a vaccine.

The shortcomings in vaccine supplies have inevitably led to a deflection of blame with vaccine manufacturers in the firing line. Questions over vaccine prices, manufacturing capacity, and the destination of supplies have beset the world's largest vaccine manufacturer. As the drug substance in production progresses to become the drug product that can be put into suitable containers and used, "fill and finish" takes place to transport the vaccine in multidose vials packaged into cartons. Virologists and pharmaceutical experts have said that the Indian government involved more companies in manufacturing to cope up with current COVID vaccine shortages.

19.6.2 Quality Control

Many methods of quality testing are used throughout the manufacturing and production process. These are designed to ensure high quality by the following:

- Checking that the vaccine carries the correct instructions to the body.
- Assessing vaccine purity.
- Measuring vaccine concentration to ensure the amount of vaccine required is present.

The next crucial step is to ensure consistency and quality of vaccine manufacture by pharmaceutical companies. It includes various key processes like testing of raw materials, conducting quality control tests throughout vaccine production, site visits and virtual tours by central personnel, electronic monitoring to ensure careful control of temperature for storage and transport and use of virtual technology to provide real-time technical support, and coaching for production teams on site. By putting these types of processes in place, vaccine production and manufacturing can progress rapidly without compromising safety, quality, or effectiveness.

19.6.3 Packaging

Once the vaccine has been made in bulk quantities, it is bottled in glass vials and then carefully packaged for safe cold storage and transport.

Vaccine packaging must be able to withstand extreme temperatures, as well as the risks involved in being transported globally. Therefore, vaccine vials are most commonly made from glass, as it is durable and able to maintain its integrity in extreme temperatures.

19.6.4 Storage

Most of the vaccines require refrigerated storage at between 2 °C and 8 °C because at too hot or too cold temperature, it becomes less effective or even inactive. However, some vaccines require temperatures as cold as −20 °C, but some of the newer vaccines need to be kept ultracold at −70 °C. Hence, specialized medical refrigerators are required for these precious products.

19.6.5 Shipping

Vaccines are shipped in the destination country by using specialized equipment that does not compromise the integrity of the product. From the warehouse, portable iceboxes are used to transport vaccines to regional centers or the place outside of the regional center, where they are stored in refrigerators. Nowadays, some portable devices are used that can keep vaccines at their cold temperature for several days without needing electricity.

Once vaccines start being administered, national authorities and WHO constantly monitor for and establish the severity of any possible adverse side effects and responses from people who have received the vaccine. The safety of the vaccine is paramount, with regular assessments and postapproval clinical studies to report on its safety and effectiveness.

19.7 Key Challenges to Scale Up Vaccine Production

Typically, a good workforce and more than 200 individual components are required for the large-scale production of a vaccine. The components include glass vials, filters, syringes, tubing, disposable bags, and stabilizing agents. These components are often manufactured in different countries, and hence, the travel restriction on

people and shortage of supply of any of the components may result in break of supply chain of the vaccine.

Another key challenge is to develop an affordable vaccine for low- and middle-income countries (LMICs). Several companies, such as AstraZeneca and Johnson & Johnson, which rely on public sector investments, have claimed to sell their vaccine globally at a low cost during the pandemic to improve accessibility. Indian manufacturers have stated that they have the capacity to meet the country's future needs for COVID-19 vaccines. The Indian government has taken urgent measures to expand the country's vaccine manufacturing capacity and has also developed an efficient digital system to address and monitor all the aspects of vaccine administration (Kumar et al. 2021). Serum Institute of India (SII), Pune, has signed agreements with a few manufacturers such as Oxford–AstraZeneca, Codagenix, and Novavax. It is now producing at a large scale, the Oxford–AstraZeneca adenovirus vector-based vaccine AZD1222 (which goes under the name "Covishield" in India), and it has stockpiled about 50 million doses (Voysey et al. 2021). Covaxin™ is an inactivated virus vaccine, developed in Vero cells. The inactivated virus is combined with Alhydroxiquim-II (Algel-IMDG), chemosorbed imidazoquinoline onto aluminum hydroxide gel, as an adjuvant to boost immune response and longer-lasting immunity (Kumar et al. 2021).

19.8 Overcoming Key Challenges Related to Vaccine Scale-Up

According to a special report published in *Nature*, researchers explain that to accelerate vaccine production, collaboration with multiple supply partners and analytical testing sites is essential in different countries. Recently, Martin Friede, head of vaccine development at the WHO, stated that WHO has recognized many organizations around the world which provide matchmaking services by connecting distributors of vaccine components and major manufacturing companies. For example, AstraZeneca has collaborated with multiple manufacturing companies across the world where each and every process is governed and technically guided by the company to support each stage of production.

In many organizations, across multiple industries, data sharing remains a key issue which can lead to the generation of data silos especially in pharmaceutical companies. Besides clinical trial data on vaccine candidates, reports on monoclonal antibodies and epidemiological modeling studies should be shared widely among biopharmaceutical companies in advanced countries as well as in LMICs (low- and middle-income countries). Therefore, pharmaceutical industry is taking steps toward solving the problem by "connecting" siloed data. Further, multiple technology transfer hub is established by WHO and its partners to scale up the vaccine production capabilities of LMICs (low- and middle-income countries).

Another key area that can help in the large-scale manufacturing of vaccine is optimization and quality checking. Environmental conditions, such as heat, light, and radiation, can affect the quality and purity of vaccines. Different technologies like polymerase chain reaction (PCR), high-performance liquid chromatography (HPLC), anion exchange chromatography (AEX), and affinity chromatography are being used to evaluate vaccine quality. To ensure stability, shelf life, and safety, researchers will continue to assess and refine storage and handling conditions, at each phase of the vaccine production. In the current scenario where production rate is extremely high, quality and stability testing are being carried out side by side with the manufacturing process.

In the process of meeting the unprecedented demand for COVID-19 vaccines, manufacturers whole over the world have had the opportunity to innovate and streamline vaccine production on a large scale. Such optimization may yield rich dividends to the manufacturing companies in the future.

19.9 Business Plan for Novel Corona Vaccine

With promising COVID-19 vaccines from companies like Pfizer, Moderna, and Johnson & Johnson now being distributed across the USA, business owners might want to start thinking about how their operations could change. The vaccines, which were approved for emergency use by the government in December 2020, will likely have far-reaching impacts on both employees and customers, so it is important to get planning.

The most important point for business is to start thinking how their employees align with the government's latest recommendations for receiving the vaccine first. Just because vaccines are available, it does not mean every eligible individual will be lining up to receive their doses. Employers have a unique role to play in encouraging widespread vaccination and dispelling any hesitancy among their workforce. To substantially reduce morbidity and mortality from COVID-19, an efficacious and safe vaccine must be delivered swiftly and broadly to the public as soon as it is available. However, the mere availability of a vaccine is insufficient to guarantee broad immunological protection; the vaccine must also be acceptable to both the health community and general public (Schafer et al. 2021). Vaccine hesitancy is a major barrier to vaccine uptake and the achievement of herd immunity, which is required to protect the most vulnerable populations. Depending on varying biological, environmental, and sociobehavioral factors, the threshold for COVID-19 herd immunity may be between 55% and 82% of the population (Sanche et al. 2020).

Given that certain individuals will be ineligible for COVID-19 vaccination due to age, immune compromise, and other preexisting medical conditions, a vaccine refusal rate greater than 10% could significantly impede attainment of this goal. Recent surveys, that included 493 and 2200 individuals, suggest only 3 in 4 people would get vaccinated if a COVID-19 vaccine were available, and only 30% would want to receive the vaccine soon after it becomes available (Trujillo and Motta

2020). Confidence in vaccines lies along a spectrum, and individuals who have hesitation about routine childhood vaccines have expressed various concerns (Edwards and Hackell 2016). In their report on vaccine hesitancy, Edwards and Hackell identified 3 broad categories of parents' concerns regarding childhood vaccines: (1) the necessity of vaccines, (2) vaccine safety, and (3) freedom of choice (Edwards and Hackell 2016). This viewpoint describes these categories of concerns with regard to a future COVID-19 vaccine and presents suggestions to enhance the likelihood of rapid, widespread vaccine uptake in the United States.

Frontline healthcare workers will play a central role in encouraging COVID-19 vaccination. Many studies have found that physicians are the most important influencers of vaccine decision making (Edwards and Hackell 2016). Thus, strong physician recommendations can bolster public and individual support for a COVID-19 vaccine. Physicians who share personal anecdotes about being immunized and immunizing their family members are effective in encouraging vaccine uptake in vaccine-hesitant families (Kempe et al. 2015). As such, achieving a high vaccination coverage level of healthcare workers early on not only ensures an adequate workforce to treat infected patients, but also allows medical authority figures to share their positive vaccination experiences with patients. While most studies have focused on the role of physicians, the influence of nurses and allied health professionals on patients' vaccination attitudes and beliefs also is important. Healthcare workers are exposed to the same media stories as the general public and may be subjected to the same cognitive biases that can lead to excessive reliance on anecdotal evidence and false conclusions. Ensuring that all individuals who interface with patients in the clinical setting are confident about the safety and effectiveness of a future COVID-19 vaccine is critical for presenting a unified message of strong vaccination support from the medical community.

19.10 Conclusion

In India, free vaccination against COVID-19 has begun on January 16, 2021, and the government is advising all of its citizens to be immunized and is expected to be the largest vaccination program in the world. India, which has a robust vaccine development program, not only plans for domestic manufacture of COVID-19 vaccine but also plans for the distribution in countries that cannot afford to buy vaccines. While obtaining the vaccine is the first requirement, distribution and vaccination of the huge Indian population present a significant logistic challenge. The purpose of this perspective was to highlight the overall crux of the vaccine development and vaccination strategies that were implemented during a pandemic in a densely populated country. This report can be viewed as a baseline document for future pandemic preparedness and to effectively tailor and refine the strategies that will help the population at large. These data analyses can hold the keys to the future effective public health management of COVID-19. India's experience in

immunization for COVID-19 offers tips for strategy preparation, not only for countries with similar economic strength and health facilities but also for the world at large.

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