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Ahmet Aksoy · Muhammad Sajid Aqeel
Ahmad *Editors*

# Phytoremediation for Green Energy

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# Chapter 1
# Energy, Environment and the Future of Mankind

**Yuan Tseh Lee**

**Abstract** Charles Darwin who was born 200 years ago did remind us that "It is not the strongest of the species that will survive, or the most intelligent; it is the one most adaptable to change." If the environment changes faster than the time required for a given species to evolve, the likely result will be extinction. With the fast changing climate and rapidly deteriorating ecosystem today, human species, with a life cycle of 30–40 years, are not likely to evolve and adapt as quickly. Unless humans manage to slow down the change of environment, the fate of extinction might be inevitable.

**Keywords** Energy crisis · Environmental degradation · Energy efficiency · Future of mankind

## 1 Recent Development of Human Society on Earth

After the appearance of our ancestor on the heavily forested planet a couple of million years ago, the development of the human society as a whole, was in harmony with nature. Mankind was indeed a part of nature, reliant on the sun for the creation of most of what was needed to survive. Since the population of mankind was small, for a long period of time their limited activities seemed to have affected neither the biosphere nor their living environment to any great extent.

The development of mankind took a new turn after the industrial revolution, which began about 250 years ago. As mankind learned to transform energy from one form to another—from chemical, thermal, electrical to mechanical—and invented various machines that could perform work thousands of times more powerfully, more precisely and more reliably than could be possibly done with human and animal labor, the productivity of mankind increased immensely and an unprecedented improvement of living standards was achieved. The success of mankind on the surface of the earth had been quite remarkable. But, during this process, mankind became addicted to the use of a large amount of energy, and since the energy from biomass created by sunshine no longer satisfied our need, we began to

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depend more and more on fossil fuels—coal, natural gas and petroleum—which were buried under the ground and took millions of years to accumulate. In USA in 1850, 90% of the energy depended on wood burning, but 80 years later, by 1930, 90% of the energy came from the combustion of fossil fuel. Fossil fuels also provided energy and feed stock needed for the production of various new materials, such as plastics, fertilizer, synthetic fibers, steel and cement, and regrettably man had drastically changed the intimate relation between man and the nature. Harmonious relations between man and the biosphere was disrupted, and the important role played by the sun in the development of mankind, or the philosophical view of Confucius: "Man and Nature are but one", somehow seemed to have been forgotten.

As we entered the twenty-first century, we began to realize that the current development patterns of human society are not sustainable. Problems related to population explosion, natural resource depletion and the damage done to the living environment have become quite serious. In a sense, the earth was once regarded as "infinite" or "unlimited" for mankind, not only because of the resources available, but also due to the ability of the earth to digest all the waste produced by mankind. However, from the point of view of the damage done to the ecosystem or the living environment, the earth as a whole should be considered "limited" and "overdeveloped" at present. For example, carbon dioxide produced by human activities is far exceeding the capacity of the earth to absorb through the growth of the forest or coral reefs and other mechanism, and the global warming trend is threatening the very existence of human beings on earth. It is quite ironic that during the twentieth century, not only are the "developed" countries overdeveloped, the so-called "developing" countries are also overdeveloped. It is unfortunate that the so-called "developing" countries are following in the footsteps of the "developed" countries, and marching along the unsustainable path established by "developed countries" in the past, when the earth was still "unlimited".

It is extremely important for mankind to wake up immediately and accede to the fact that human society as a whole is living beyond its means. We must learn to work together as a community to find new, sustainable ways to re-establish an intimate relation with the biosphere, live in harmony with nature, and to return to a more direct relationship with the mighty power of the sun. After all, it was the sun that brought us altogether here on the surface of the earth.

## 2 Issues on Energy and Environment

One of the most urgent problems man faces today is the problem related to the relationship between energy and the environment, especially the global warming trends caused by the emission of greenhouse gases, and the energy crises caused by the widening gap between the limited supply and rapidly growing demand for petroleum and other fossil fuels. The other problem, which menaces to wipe out large portions of humanity in a short time, is the spread of infectious diseases, like those caused by virus $H_5N_1$.

It is comforting to know that, at present, energy received by the surface of the earth in one hour is approximately equal to the total energy consumption of the entire world in a year. In other worlds, the amount of energy the surface of the earth received is approximately ten thousand times the energy consumed by human society. It means that if we were clever enough, we can depend entirely on solar energy. For example, if an inexpensive and practical photovoltaic cell, that converts 10% of solar energy to electricity becomes available, it will only take 1% of the planet's land area to generate enough electric energy to satisfy the energy needs of the entire world. If the electrical energy generated by a photovoltaic cell could be effectively stored or used to electrolyze water into hydrogen and oxygen—or to even more directly dissociate water by using a combination of photovoltaic cells—it is not inconceivable that countries with large land masses could become energy exporting countries, nor that hydrogen gas might then become a major energy source as we enter the age of the "hydrogen economy". If we learn to develop biofuel more efficiently, or to invent efficient "artificial leaf", photosynthesis might provide enough biomass on earth to satisfy the need for liquid fuel and other chemical feed stocks now provided by petroleum.

To make it possible for the world to achieve sustainable development, we must do the following things to reduce our dependence on fossil fuel.

1. Increase of our energy efficiency, and improve the recyclable usage of materials.
2. Develop efficient renewable energy sources, e.g. photovoltaic cells, wind power generators, geothermal, ocean current and thermal energy conversion, and various biofuels.
3. Develop a new generation of safe nuclear reactors and appropriate waste disposal technology and fusion reactors.
4. Examine our population policies and the way of life. We need to learn to live simpler and more frugal lives.
5. Protect our living environment and ecosystems, and maintain biodiversity.

Although our current scientific knowledge and technology enables us to get it started, there are many challenging scientific problems awaiting a solution. For example, in photosynthetic processes, most of the solar energy is stored in the fiber plants rather than carbohydrates. Although the production of alcohol from sugar cane or corn has been effective and successful, the challenge lies in the effective production of alcohol from fiber through hydrolysis and fermentation. For harvesting energy from geothermal, ocean flow and thermal energy conversion, new engineering technologies need to be developed.

With concerted efforts, which include the development of various renewable energies, changes to our way of life and social structure, 40–50 years from now, we could become largely free from the use of fossil fuels. We will be again like our ancestors, directly reliant on the power of the sun; perhaps supplemented by new generations of nuclear fission reactors or micro suns, in the form of fusion reactors.

But during the transition period of the next 30 years, especially before fusion reactor becomes successful, while nuclear fission reactors still play a role, we

probably will continue to depend on coal to a great extent, and the sequestering of $CO_2$ will remain a problem in need of a solution.

In recent years, the long neglected development of vaccine for infectious disease is finally picking up some momentum with international efforts. The race lies between the perfection of $H_5N_1$ vaccine and the mutation of $H_5N_1$ virus, which initiate the transmission from people to people. More research works need to be carried out in this area. However, we do have to pay attention to the fact that in the past, the funds spent for medical research globally has only been targeted at problems related to 10 % of the population. It is quite obvious that if we do not pay more attention to the deteriorating situation in developing countries, there is no way we can combat infectious decease effectively.

## 3 The St James's Palace Memorandum

In May 2009, after the St James's Palace Symposium of Nobel Laureates in London, a memorandum was issued which calls for "Action for a Low Carbon and Equitable Future", with the following content.

> The St James's Palace Memorandum calls for a global deal on climate change that matches the scale and urgency of the human, ecological and economic crises facing the world today. It urges governments at all levels, as well as the scientific community, to join with business and civil Society to seize hold of this historic opportunity to transform our carbon-intensive economies into sustainable and equitable systems. We must recognize the fierce urgency of now.

### *3.1 The Fierce Urgency of Now*

Climate risk avoidance, energy security, sustainable land use, population growth and equitable economic development constitute a key set of interacting challenges for humankind in the twenty-first century. The evidence is increasingly compelling for the range and scale of climate impacts that must be avoided, such as droughts, sea level rise and flooding, leading to mass migration and conflict. The robust scientific process, by which this evidence has been gathered, should be used as a clear mandate to accelerate the actions that need to be taken. Political leaders cannot possibly ask for a more robust, evidence-based call for action.

In a time of financial and economic crisis, the participants of the St James's Palace Symposium emphasize that without directing current economic recovery resources wisely, and embarking on a path towards a low carbon economy, the world will have lost the opportunity to meet the global sustainability challenge. Decarbonising our economy offers a multitude of benefits, from addressing energy security to stimulating unprecedented technological innovation. A zero carbon economy is an ultimate necessity and must be seriously explored *now*.

## *3.2 Milestones of the Great Transformation*

Building on the Potsdam Memorandum and recent advances in the scientific understanding of climate change, the participants of the St James's Symposium identified as key requirements **an effective and just global agreement on climate change, low-carbon energy infrastructure and tropical forest protection, conservation and restoration.**

### 3.2.1 Delivering an Effective and Just Global Agreement on Climate Change

Firm political leadership is *now* crucial. Leadership is primarily required from developed countries, acknowledging their historical responsibility as well as their financial and technological capacity. However, all countries will need to implement low carbon development strategies. *In this spirit of trust, every country must act on the firm assumption that all others will also act*.

A long-term commitment under the United Nation Framework Convention on Climate Change (UNFCCC) is now urgently required. The global agreement in Copenhagen must include the following elements:

1. Acknowledging the compelling evidence of science, we should confine temperature rise to 2 degrees Celsius to avoid unmanageable climate risks. This can only be achieved with a peak of global emissions of all greenhouse gases by 2015, and at least a 50% emission reduction by 2050 on a 1990 baseline. This in turn means that developed countries have to aim for a 25–40% reduction by 2020. A robust measure of assessing the necessary emission reductions is a total carbon budget, which should be accepted as the base for measuring the effectiveness of short-term (2020) and long-term (2050) targets;
2. The creation of carbon prices adopted across large parts of the global economy combined with measures to lower the price of low carbon energy, especially in developing countries. Funds raised should be used to provide the necessary financial support for adaptation;
3. The agreement must acknowledge the priority of developing countries to overcome poverty while ensuring sustainable development.

### 3.2.2 Delivering a Low Carbon Energy Infrastructure

Decarbonising our society requires an increase in energy conservation and efficiency, and a revolution in our energy infrastructure *now*. "*The required technological innovations will not be achieved without an unprecedented partnership between government and business*".

Actions in the following areas are needed:

1. Clear policy frameworks aimed at fostering innovation and the demonstration, scale up and roll out of low carbon technologies, including globally coordinated investment frameworks linked to economic recovery, with the emphasis on ‘green growth’;
2. Developed countries should commit to a significant increase in investments for research, development and deployment;
3. Technology sharing and financial support, through mechanisms such as globally supported feed-in-tariffs for renewable energy, are required to help developing countries leapfrog to a low carbon economy;
4. The establishment of “smart grids”—connecting renewable energy sources over large areas and implementing novel energy storage technologies.

### 3.2.3 Delivering Tropical Forest Protection, Conservation and Restoration

Tropical forests provide the ecosystem services essential for human well-being and poverty alleviation. In addition, deforestation and forest degradation are substantially contributing to climate change and global biodiversity loss at the genetic, species and landscape level. Both locally and globally, protecting boreal and tropical forest cover is an essential tool for mitigation of, and adaptation to, climate change. *Without a solution to rainforest protection, there is no solution to tackling climate change.*

An emergency package is needed *now* to provide substantial funding to tropical forest nations to help them halt deforestation and embark on alternative economic development paths, including:

1. Accelerating a long-term UNFCCC agreement on halting deforestation and on forest restoration, including innovative financing mechanisms from public and private sources;
2. Building capacity as well as mechanisms for verification and national governance structures that can support and reward the maintenance of rainforest regions. Developing countries need to take their own responsibility in tropical forest protection, conservation and restoration.

## *3.3 The Contribution of Science*

The solutions to the extraordinary environmental, economic and human crises of this century will not be found in the political arena alone. Stimulated by the manifesto of Bertrand Russell and Albert Einstein, the first Pugwash gathering of 1957 united scientists of all political persuasions to discuss the threat posed to civilization by the advent of thermonuclear weapons. Global climate change represents a threat of similar proportions, and should be addressed in a similar manner. There should be an acceleration and integration of global sustainability studies, to encourage the

active involvement of *all* scientists in these matters, championing the process of robust scientific study. All scientists should be urged to contribute to raising levels of public knowledge on these threats to civilization, and engage in a massive education effort to popularize the principles in this Memorandum.

> We know what need to be done. We cannot wait until it is too late.
> We cannot wait until what we value most is lost.

What is stated in this Memorandum is extremely important and worth paying great attention to by all.

## 4 Dilemma of Living in a Partially-Globalized World

Although we have witnessed the globalization of human society during the last few decades, the process is only half complete, and because of this, we are suffering from the consequences. Owing to highly-developed transportation and communication technologies, our world is relatively shrunken than it once was, and it appears that the concept of global village is slowly taking root as a number of human activities, most notably in the economic sphere, become globalized. The spread of disease around the world is another example. With thousands of airplanes daily crossing oceans and continents, loaded with people and goods, disease causing bacteria, viruses, and other microbes certainly will not be confined to specific locations. Similarly, environmental problems such as the depletion of the ozone layer by chlorofluorocarbons, and global warming trends caused by greenhouse gases, are problems that must be addressed on a global scale. On the other hand, in spite of the increased international collaboration in the areas of science and technology, high-tech based economic competition is still largely carried out on a national basis. Currently, in the partially globalized world, it is quite clear that only those people who are able to stage their activities on a global scale are benefiting enormously. For that reason it is not surprising that we will have to tackle such problems as the widening gap between rich and poor, both among countries and people in a country, nor that threats to solve problems by military force have not disappeared. These problems might be avoided if the entire world were to become "one community".

We should also realize that, though the globalization of the world economy is driving us toward a borderless society, it will not reduce the differences among peoples in various regions overnight. The establishment of a new, common global culture, together with more effective ways of communicating among all peoples, will certainly take time. The differences among cultural heritages, languages, and religions that make this world so rich and colorful will not, and should not, be made to disappear. As the world shrinks in relative terms, and contact between peoples becomes more frequent, whether or not the difference in civilization are likely to cause an inevitable crash (as suggested by the well-known scholar Huntington), would seem to be entirely dependent on how well people around the world learn to communicate and to understand, appreciate, and respect cultural heritage. To

become good citizens of the global village, we need to learn quickly and also to teach our young people to take a global view and to respect, appreciate, and understand the different cultures of different peoples. In this aspect, scientists certainly can lead the way.

## 5 Science and Technology in Society Forum in Kyoto

In the fall of 2004, Mr. Koji Omi, the former Minister of Finance of Japan, organized a very important annual forum in Kyoto, with the title of "Science and Technology in Society forum". More than six hundred leading scientists, business leaders and policy makers were invited every year from all over the world to discuss problems related to the subject matter of the forum. The forum aroused great enthusiasm among participants, and has since become a very successful and important annual event. During the past October, the fifth forum was held with more than 600 attendees.

Mr. Omi made two important points when he described the fundamental concept of this forum in the opening ceremony of the first forum. He mentioned positive and negative aspects of the rapid progress of science and technology, and noted that the benefits of science and technology have not yet reached everyone equally, which, as he said, "is really what symbolizes the lights and shadows of science and technology." While the negative aspects must be properly controlled, the positive features of science and technology should be promoted.

Mr. Omi's other important point was stated thus: "Today's problems are global and can not be solved by any single country or by scientists alone." He went on saying that "Boundaries between nations are merely lines on a map; nature makes no such distinctions. We should think of ourselves as members of humankind, whose very existence will be at risk if we do not live in accordance with the principles of Mother Nature." Indeed, as an astronaut observes the beautiful earth from the spacecraft, the astronaut will not find any national boundaries.

I believe most of us sitting in this room would support this idea without hesitation. However, if we do not try to answer some other questions related to the fact that the earth is "limited" and the world is only "partially globalized", our efforts to find solutions might encounter some difficulties. For example, we must also ask, "How many people can the planet support if we were to extend the living standard of the people in the so called "developed countries" to everyone on earth?" It is interesting to note that when India became independent, in response to the question of how the people in that country could catch up with the living standard of the people in Great Britain, Gandhi, rightfully recognized that it would take the natural resources of many Planet Earths, if the people in India were to have the British way of life. It is just impossible.

If we do not fully appreciate and understand the boundary conditions of the earth, the rules of the game and the consequences of competition in a globalized market-driven economy, practicing the so called "good sciences" for the greater

good can still produce miserable losers among us, when these "good sciences" are used mainly as a tool for global economic competition, especially when science and technology are used for the domination of some countries over others. Scientists as a whole should take full responsibility to ensure that science and technology bring benefits to everyone equally. If we are not careful, we might predict that, even if science and technology were to advance in faster pace along with excellent material comforts and improvement in healthcare, the continuing population explosion and excessive usage of natural resources might overload the planet, and then sustainable development might not be possible.

## 6 Concluding Remarks

Many of the problems we face today are problems that cannot be solved with current scientific knowledge and technologies—they await the accumulation of new knowledge and the development of new technologies. That is why it is so important to continue our efforts for the advancement of science and technology, and for the education of a new generation of creative scientists.

During the long history of mankind, our ancestors invented various technologies in order to survive better or to improve the quality of life. Their curiosity and their desire to understand natural phenomena were the basis of the advancement of science. Until about one hundred years ago, the advancement of science was driven by the available technology; only during the last century have technological advances been led by the results of scientific research.

In recent years, we have observed encouraging improvement in international scientific collaboration. Many projects were initiated, many agreements were signed. Year after year, we have discussed the "capacity building" of science, technology and education for developing countries, but the worsening situation of the entire world has yet to find its turning point. For example, the rain forest, which is often compared with the lung of a human body, is continuing to disappear from the surface of the earth. For the past decade, every summer we witnessed the thick dark smog generated by the forest fire in Indonesia contaminate not only the air in Indonesia, but also their neighboring countries. It is not realistic to blame or to expect Indonesia to be able to keep their rain forest from disappearing. Unless we consider the protection of the rain forest in Indonesia as "our responsibility", and raise enough funds to help Indonesia establish a protected "global rain forest", no matter how serious we engage in international scientific collaboration, the rain forest will continue to disappear.

We should all recognize the fact that the increasingly interconnected world cannot be a safe place if a large portion of its population still suffers from grinding poverty, disease stricken, illiteracy, derived of education, unemployment, and other barriers to survival. Scientists can play key roles in finding solutions to these problems. Especially if we learn to solve problems together, learn to share knowledge, new technological options and the limited resources available, learn to respect and

understand different cultural heritages, then it will be possible to realize the establishment of a genuine global village that enables sustainable development for all.

In order for science and technology to solve the problems man faces in the twenty-first century, it is not enough to advance science and technology at a faster pace. The advancement of science and technology certainly will solve many problems we are facing today and will also shape the development of human society of the future. However, unless we pay special attention to the roles play by science and technology in this "finite" and "half-globalized" world, and learn to work together beyond the national boundaries, and pay more attention to our "global competitiveness" for solving problems of the entire world, rather than continue to worry about "national competitiveness" for their own countries, the serious problems related to the sustainable development will not be solved.

At present, the entire world consists of more than one hundred nation-states. One of the duties of the government of a nation-state is to collect taxes from their citizen and business to solve their problems and redistribute wealth. As the world became more and more globalized, it became obvious that there is a need to have some sort of a "global government", which can resolve conflicts between the interests of nation- states, and the interest of the entire world.

The best way to work together beyond national boundaries is to make national boundaries disappear all together. Although it might take a long time, our future certainly will depend on how soon all of us in different countries learn to operate as "one community" for the entire world, and we do not have much time to waste. Perhaps the European Union is a step toward that direction, and half way through the twenty-first Century, the formation of the "Global Union of the Planet of Earth" might become a reality, then the sustainable development of the entire world might become possible. Otherwise, the solar system might send a farewell message to mankind on earth in the not too distant future.

# Chapter 2
# Bio-fuels: A Blessing in Disguise

**O. Surriya, Syeda Sarah Saleem, K. Waqar, A. Gul Kazi and M. Öztürk**

**Abstract** Biofuels are part of the bio energy family that can be transformed into fuels for both mobile as well as stationary incentives. Bio-fuels obtained from various forms of bio-mass are considered environmentally safe and economically efficient candidates for complete replacement of natural oil in the twenty-first century. Depending on their future accessibility, geologists categorize bio-fuels into three generations, namely; first, second and third. According to research analysts, energy demand will increase with alarming celerity until late 2020–2030, up to more than 50%. Because of the emerging economies of the developing countries in recent years, energy consumption will directly enhance the demand for renewable, cost effective energy generation sources. The depleting life expectancy of natural fossil fuels in the world market has led research institutes, policy makers and enterprises to discover alternative means of generating transportation fuel. One such prominent and promising alternative is "Biofuels" which not only contributes to diminishing the increasing bubble of global warming but also generates substantial amount of energy in a less cumbersome manner.

**Keywords** Biofuel · Biodiesel · Bioethanol · Biomethanol · Biohydrogen · Algae biofuels

## 1 Introduction

Biofuels refer to combustible materials which are directly or indirectly derived from biomass. Most common biomass includes wood, agricultural crops, aquatic plants, forestry products and animal wastes (Keck 2001; Öztürk et al. 2006, 2007, 2010; Öztürk 2010; Abideen et al. 2011). Biofuels are part of the bio energy family that can be transformed into fuels for both mobile as well as stationary incentives. These

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**Table 2.1** Detailed classification and characteristics of biofuels on generation basis

| Biofuels | Basic technology | Feedstock |
|---|---|---|
| *First generation* | | |
| 1. Bio-diesel | Transesterification of fats and oils | Rapeseed, sunflower oil soya |
| 2. Plant oils | Transesterification, pyrolysis, micro emulsification | Coconut oil, jojoba oil, sunflower oil, rapeseed oil |
| 3. Bio-gas | Microbial metabolism via fermentation | Energy crops, waste material, proteins, carbohydrates, cellulose, fats |
| *Second generation* | | |
| 1. Bio-alcohols | Breakdown of cellulosic biomass in several steps including fermentation and hydrolysis | Wheat, wood, sugar cane and bagasse |
| 2. Wood diesel | Fischer tropsch process, gasification | Woody biomass |
| 3. DMF | Conversion of carbohydrates to DMF via acid catalyzed dehydration process reaction | Lignocellulosic biomass, waste biomass carbohydrates |
| *Third generation* | | |
| 1. Algal fuel | Use of bioreactors, pyrolysis, transesterification | Marine or fresh water micro algae |

can be in the form of solid, liquid or gases. Wood, charcoal and bagasse (sugar cane after juice extraction) are some useful examples of solid biofuels which are extensively consumed for domestic purposes such as cooking in rural areas of most third world countries. Waste bagasse; the fibrous material obtained from the processing of sugar cane is widely used for power generation in raw sugar mills. Liquid biofuels like ethanol, methanol, plant oils and methyl esters produced from these oils are biodiesel, whereas methane and producer gas are forms of gaseous biofuels. According to the research analysts, energy demand will increase with alarming celerity up to more than 50% between 2020 and 2030. Because of the emerging economies of the developing countries in recent years, energy consumption will directly enhance the demand for renewable, cost effective energy generation sources.

The depleting life expectancy of natural fossil fuels in the world market has led research institutes, policy makers and enterprises to discover alternative means of generating transportation fuel. One such prominent and promising alternative is “Biofuels” which not only contribute to diminishing the increasing bubble of “Global Warming” but also generates substantial amount of energy in a less cumbersome manner. Biofuels are categorized according to their current and future availability as first, second or third generation biofuels as shown in Table 2.1. First generation biofuels are manufactured using conventional technology. Starch, sugar and vegetable oils are the main substrates used to provide the synthesis of first generation bio-fuels. Some common examples of first generation biofuels include biodiesel, biogas and vegetable oils. Second generation biofuels are considered more preferable over the first generation biofuels, since they are obtained from biomasses produced by non-food crops, cellulosic materials like wood and waste materials of animals. Wood diesel, dimethyl ether and bio-alcohol are a few examples of second generation biofuels.

Third generation biofuels are relatively the cheapest and most energy producing of all the three types of biofuels. Algal oil obtained from algae is utilized for the synthesis of third generation biofuels. They may also be called as oilgae.

## 2 Historical Perspective

The use of biofuels dates back to the early sixteenth–seventeenth century. Humans have consumed ethyl alcohol since before it was chemically discovered. In prewar America the same alcohol was utilized as fuel for lightening a lamp. In the World Fair of 1900 in Paris, biofuel in the form of peanut was used for the very first time as engine fuel by Rudolf Diesel; since then the French government has been exploring the possibilities of bio oils for the generation of transportation fuel.

Henry Ford, an American industrialist and the founder of Ford Motor Company, made his first automobile using ethanol in 1916. America, one of the earliest growing economies of the world began working on promoting bio-alcohols during mid-nineteenth century. Later towards World War II, when scarcity of fuel hit the military camps, ethanol was considered as a viable alternative. But it was not until 1970 that bio fuels successfully captured the lime light for being the second most preferable source of engine fuel after diesel. American Congress passed the Energy Tax Act of 1978 that enforced incentives and economic subsidies for the development of ethanol. Much to automobile industries amazement, The Clean Air Act Amendments of 1990 and Energy Policy Act of 1992 issued a compulsive authorization for the use of ethanol fuel in trucks and bus fleets. These laws were the catapult force behind expanding the popularity of biofuels.

## 3 First Generation Biofuels

### *3.1 Bio Diesel (FAME)*

Bio diesel is a fuel encompassed with mono alkyl esters derived from vegetable or animal fats, of long chain fatty acids. It is a renewable transportation fuel consisting of fatty acid methyl esters (FAME), mainly produced by trans-esterification of vegetable oils and animal fats (Wang et al. 2006). Engine carbon monoxide emission is reduced when biodiesel proportion in diesel fuel is increased. For example using the blend B50, the carbon monoxide emission is lessened to 31% compared to the neat diesel fuel (Wang et al. 2006).

The interest in biodiesel production increased because of some important reasons:

1. Concerns to reduce greenhouse gas emission for maintaining a stable climate.
2. A desire for renewable energy source.
3. Interest in developing domestic and more secure food supplies (Vyas et al. 2010).

**Table 2.2** The feed stock types with their examples used for biodiesel production

| Feed stock type | Examples |
|---|---|
| Plant oils | Cottonseed, jatropha oil, soya bean |
| Animal fats | Tallow, lard, grease |

Some of the advantages of biodiesel are that it is a renewable energy source, can decompose easily under natural conditions, has high combustible value, is safe, easily transportable and less toxic as it has less sulphur compounds in it.

The disadvantages include high viscosity, high surface stress, easily subjected to oxidation and expensive raw material used for its production (Wang et al. 2006).

### 3.1.1 Feed Stocks Used for Bio-diesel Production

Biodiesel can be produced by using various feed stocks. The main biodiesel feed stocks are classified into three types; plant oils, animal fats and waste cooking oils with industrial wastes. Table 2.2 presents some examples of plant oils and animal fats used in this connection. Each country develops feed stock for biodiesel production in accordance with their geographical conditions. The United States generally uses soya bean which is genetically modified while Canada and other European countries use rapeseed to produce biodiesel. Indonesia and Malaysia own plentiful palm oil so they use it for biodiesel formation (Vyas et al. 2010).

China is a big agricultural country because of which it faces a lot of problems regarding food supply. In order to produce biodiesel they use a principle by which they never get to compete with food grains as they use waste cooking oil for biodiesel production.

### 3.1.2 Bio-diesel Production via Trans-esterification

The main chemical composition of animal fats along with vegetable oils is triglycerides. The industrial method used for the production of biodiesel is trans esterification also called alcoholysis (Wang et al. 2006). Trans esterification is a process which involves a reaction of an oil or fat with an alcohol (with or without using a catalyst) to produce esters and glycerol. This reaction is extremely reversible so additional amount of alcohol is used to shift equilibrium towards the product production side (Fukuda et al. 2001). *Jatropha* is a good source of biodiesel formation as it grows on waste land with really less amount of water and minimum need of fertilizer. This oil is non-edible because of toxic phorbol esters (Tan et al. 2010).

This reaction takes place in three steps. These three steps are shown in Fig. 2.1. It involves the alteration of triglycerides as a result of which diglycerides are formed, which is trailed by transformation of glycerol through various high glycerides. The whole process yields one methyl ester molecule from each glyceride at every stage. This reaction proceeds by mixing the reactant and it may accelerate with the presence of a catalyst (Fukuda et al. 2001).

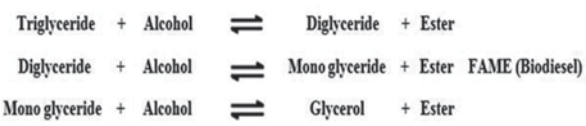


**Fig. 2.1** The three main steps of trans esterification

There are various types of trans-esterification reactions. Some of these are:

i. **Homogeneous alkali catalyzed trans-esterification:**
Trans-esterification can be catalyzed by using homogeneous and heterogeneous catalyst. This is carried out in the same three steps. The alkali catalyzed reactions for trans-esterification are faster than acid catalyzed reactions (Vicente et al. 2004)

ii. **Homogeneous acid catalyzed trans-esterification:**
The liquid acid catalyzed reaction of trans esterification is not popular as compared to base catalyzed reaction. This process is about four thousand times slower as compared to base catalyzed reaction. The performance of this process isn't affected much due to the presence of free fatty acids in feed stock. It can catalyze both esterification and trans esterification. The great advantage of using this process is direct formation of biodiesel from low cost lipids feedstocks, having high free fatty acid concentration which is greater than 6%. The common catalysts for acid catalyzed reactions are hydrochloric acid, sulphuric acid, phosphoric acid, boron tri fluoride and organic sulphonic acid (Lotero et al. 2005).

iii. **Heterogeneous acid and base catalyzed transesterification:**
The former reaction has greater performance for trans-esterification to gain bio diesel but they consume high amount of energy. Heterogeneous catalyst in comparison utilizes less energy, can be separated from the product more easily, avoids undesired saponification reactions and can use high free fatty acid feed stocks. Biodiesel synthesis by using solid catalyst can lead to a cheap manufacture cost because the catalyst can be re-used and esterification can be carried out along with trans esterification simultaneously (Di Serio et al. 2007; Garcia et al. 2008).

iv. **Enzymatic trans esterification:**
Enzymatic trans esterification using lipase has promising outcomes such as ease in product separation, negligible water surplus requirements, ease in glycerol regaining and nonexistence of side responses. It also faces some difficulties like infectivity of product due to left over enzyme action and market price. To fix these problems enzyme is used in immobilized condition hence allowing to be used numerous times to decrease the cost and improve the quality of product formed. When free enzymes are used in this process the enzyme activity is incompletely recovered but the built up of glycerol also limits the number of uses of free and immobilized enzymes (Nielsen et al. 2008).

v. **Supercritical and subcritical alcohol trans esterification:**
Trans-esterification of vegetable oils by super critical without the use of catalyst offers a novel way of making bio-diesel. By using this process the trans esterification is finished in minutes while other catalytic processes take several

hours (He et al. 2007). In this process the blend converts into a single uniform phase which quickens the reaction as there is no intermediate phase in mass transfer to limit the reaction rate. The benefit of using this procedure is that alcohol is a reactant and an acid catalyst. The main draw back of the process is great charge of apparatus due to the usage of high pressure and temperature (Yin et al. 2008).

vi. **Microwave assisted trans esterification:**
Micro wave irradiation can be used for biodiesel formation. This process activates polar molecules and ions to alcohol with fast modification in magnetic field, due to which it cooperates with dipoles and charged ions. The preparation of biodiesel using this method offers fast, easy, small response time, little oil to methanol ratio, decrease in amount of by products and cheap energy consumption (Azcan and Danisman 2007).

vii. **Ultra sound assisted trans esterification:**
Ultra sound delivers the power-driven energy for mingling and trans-esterification reaction. This process maximizes the chemical speed and harvest of the entire procedure of trans esterification of oil and fats into bio diesel. The main advantages of this process are less time, less energy consumption especially in mechanical stirring and simplicity of the process. The trans esterification of 1 kg soya bean oil using ultra-sonic and conventional stirring method consume 250 and 500 W/kg energy respectively (Singh et al. 2007).

## *3.2 Vegetable Oil*

In the colossal network of biological entities that can render the replacement of fossil fuels with immense appositeness, oils obtained from plants are surfacing the list with great celerity. According to the experts on environmental sciences, vegetable oils are evaluated for production of biofuel for the following two reasons:

1. Vegetables oils render sustainability in relation to factors like viscosity, energy content and combustion product.
2. Capital, labor and yield of biodiesel production from vegetable oil are favorable.

Some edible oils listed in Table 2.3 below have also been considered for the use of biofuels. Though due to food population ratio crisis elevating higher and higher, not much effort has been poured into this idea.

### 3.2.1 Pant Oils Used for Biodiesel

The main plants whose oils have been employed for the use of bio-diesel include:

**Table 2.3** List of edible oils suitable for biofuel production. (After Vijayalakshmi et al. 2007)

| Edible oil for biofuel | Property and uses |
|---|---|
| *Corn oil* | Abundance of crop |
| *Coconut oil* | Favorable for locations harvesting coconut |
| *Castor oil* | Lower cost than other oils. But has viscosity issues |
| *Hemp oil* | Has a high flash point but low emissions |
| *Mustard oil* | Satisfactory for biofuel |
| *Palm oil* | Very promising for a biofuel |
| *Peanut oil* | Been used in the very first demonstration for diesel engine in 1900 |
| *Radish oil* | Crop contains 48 % oil, making it very desirable for a fuel |
| *Safflower oil* | Relatively new discovered |
| *Soybean oil* | Not very economical for use as biofuels |

i. **Artichoke**
Its seed is used to extract edible oil. The oil composition constitutes of 3 % stearic, 12 % palmitic, 25 % oleic and 60 % linoleic acids. Presently continuous experiments and analysis of the crop are in full swing and deduce vast potential for producing biodiesel (Vijayalakshmi et al. 2007).

ii. **Canola Oil**
Canola seeds are rich in oil content (40 %). During the last few years, canola oil has started coming under the lime light of bio-fuels. In Australia a small group of farmers have begun producing bio-diesel from canola oil. Not much is known about its commercial success. Other countries too have started producing it (Vijayalakshmi et al. 2007).

iii. **Castor Oil**
Castor oil is one of the few vegetable oils that bears the 100 % viscosity as that of diesel fuel. It is one characteristic that has dismantled the environmentalists parallel to attracting their attention with its other few noteworthy characteristics.

iv. **Coconut Oil**
Least viscous oil and thus appears to be a good candidate for biodiesel. As opposed to castor oil, coconut oil has the largest oil content, of about 70 % (Vijayalakshmi et al. 2007).

v. **Corn Oil**
Corn has been over many decades under constant experimentation as a feedstock for biodiesel. But it was never favored, because primitive extraction methods did not favor production of wide quantity of oil suitable for processing of bio-diesel (Vijayalakshmi et al. 2007).

vi. **Cottonseed Oil**
So far USA is in function of producing bio-diesel from cotton seed but the production volumes are quite low (Vijayalakshmi et al. 2007).

vii. **Jatropha Oil**
The *Jatropha* tree has been used as a significant fuel source for a lot of years especially in Southeast Asia and India. The tree has convenient harvesting condition that allows it to grow in arid conditions and gives a significantly large oil yield (Vijayalakshmi et al. 2007).

viii. **Jojoba Oil**
Jojoba oil is a new candidate in vege oils as bio-fuel. Easy growth in saline soils and on desert lands has landed jojoba oil under the spotlight of researchers. Given the small amounts of cultivation, studies across the globe have shown that jojoba oil will make scanty impact on the production of bio-diesel (Vijayalakshmi et al. 2007).

ix. **Karanj Plant**
Native plant found in India, appears to be a good candidate for biodiesel production. It is considered less toxic and cheap as well. However there is a large room for studies and more research is needed on Karanj plant (Vijayalakshmi et al. 2007).

x. **Peanut Oil**
From the beginning in diesel shortages, peanut oil was used frequently since Rudolf ran his first diesel engine on peanut oil. But gradually its usage decreased, mostly because of economic reasons (Vijayalakshmi et al. 2007).

### 3.2.2 Production of Diesel oil from Vegetable Oil

Vegetable oils fulfill the parameters of being economical and environment friendly. However characteristics like high viscosity, low volatilities and polysaturatedness act as tangible obstacles in replacing diesel fuels with triglycerides. Methods that produce vegetable oil derivatives have been developed to circumvent this hindrance: Pyrolysis, Micro emulsification, Dilution, Transesterification.

a. **Pyrolysis**
Pyrolysis harbors the decomposition of vegetable oil via thermal energy in the absence of air. The resultant vegetable oil product is referred to as pyrolyzate and has been shown to have less viscosity, pour point and flash point but equal calorific values. Even the cetane number of the vegetable oil pyrolyzate turned out to be low. The sulphur water and sediment of the pyrolyzate remain under acceptable range alongside acceptable copper corrosion (Vijayalakshmi et al. 2007).

b. **Micro emulsification**
The formation of micro emulsification of liquids has potentially added to the lessening of vegetable oil viscosity. These are colloidal dispersions that are thermodynamically stable ranging from 100 to 1,000 A° (Vijayalakshmi et al. 2007).

c. **Transesterification**
The most popular technology employed for the production of bio diesel from vegetable oils is the transesterification of triglycerides to methyl esters of glycerin and fatty acids. It is a relatively simple method than others cited here. Duy and Patrick first discovered it in 1853 (Vijayalakshmi et al. 2007). In the transesterification of vegetable oils, a triglyceride is made to react with an alcohol alongside strong acid or base accompanying the reaction, forming a mixture of fatty acids, alkyl esters and glycerol. Various factors such as temperature, molar ratio, type of catalyst, free fatty acid content and clarity of the reactants have an impact on the course of the transesterification. The various processes involved are given below:

i. **Acid catalyzed process**
Transesterification is usually carried out using this process, with preference for sulphonic or sulphuric acids. These catalysts have high yielding capacities of alkyl esters but the reactions are relatively slow, demanding temperatures above 100 °C with cumbersome time consuming durations to reach complete conversion. For example lysis of methane in soybean oil, in the presence of 1 mol percent of $H_2SO_4$ at 650 °C takes 50 h to reach whole transformation (Vijayalakshmi et al. 2007).

The most influential factor acting on transesterification is alcohol vegetable-oil molar ratio. Copious amounts of alcohol favor the synthesis of product. But excess alcohol creates the retrieval of glycerol problems (Vijayalakshmi et al. 2007). The transesterification catalyzed by acid must be performed in the absence of water so that the competitive formation of carboxylic acid can be avoided which reduces the amount of alkyl esters produced (Payawan et al. 2010).

ii. **Base catalyzed process**
Base catalyzed reaction, on the other hand, is employed for a large scale production particularly because of its celerity and reduced corrosive troubles. The process commences with the first step mainly consisting of a reaction of the base with alcohol which results in the formation of a protonated catalyst and an alkoxide. The next step proceeds to be a nucleophilic reaction in which the alkoxide reacts with the carbonyl group of the triglyceride producing a tetrahedral intermediate that later gives an alkyl ester and diglyceride anion. The alkyl ester product deprotonates the catalyst in the end to re-initiate the cycle (Payawan et al. 2010). However a few disadvantages are associated with the base catalyzed reaction. Chief among these are the condition of the presence of water in the reaction that may react with the alkyl ester to give rise to Free Fatty acids (FFA). High content of FFA may perturb the separation, purification and washing stages of glycerol and ester. Hence modifications in the base catalyzed method have been introduced.

In a recent study by Payawan et al. (2010) on the characteristics of *Jatropha* oil as a convenient and one of the leading candidates for biodiesel is seen to contain 14 % FFA content. An amount that is largely beyond the 1 % FFA level can

be transformed into biodiesel via transesterification process through an alkaline catalyst. Alterations in the process were administered to achieve a higher FFA content in the biodiesel. Heterogeneous catalysts have been produced which can lead to higher efficiency and convenient removal of glycerol and aqueous base catalyst. Not much is discovered about the deleterious emission of biodiesel made from vegetable oils. Much needs to be experimented on its clinical and economical attributes.

## *3.3 Bio-gas*

With the rising population and urban development towards the millennium, air pollution issues have touched the peak. As the number of vehicles on roads multiplied, so did the harmful emission in the atmosphere. The urgent need to reduce the menacing emission for minimizing global warming was therefore the first catapult objective behind the idea of biogas.

Biogas is by far the most versatile renewable resource that cannot only replace fossil fuels for the use of vehicle engines but also for the use of heat and power production (Fehrenbach et al. 2008). The production mechanism of biogas via anaerobic digestion provides environmental and energy-efficient advantages over other energy sources. Methane gas is the first viable candidate for the major composition of biogas since its early production.

### 3.3.1 Biochemical Process

Methane gas is fermented for obtaining it in colossal amounts. Its fermentation is a perplex process, composing of distinct steps, namely, hydrolysis, dehydrogenation and methanation (Angelidaki et al. 1999). The first step is hydrolysis using hydrolyzing microorganisms. These secrete hydrolyzing enzymes: cellulose, amylase and lipase to breakdown the added monomers and polymers (Bagi et al. 2007). This breakdown of polymers or monomers results in the production of acetate and hydrogen and some fatty acid derivatives like butyrate and propionate.

An exclusive nexus of microorganisms are added into the culture medium for the production of methane gas. Of this consortium of microorganisms, mostly anaerobes are employed, such as *Bacteriocides, Bifidobacteria* and *Clostridia.* Some facultative anaerobes can also be employed such as *Streptococci* and *Enterobacteriacea* (Bagi et al. 2007). Subsequent to hydrolysis of polymers, the methanogenic bacteria produce methane gas using acetate and hydrogen molecules. When using methanogenic bacteria, hydrogen production is easily achieved in two stages only (Schink 1997).

The neighboring degradation steps of the anaerobic fermentation have to occur in an equilibrium proportion, if it occurs at a faster rate, the pH will fall below 7, putting the survival of the methanogenic bacteria in trouble. If the second step out

runs the first one, methane yield will suffer. Therefore special care has to be taken while designing the process to achieve minimal loss (Karakashev et al. 2005).

The next step, digestion, takes place under both mesophilic and thermophilic conditions. A constant temperature throughout the entire process is necessary for a fruitful yield of biogas. Numerous studies have shown that methanogenic bacteria work best at thermophilic temperatures and tolerate a temperature change of $\pm 3\,^{\circ}C$ (Karakashev et al. 2005). In terms of pH, methane gas is released most efficiently at pH between 6–8. However if the pH goes down below 6 or rises above 8, the production of methane gas is severely inhibited. The pH alters or reaches above 8 mostly due to accumulation of ammonia gas released upon degradation of protein substrates (Abdoun and Weiland 2009).

### 3.3.2 Growth Parameters for Microorganisms

All living microorganisms require certain macro and micronutrients for growth and reproduction. Carbon, phosphorus and sulphur are added as macronutrients for the culture medium. Micronutrients are added in considerably lower amounts (0.05–0.06 mg/l), since their need is not particularly essential (Bischoff 2009). Trace metals are added in significant amounts, as they are needed as co-factor for enzyme functions. Nickel, cobalt, selenium, molybdenum, tungsten and iron are the common trace metals added (Bischoff 2009). Nickel is utilized by the methanogenic bacteria for the synthesis of an essential cell component called $F_{430,}$ involved in the formation of methane. Iron is normally added in the concentration ranging from 1 to 10 mg/l.

### 3.3.3 Feedstock

The criteria for use of biomass as substrates for biogas production is the presence of carbohydrates, cellulose, proteins, lipids, fats and hemicelluloses as main components. According to researchers, the biogas yield depends upon the content of carbon, proteins and fats. Wood, a strong lignified organic biomass is not encouraged for use as substrates in biogas production mainly because its anaerobic decomposition is extremely slow. Different biomasses produce different amounts of biogas concentrations, depending on the content of their organic substances. For instance fats yield the most biogas amount but require long retention time, whereas proteins and carbohydrates have faster rates of conversion but low amount of gas yields.

Crop plants are the most paramount, biogas producing substrate candidates. Figure 2.2 shows the potential of crop plants and organic wastes for usage as biomass for biogas production in Germany. About 50 % of biogas energy is synthesized from using crop plants as co-substrates. The net energy yield per hectare of crop plants is phenomenal. Maize, forage beets, perennial grass and cereal crops provide high gross energy per hectare. Certain parameters of crop plants such as harvesting and the frequency of harvesting are to be kept in consideration while choosing the

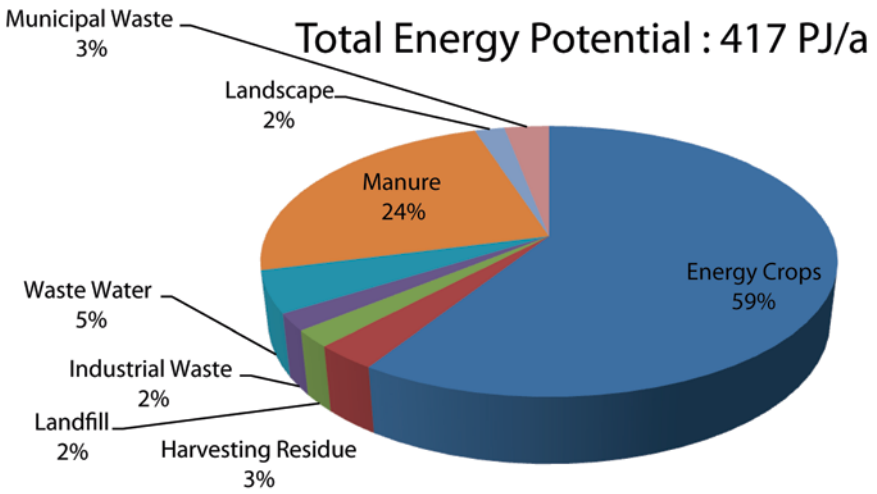


**Fig. 2.2** Usable biomass potential for biogas production in Germany

variety of crop as biomass. Forage crops are thus mostly suitable for biogas production because of small harvesting periods (Weinberg et al. 2003).

The crop residues and the crops themselves are stored via a process called ensiling. In the biochemical process of ensiling, the soluble carbohydrates in plant crops are converted into butyrate, acetate, lactic acid and propionate. The production of these organic compounds inhibits the growth of contagious pathogens by lowering the pH to 3–4 (Banemann and Nelles 2009). However one downfall of energy losses of up to 8–20 % accompanies the process of ensiling. To minimize the energy loss by biomass degradation, the energy crops are put in silos and wrapped in plastic wraps (Weiland 2006).

## 4 Second Generation Biofuels

### *4.1 Bio Alcohols*

Alcohol is a very important source of energy because it can be produced chemically and biologically. Alcohol produced by the traditional chemical methods is now being replaced by the bio alcohols produced by microorganisms and enzymes using renewable energy sources (Weber et al. 2010). Out of all the other biofuels, bio alcohol fuels proposes the most verified and attainable substitute for the gasoline, which is three fourth of on-road fuel practice, over one-half of all transportation energy use especially in the USA (Weber et al. 2010). Main bio alcohols used for the production of energy are methanol, ethanol, propanol and butanol. The main reason why bio alcohol based fuels are essential is their advantage to hold a high

octane number which is very important for efficiency and balancing the low energy density of the fuel. Out of all bio alcohols, bio methanol and bio ethanol are most important (Agarwal 2007).

### 4.1.1 Bio Methanol

Bio methanol is an emerging biofuel. Some of the properties which make bio methanol really attractive as a biofuel are that it is a liquid fuel which can be combined with gasoline and ethanol and used with vehicle equipment at minimal expenses. It is a high octane fuel having combustion properties that permit engines specifically designed for methanol fuel to work at the best efficiencies and regulating pollutant release. It can be produced from renewable biomass, is a safe fuel though toxic but the toxicity is comparable to or better than gasoline, and biodegrades quickly in case of a spill and doesn't persist in surface waters. Bio methanol is a greenhouse gas reduction fuel as the fuel just returns the carbon back into the surroundings when formed from a renewable resource (Agarwal 2007).

**The Partial Oxidation and Gasification Using Water and Oxygen** The main method used for the making of bio methanol via biomass is by its partial oxidation and gasification. The plant comprises of two chief parts in which the process is carried out. First is a biomass gasifier in order to transform the feedstock to synthesis gas (syngas) and second one is a methanol production plant. Biomass resources usually used are wood components including wood wastes. The oxygen required for the conversion of biomass into gas is obtained from electrolysis of water by consuming electricity. About 10.32 kt of oxygen gasifies 10.1 kt of biomass yearly. The complete output of biomass derived methanol can be improved by more electrolytic oxygen supply. The fume from the gasifier consists of elements like tar, alkali, sulphur and chloride complexes. These particles can lead to poisoning of the catalysts and cause corrosion of the equipment being used. Hence the minor particles using the gas are formed, which are its principal investments, compulsory for the treatment of the syngas (Demirbas 2009a, b).

The presence of nickel based catalyst reforms natural gas, tar and additional hydrocarbon compounds into carbon monoxide and hydrogen gas at extreme temperature. The addition of hydrogen helps to regulate the appropriate hydrogen:carbon monoxide percentage for methanol production. 1.29 kt amount of hydrogen is used for the manufacture of 12.2 kt of methanol annually (Cifre and Badr 2007).

Methanol processer uses catalytic conversion of syngas for methanol manufacture. The key benefits of this technology are low production cost and improved working reliability. The unpolished methanol is treated in a distillation chamber to attain good quality. The gas turbine can be used in order to consume the residual gas for electricity production (Cifre and Badr 2007).

Bio methanol manufactured using this process is at least 2–3 times extra costly than methanol formed by fossil fuel. Methanol competes with the fossil fuels only under a green assessment based on pollutant released, mainly involving carbon dioxide, which favors the manufacture of methanol using biomass. Some new

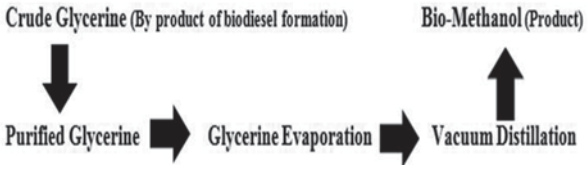


**Fig. 2.3** Methanol production using Bio-MCN process, the crude glycerin processing leads to bio methanol production

methods propose the usage of renewable hydrogen for methanol production. Methanol production using biomass is inexpensive and more effective compared with that formed from carbon dioxide. Methanol production from carbon dioxide reduces the emission of this greenhouse gas. Numerous choices are being examined for utilization of carbon dioxide taken from flue gases of power stations (Cifre and Badr 2007).

i. **Flue gas carbon dioxide**
The methanol can also be formed using carbon dioxide. This process increases the yield of methanol, controls the greenhouse effect and recycles carbon dioxide emitted from various sources as a hydrogen carrier. Once carbon dioxide is obtained, it is converted into liquid phase and is taken to the hydrogen manufacture unit. The water electrolysis is done using electricity. The manufacture of methanol using reprocessed carbon dioxide along with hydrogen gas comprises of two stages: methanol production using a catalyst and methanol decontamination (Cifre and Badr 2007). Methanol manufactured using biomass and flue gases is about 428.26 and 576.42 €/t respectively, while natural gas source produces least amount of methanol which is 101.91 €/t (Cifre and Badr 2007).

ii. **Bio-MCN method**
Another method for the production of biomethane is using crude glycerin. Bio-MCN is the first corporation in the whole globe to manufacture and sell large amount of bio methanol having extraordinary quality. The Fig. 2.3 shows the flow diagram of the process of methanol production by Bio-MCN process (Van Bennekom et al. 2012). In this process, the crude glycerin as a byproduct from biodiesel manufacture is converted into bio methanol, which is disinfected and converted into gaseous phase. Its purification is done by vacuum distillation unit, where it is evaporated and the contaminations are removed. The glycerin vapors are introduced into the steam to remove water, alkanes and heavy fractions. The resultant methanol formed via this process is 99.85 %, having the same purity as from methane (Van Bennekom et al. 2012). The low amount of greenhouse gases using Bio-MCN process for methanol production is seen when compared to gasoline. The Fig. 2.4 tells that gasoline causes about 80 % of greenhouse gases emission all around the globe. In comparison Bio-MCN bio methanol resulted in 50 % greenhouse gases emission in 2011.

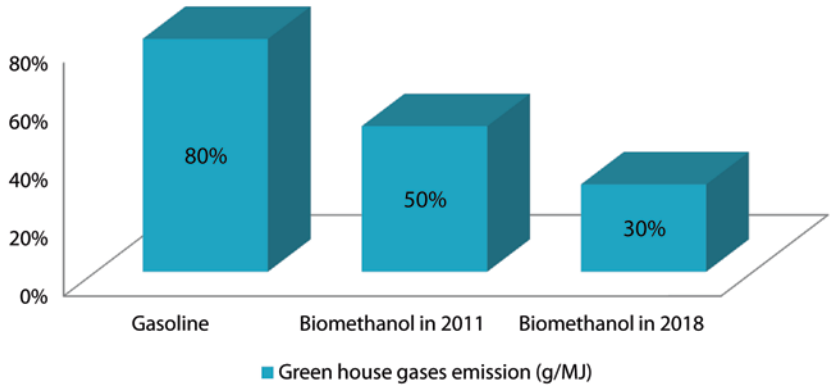


**Fig. 2.4** The estimated greenhouse gas emission by gasoline production and bio methanol synthesis using BioMCN process

It is estimated that by 2018, the emission of greenhouse gases will be reduced to 30%. This is because the reuse of the byproduct of biodiesel production (glycerin) reduces the amount of carbon dioxide in the atmosphere (Van Bennekom et al. 2012), thereby reducing the greenhouse effect and global warming by controlling the over production of carbon dioxide in the atmosphere. This process is environmental friendly. Bio-methanol is used as a chemical building block for a range of future-oriented products, including bio-methyl, tertiary-butyl ether, bio-dimethyl ether, bio-hydrogen, and synthetic biofuels etc. Bio-methanol is environment friendly and helps limit global warming by reducing carbon dioxide emission by about 70% in comparison with conventional methanol production technologies (Cifre and Badr 2007; Van Bennekom et al. 2012). The Nobel Laurate George Olah suggested whole methanol budget instead of hydrogen economy because using bio methanol only, slight changes will be required in filling stations and car engines, unlike hydrogen gas which would require an entirely novel setup (Cifre and Badr 2007).

### 4.1.2 Bio Ethanol

Fossil fuels produce about 73% of the $CO_2$ and release it into the environment. Production of bio-ethanol using biomass reduces the use of crude oil along with environmental pollution. Bio-ethanol is used in the car engine because of its great octane and cetane ratings, which delays self-ignition in an engine (Lokhorst and Wildenborg 2005). Some drawbacks of bio ethanol include low energy density compared to gasoline, corrosive nature, little flame radiance, low vapor pressure that make cold starts challenging, miscibility and harmfulness to environment (Luque et al. 2010). It can be produced from cellulosic feedstocks; but the main issue with it is the handiness of raw materials. The accessibility of feedstock for this can vary greatly from one season to another and is dependent on geographical localities. For planning bioethanol manufacture procedures, evaluation of consump-

**Table 2.4** Various feed stocks used for ethanol formation and their relative production value. (Luque et al. 2010)

| Soure used | Ethanol obtained (I/ton) |
|---|---|
| Sugar cane | 70 |
| Sugar beet | 110 |
| Sweet potato | 125 |
| Potato | 110 |
| Cassava | 180 |
| Maize | 360 |
| Rice | 430 |
| Barley | 225 |
| Wheat | 340 |

tion of various feedstocks is required as they hold big share in bioethanol costs (Dien et al. 2003).

i. **Feedstocks of bioethanol manufacture**
There are many types of feedstock for the production of bioethanol, some of which are shown in Table 2.4.

The bioethanol feedstock can be mostly classified into three kinds: sucrose, starch and lignocellulosic biomass. These are discussed below:

a. **Sucrose containing feedstock**
This includes sugar cane, sugar beet, sweet sorghum etc. but bioethanol is chiefly formed from sugar beet and sugar cane (Dhavala et al. 2006). About 2/3 sucrose is made from sugar cane while 1/3 from sugar beet. Brazil is the chief manufacturer of sugar cane having worldwide manufacture of about 27% (Kim and Dale 2004). Bioethanol formation by sugar cane is very cost-effective especially in Brazil; since Brazil lowered the sugar cane prices in order to support the bioethanol industry. This significantly dropped the price of the feedstock and generated a petition for the maintained worth of bioethanol (Cardona and Sanchez 2007). In European countries, sugar beet molasses is the best used feedstock. The benefits of using sugar beet include: minimum rotation of crop production, greater harvest, and great resistance towards climatic variations, less water and fertilizer necessity. Sugar beet requires 35–40% less amount of water and fertilizer as compared to sugar cane (Dhavala et al. 2006; Luque et al. 2010).

b. **Starch containing feedstock:**
Starch is a biopolymer. In order to obtain bioethanol from this feed stock it is necessary to break it down, which can then further be transformed into bio ethanol by yeasts. This process is mostly followed for bioethanol production in Europe and North America (Luque et al. 2010). Wheat and corn are largely engaged for these procedures (Balat et al. 2008). USA owns a large corn bioethanol industry producing over 15 billion tons per year. The single factor increasing the cost of the manufacture of bioethanol from corn is the rate of the corn itself. Corn prices vary from 1 year to another. The price previously ranged between 1.94 and 3.24 $ per bushel (De Oliveira et al. 2005). The value of corn now is four

dollars per bushel, which is extremely high (Luque et al. 2010). The prices of corn will also vary in various places due to shipping expenses. USA bioethanol manufacture is 1.1, which is visibly less than the percentage of 3.7 for bioethanol produced in Brazil using sugar cane (De Oliveira et al. 2005). Other reasons for the high cost of starch based bioethanol are that the yeast *Saccharomyces cerevisiae* is not able to utilize starch, so large amount of amylytic enzymes are needed and the starchy material is essentially cooked at a quite high temperature to acquire a great bioethanol harvest. The two-step enzymatic hydrolysis of corn meal at a lower temperature yields bioethanol of more than 80% just after 4 h (Mojovic et al. 2006).

c. **Ligno cellulosic biomass**
The chief structure of all lignocellulosic biomass is: cellulose, hemicelluloses and lignin. This feedstock is used for bioethanol fuel production since it is the most abundant resource all around the globe (Luque et al. 2010). Lignocellulosic biomass could go up to 442 billion/year. The entire possible bioethanol making from crop left over and crop wastes is 491 billion/year, which is 16 times greater than the present world bioethanol production (Karimi et al. 2006). The highly overflowing lignocellulosic waste material in the world is rice straw. It has the potential to produce about 205 billion bio-ethanol (Hamelinck et al. 2005). Lignocellulosic perennial crops are a good feedstock as they give excessive yield, least expenses, worthy suitability for low quality land, and great environmental resistance. Pine has the highest collective sugar content, involving the extreme potential of bioethanol production. The lignin content is about 27% for most feed stock while grasses contain visibly lesser amount and thus result in less electricity production (Hamelinck et al. 2005). The bioethanol formed using lignocellulosic materials has a moderately greater cost, based on the chief encounters and at hand technologies, such as high price of the hydrolysis process (Dhavala et al. 2006). The feedstock can signify 440% of all process charges; a money-making biomass-to-bioethanol process analytically relies on the fast and effective transformation of the sugars into both cellulose and hemicellulose portions (Hamelinck et al. 2005). Lignin fermentation, which is coproduced in bioethanol prepared from lignocellulose, can actually produce 458 terra-watt-hours of current and 2.6 EJ of steam unit (Weber et al. 2010).

ii. **Thermochemical method of bioethanol production:**
Two leading methods for bioethanol production, which use thermochemical reactions in their processes are:

a. **Hybrid biological and thermochemical system**
In this process cellulosic biomass is first thermochemically converted in gas phase and the synthesis is bubbled through specifically aimed fermenters (Dhavala et al. 2006). Also a microorganism with the ability of converting syngas is present in the fermentation containers thus permitting bioethanol to get fermented (Jansson et al. 2009).

b. **Bioethanol production thermochemically without microorganisms**
Biomass is mainly thermochemically converted into gas and then the syngas is passed through a unit comprising of catalysts, which allow the gas to be converted into bioethanol. Bioethanol yields up to 50% have been achieved using syngas—bioethanol way. The quest for an economical thermochemical process has been problematic (Dhavala et al. 2006). Thermochemical is encouraging than biological selection for the transformation of lignin of cellulosic biomass, which can have an unfavorable consequence on enzymatic hydrolysis but also helps in processing energy and formation of possible byproducts with significant profits (Jansson et al. 2009).

iii. **Lignocelluloses to bioethanol:**
There are numerous choices for bioethanol production using this feed stock irrespective of which one is selected. The following points need evaluation in contrast with various well-known feed stocks for bioethanol making (Luque et al. 2010): effective de-polymerization of cellulose and hemicellulose; effective fermentation of a mixed-sugar hydrolysate; innovative method used in order to lower procedure energy claim, less lignin content of feedstock which reduces the price of bioethanol.
The benefits of using lignocelluloses are the chances to create a bio plant, generating byproducts along with the fuel, bioethanol. For example, sugars when exposed to bacterial fermentation in the absence or presence of oxygen produce a variation of products like lactic acid, which may be managed into plastics and other products (Dhavala et al. 2006). The treatment of lignocelluloses to bioethanol comprises of four leading units: pretreatment, hydrolysis, fermentation and separation/distillation of product (Luque et al. 2010).

a. **Pre-treatment:**
Pretreatment and size reduction is the first step in lignocellulose to ethanol bioconversion. The purpose of pretreatment is to change and eliminate mechanical and compositional obstructions to hydrolysis so that rate of enzyme hydrolysis increases leading to more fermentable sugar from cellulose and hemicellulose. A popular pretreatment should fulfill the requirements like; increase sugar formation, allow least degradation of carbohydrate, evade the production of hydrolysis and fermentation inhibitory byproducts and should be cost effective (Luque et al. 2010).

b. **Hydrolysis:**
When pre-treatment is complete, the cellulose undergoes hydrolysis. This includes acid hydrolysis and enzymatic hydrolysis. Acid hydrolysis can be of two types, dilute and concentrated. The dilute acid hydrolysis is for transforming cellulose biomass to bioethanol. The hemicellulosic part is depolymerized at lower temperature than cellulose. Usually dilute acid procedures are restricted to 50% regaining of sugar. The current task is to raise sugar recovery to as high as 70% in a favorable industrial use (Luque et al. 2010). Enzymes are naturally occuring in nature used for several chemical reactions and both bacteria and

fungi can be evaluated in this direction. In this procedure two technologies are used: (1) direct microbial transformation method and (2) enzymatic transformation method. It has numerous advantages like less cost, insignificant conditions needed and improved yields (Luque et al. 2010).

c. **Fermentation:**
The process of fermentation comprises of microbes which consume sugar as food and in the process lead to the production of bioethanol and other varieties of products. The microbes are termed as the ethanologens, as these convert a portion of sugar to ethanol. The microorganisms used are compatible with the fermentation conditions i.e. pH, temperature, growth rate, tolerance to inhibitory compounds, output result, osmotic tolerance, specificity, yield, stability etc (Luque et al. 2010). The fermentation can be done in batch, fed batch and continuous process. The selection of the method depends on properties of microbes and nature of lignocellulosic hydrolysate other than economic aspects. Fed batch containers are extensively used on the industrial level since they have both the benefits of batch and continuous process. The key benefit when compared to batch is, ability to increase the cell viable concentration, longer culture life and more product accumulation (Luque et al. 2010).

d. **Product and Solid recovery:**
Fermentation products are unstable, so distillation is widely used equipment for the recovery of the bioethanol and other products from a number of impurities. The distillation separates bioethanol from water; the virgin ethanol has 80% water in it. Large amount of energy is needed for the concentration of bioethanol to 95.6% (Luque et al. 2010). At first bioethanol is recovered from water which has high moisture content. The bioethanol (37%) is concentrated in the rectifying column and is adjusted below a zeotrope, reaching 95%. The residual product is fed to stripping column for the removal of extra water (Luque et al. 2010). Bioethanol is recovered in the distillation unit which is stabilized to be about 99.6% to lessen the bioethanol loss (Garcia et al. 2008). Solids are dispersed by centrifuge and dried over rotary dryer. About 25% waste is reprocessed to fermentation while the remaining is sent to evaporator. The concentrated solution comprises of 15–20% weight of total solid (Luque et al. 2010).

## *4.2 DMF (2, 5-Dimethylfuran)*

Until recently scientists perfectly were confident on “Bio-ethanol” as the only satisfying bio-fuel in the energy market across the globe. The advent of advancement in methods like catalytic systems for the production of 2,5 Dimethylfuran from biomass opened a new window of research and hope in the world of biofuels. With its infallible chemical characteristics fulfilling particularly all requirements as an ideal candidate for biofuels, DMF was largely considered an auspicious bio-fuel for the future of power generation and internal combustion engines.

i. **Structural Chemistry of DMF**
Dimethylformamide is a colorless, polar, high boiling point liquid with a distinctive odor having a molecular formula of $C_3H_7NO$. It is immune to decomposition upon distillation procedures and even at elevated temperatures. It is freely miscible in water, ketones, esters, alcohols and ethers. However its rate of hydrolysis increases in the vicinity of acids and alkalis. DMF is one of the rare liquids that has a high dielectric constant and low volatility which qualifies DMF as an excellent universal solvent, particularly for chemical reactions that require a high solvency power.

**Physical Properties:** Density −0.949 g/cm$^3$; Distillation Range- 760 mmHg; Temperature—347 °C; Boiling Point- 153 °C

a. **Production of DMF from Biomass: Carbohydrates**
Previously, DMF was widely employed in a number of industries but at a high cost and low production yield. In 2007, biochemists from University of Wisconsin (USA) developed a new technique for the convenient conversion of carbohydrates to DMF. Earlier, carbohydrates were enzymatically broken down to fructose which was then deoxygenated to DMF. It was proposed that the production of bio-fuel with high yield and low energy consumption from biomass is possible if 5 oxygen atoms are removed from hexose for the production of DMF. This step can be carried out in two steps. The first step maneuvers the removal of 3 oxygen atoms via the process of acid catalyzed dehydration reaction with a solvent having low boiling point to produce 5-hydroxymethylfuran, following it butanol solvent is used for quick extraction of DMF, the quicker the extraction, the bigger the yield. The second step includes removal of two oxygen atoms via copper catalyzed hydrogenolysis involving the production of two intermediates: 2 methyl furan and 2-methyl,5-hydroxymethylfuran (Leshkov et al. 2007). These methods were modified later on by rendering the need of acid based catalysts as non-imperative. This led to an augmented interest towards DMF as a prospective gasoline alternative biofuel. Engineers from the automotive community began paying attention to DMF as an alternate automotive fuel. The first study on the capability of DMF as a fuel, focusing on its emission and combustion performance commenced in Birmingham University of UK. The factors under observation viable for required performance of a fuel were spray characteristics, laminar burning velocity and unregulated engine emission (Leshkov et al. 2007).

b. **Fuel Spray Characteristics**
A desired engine performance fuel spray characteristics tremendously influence the fuel-air mixture generation and combustion manner. A number of comparative studies between spray characteristics of ethanol, gasoline and DMF have been conducted through use of Optical methods such as Phase Doppler Particle Analyzer. Results of the studies have shown that spray characteristics of DMF are preferably favorable over those of ethanol and gasoline. The spray pattern of DMF was not very different from that of gasoline. DMF spray velocity turned out to be greater than that of ethanol and DMF spray droplet size very small than the large ones of ethanol. These findings rendered DMF a considerably suitable engine fuel given its extreme similarity to gasoline behavior.

c. **Laminar Burning Velocity**
Laminar burning velocity is another essential fuel engine characteristic responsible for good simulation work. A variety of methods were employed, prominent one being Schlieren techniques for cost effectiveness, accuracy and ease. Ethanol exhibited higher potential in laminar burning velocity. About 30% difference was measured between the burning velocities of ethanol and DMF. The laminar burning velocity of ethanol, according to the results, came out to be 62 cm/s, whereas the burning velocity of gasoline is 36 cm/s which is homologous for DMF. In summary, the results clearly promoted the adoption of DMF in spark ignition engines due to its yet again similar behavior to gasoline in laminar burning velocity.

d. **Unregulated Emissions**
The particulate matter emission of DMF (presently an unregulated requirement in USA and Europe) proved to be comparable to that of gasoline. This research is ongoing and further results are likely to appear in publications. Such results will provide explicit details of the toxic compounds, thus helping to better understand the possible effect to the environment.

ii. **DMF Future Prospect: Positive or detrimental?**
High energy density of DMF has earned bio-DMF a golden ticket under the lime light of researchers in the major commercial industries in developed countries. With an energy density 40% greater than the previously reigning bio-fuel: ethanol, DMF has proved itself spectacularly suitable as automotive fuel. With august chemical properties like high boiling point that allow DMF to blend sufficiently with gasoline (as fuel additive), DMF has out raced ethanol in behaving like a more preferable fuel. Moreover DMF consumes only one third of its energy during the second stage of its production as opposed to the fermentation of ethanol. The catalyzed production of DMF from carbohydrates and cellulose has augmented the efficiency in amount of yield and lessened the production cost. So far no adverse effects on the engine have been observed from DMF. Unregulated emission of DMF and gasoline do not vary much; scientists are still working on eliciting the precarious emission of gases to the environment.

Aside from a potential promising biofuel, DMF is under clinical trials for any debilitating effects. Not much is known yet of the toxicity of DMF to human health because of scanty environmental testing. Recently however, *in vitro* studies on erythropoietic micronucleus assay exposed to 0.1 mM DF for an hour, showed an increase in micronuclei, suggesting a genotoxic effect on the bone marrow (Fromowitz et al. 2012). There is still vast room for toxicological studies on DMF to ensure environment safety.

## *4.3 Wood Diesel*

One of the leading sustainable "Second Generation" biofuels of the twentieth century is biomass derived from trees. The cellulose, lignin and hemicelluloses extracted

from the wood used for conversion to ethanol fuel is industrially known as wood diesel. Wood biomass may be regarded as a second generation biofuel but it is not a new candidate to human understanding and awareness. It was in 1819 when a scientist discovered that the addition of concentrated acid solution to cellulose present in wood can be dissolved to yield sugar; a precursor for production of ethanol. This process of dissolving cellulose in dilute acidic solution began contributing to the production of ethanol in Germany during World War I and II. From then onwards wood hydrolysis is being used in many developed countries (Lynd et al. 2005). For numerous decades biofuels from wood were not very economical. Over the years, as the demand for energy sources and environment friendly fuels aggravated, a few of the developed countries began working on modifying methods for processing engine fuel from wood at an opposite cost scale.

### 4.3.1 Production of Lipid Fuel via Gasification of Woody Mass

The most significant lipid fuels, definitely obtained from wood are ethanol, methanol and diesel fuel. Other potential candidates on which studies are yet to be conducted include mixed alcohols, tert-amylmethylether, tert-butyl alcohol, iso-propyl alcohol and sec-butyl alcohol. The main contents vital for qualifying wood as sources of bio-diesel are basically: cellulose, lignin and hemicellulose. The distribution of each of these contents varies according to the species (Pauly and Keegstra 2010). For instance there is 45% cellulose, 20% lignin and 30% hemicellulose in Angiosperm wood. The distribution of contents in gymnosperm wood is crudely 42, 27 and 28% cellulose, hemicellulose and lignin respectively (Singh et al. 2010). The decomposition of wood is distributed mainly into three categories: pretreatment, hydrolysis of cellulose into sugar and fermentation of sugar to give alcohol. The pretreatment step is essential for the proper function of the second step, because in the pretreatment step, the lignocellulosic matrix is disrupted to provide easy access for the enzymes or chemicals to cellulose (Mosier et al. 2005). Recently, many developed as well as developing countries have begun taking greater interest in gasification of woody biomass for a successful alternative to fossil fuel biofuels, chief among them are countries like Thailand, Japan and USA. In the countries where the industrial and the transportation sector is developing at an astounding rate, the fossil fuel derived energy resources are depleting, leading to an oil crisis.

Thailand is a tropical country with a large percentage of area covered by forests and lakes. There are three types of wood in Thailand as will be clear from Table 2.5, which can be and are considered as wood fuel, namely, rubber wood, palm oil tree and eucalyptus. The woody biomasses from these plants are separated into bark from saw mills followed by wood processing (Laohalidanond et al. 2006). Transportation fuel is processed by two ways in Thailand. One is transesterification and the other is fermentation. Similar to production of bio-ethanol, wood diesel is also manufactured via biomass gasification through the Fischer Tropsch synthesis process (BG-FT). The BG-FT process has three steps (Laohalidanond et al. 2006): **Biomass Gasification**, **Gas cleaning**, **Fischer Tropsch Process.**

**Table 2.5** The potential of woody biomass produced in Thailand. (Laohalidanond et al. 2006)

| Source | Reside | LHV (kJ/kg) | Potential (kt/year) | Potential (10˚KJ/year) |
|---|---|---|---|---|
| Rubber wood | Saw mill | 139,62 | 1373.44 | 19.18 |
| | Bark | | 2746.89 | 38.35 |
| Eucalyptus | Saw mill | 6,300 | 0 | 0 |
| | Bark | | 4649.25 | 29.29 |
| Palm oil tree | Front | 7,540 | 14355.86 | 108.24 |

In the first step the woody biomass material is transformed into syngas made of water gas, at a temperature of 700–1,500 ˚C. In the next step of Fischer Tropsch process, the synthesis gas is cleaned of all impurities such as tar and catalyst poisoning substances. Once the gas is cleaned, it is converted into long chain hydrocarbon product in the presence of high pressure and high temperature and iron or cobalt based catalyst. Eventually these long chains of hydrocarbons are distilled, upgraded and hydro cracked before being packaged as engine fuel (Laohalidanond et al. 2006)

In Japan, the automotive industry has also begun replacing gasoline with biofuel diesel. Toshiaki 2010 explains in his bench scale experimentation the successful manufacture of liquid fuel from woody biomass using the process of gasification. The volume of the product made from biomass to liquid (BTL) plant is 1.9 L of hydrocarbon liquid from 13 kg biomass of wood. The methodology of gasification, cleaning and Fischer Tropsch are integrated. The gasification step leads to oxygen enrichment, with the volume augmented from 21 to 31.5 %, leading to an increase in the carbon conversion from 91 to 96 %. The use of 26.7 % of oxygen as the gasifying agent results in a product containing 29 vol% of CO, 22 vol% of $H_2$, 11 vol% $CO_2$, 2 vol% $CH_4$ and 35.5 vol% $N_2$. COS and $H_2S$ concentrations are decreased to less than 5 ppb in gas cleaning stage. Once the gas is cleaned, it is compressed to 12.6 MPa to acquire the feed gas for the Fischer Tropsch production (Hanaoka et al. 2007).

Researchers in Medicinal Plant Cultivation Research Center Beijing, China have shown that more amount of cellulose with less crystalinity, lignin and hemicellulose can improve the yield of ethanol from woody biomass. Stupendous efforts have been employed to achieve this by genetic modifications in the woody feedstock (Lu et al. 2010).

## 5 Third Generation Biofuels

### *5.1 Algae*

Algae are a promising biofuel resource because of their ability to transform the energy from the sun into chemical energy. The most important of all algae are the microalgae. They are a possible foundation of renewable energy production such

as, bio-oil and gas by thermochemical reaction while using biochemical reaction for the production of ethanol, biodiesel and bio hydrogen (Kwiatkowski et al. 2006; Deirue et al. 2012). The production of algal biomass is a lot more cost worthy than the crops; this is because photosynthesis requires light, carbon dioxide, water, specific range of temperature (293–303 K) and inorganic salts. So in order to minimize the expenses, the production of biodiesel must mainly rely on the free available sunlight (Liu et al. 2012). The benefits of consuming microalgae as a source of production of biofuels are removal of carbon dioxide from industries and environment by algae bio-fixation, reducing the GHG emission while producing biodiesel, allowing algae to develop as consumers of water wastes as nutrients present in wastewater, for the removal of ammonium, nitrate, phosphate. The oil harvested from the subsequent algae biomass can be processed into ethanol, methane, livestock feed, which can be useful as carbon-based fertilizer or energy cogeneration (Chisti 2007; Amin 2009). Microalgae can produce high quality organic products with numerous probable profitable claims. These can possibly transform a bulk of biotechnological regions containing biofuels, pharmaceuticals, cosmetics, nutrition and food flavors, aquaculture, and pollution avoidance (Raja et al. 2008; Rosenberg et al. 2008; Wang et al. 2008).

Some of the important microalgae culture systems are:

i. **Open culture system or ponds:**
Open culture systems are the simplest and oldest technology used for the mass cultivation of micro-algae, also called algae farms, possessing a raceway configuration. They are usually made up of concrete or simple dug in the ground and out lined using plastic, preventing the liquid from seeping into the ground. The wheel paddle mixes and circulates the algal cells and nutrients. The system is worked in unceasing style, fresh feed is continuously placed and algal broth is harvested (Mata et al. 2010). The water in open ponds is shallow, thus allowing the algae exposure to sunlight. The efficiency is calculated in terms of biomass. These ponds under proper pH control and other physical condition can utilize up to 90 % of the waste $CO_2$ injected in the pond, thus helping in $CO_2$ fixation. The efficiency is reduced by infection with unwanted algae and microbes that consume algae. They increase the problems of land usage price, water accessibility and climatic circumstances (Richmond 2004; Mata et al. 2010).

ii. **Photo bioreactors:**
Photo bioreactors are closed arrangements in which algae are allowed to grow (Richmond 2004). They prevent direct fall out, avoiding chances of contamination but are expensive compared to open ponds. They have higher efficiency, greater biomass concentration, short harvest time and higher face to volume ratio than open ponds. The highest cost associated with closed arrangement is associated with energy price beside the mixing apparatuses (Richmond 2004; Amin 2009).

There are many different types of closed systems, some of them are:

a. **Tubular photo bioreactors:**
Tubular photo bioreactors consist of flexible plastic or glass tubes commonly positioned parallel to one another or flat beyond the ground to increase surface to volume percentage of the container. The pipes allow the penetration of light and the developmental phase mingles from reservoir to reactor and back to the reservoir. The blustery movement in the reactor permits the delivery of nutrient, increases gas exchange, lessens the cell sedimentation and flow biomass for equal enlightenment (Lee 2001).

b. **Flat plated photo bioreactors:**
These have large surface area which allows greater photosynthesis, low buildup of oxygen being dissolved, and immobilization of algae. They are cheap but great surface area roots for highly unacceptable problems, such as temperature regulator, $CO_2$ diffusion frequency and affinity of the algae to wall adhering (Lee 2001).

c. **Inclined triangular tubular photo bioreactor**:
Inclined triangular tubular bioreactor is similar to power plant using flue gas. Flue gas enters reactor from the bottom. Gas bubbles move in the pipe producing eddies for mixing and avoidance of entangling. The upper surface of pipe permits absorption of solar energy. The mixing of the flue gas and the algae culture allows growth to the extent that 15–30 % algae can be harvested each day (Ugwu et al. 2008).

d. **Continuous stirred tanks:**
Continuous stirred tanks have wide, hollow, capped pipe, cylindrical in shape which has low contamination risk and can be used both indoor and outdoor. The light source and mechanical stirrer are placed at the top while draining canals and gas injectors are placed at the foot of the reactor. The blustery movement allows growth and avoids fouling (Kwiatkowski et al. 2006).

iii. **Hybrid systems:**
The hybrid system uses open and closed system in combination to obtain better results. The hybrid system is the most reasonable option for cheap and more crop growing strains for biofuel production. Open ponds are injected with wanted strain of algae and is cultivated in bioreactor. The size of inoculum should be larger than undesired species. To avoid contamination, cleaning and flushing ponds must be a portion of aqua culture and are considered as batch cultures (Kwiatkowski et al. 2006; Sananurak et al. 2009).

## 5.2 Harvesting of Microalgae

Separating algae from its medium or algal biomass concentration is called harvesting (Rosenberg et al. 2008). It is a chief difficulty since algae are typically found in water, so collection of algae is problematic and energy consuming procedure (Schenk et al. 2008). The common methods used for harvesting are centrifugation, foam fractionation (Haesman et al. 2000), flocculation (Csordas and Wang 2004), membrane filtration (Knuckey et al. 2006) and ultra-sonic separation (Rissignol et al. 2000). Harvesting contributes to 20–30 % of total expenditure (Bosma et al. 2003). The harvesting method depends on algal species used, cell density and culture conditions (Mata et al. 2010). The four main steps of harvesting are given below:

i. **Screening:**
Screening is the first step in harvesting. The main principle is to introduce algae biomass to a screen having specific aperture size, whose efficiency depends on alga size and screen opening (Grima et al. 2003).

**a. Micro straining:**
Micro strainer has a rotator drum which is covered by straining fabric, polyester or stainless steel. The small algae aren't screened properly so there is a need of dewatering as harvest is low (Bosma et al. 2003). The problems encountered by micro staining are particle fluctuations and low harvest, which are solved by specific speed of drum rotation. The algae and bacteria also form a biofilm on the fabric whose periodic cleaning is very essential for the process to occur effectively (Bosma et al. 2003; Grima et al. 2003).

**b. Vibrating screen:**
Vibrating screen is mainly used for *Spirulina* harvest, which is a multicellular and filamentous microalgae used as a food source. This process removes high number of algae (90 %) for harvesting up to 21 $m^3$ $h^{-1}$. Algae slurry of 8–10 % is formed, which covers 1/3 of area only when compared to inclined screens, which cover 2–4 $m^2$ per unit (Bosma et al. 2003).

ii. **Thickening:**
Thickening is the process which increases the solid concentration of algae so that it may be further treated. This process reduces the volume, which reduces downstream treatment cost. Some important methods are briefly described below:

**a. Coagulation- flocculation:**
Coagulation flocculation causes aggregation of algal cells into clumps for further treatment. This process uses both organic and inorganic coagulants. Chitosan; a water purification product; can also be used for this purpose. The chemical flocculation can be done as well but the main problem is that it is expensive. The coagulant dosage used is very important and determined by bench scale jar tests (Bosma et al. 2003; Grima et al. 2003).

b. **Gravity sedimentation:**
Gravity sedimentation process involves the solid-liquid separation, separation of effluent as a clear liquid and feed suspension into slurry having high concentration. It separates algae in which clarity of overflow has primary importance and algal feed suspension is dilute or concentrated when thickening of underflow is done. This works best with heavy algae suspension (Bosma et al. 2003).

**c. Flotation:**
Flotation is gravity thickening but upside down. The liquid-solid separation is done by bubbling at the bottom of the tank rather than waiting for the particles to settle. Once the particles reach the top of tank, thick layered slurry is formed which can now be collected by skimming operation. This process uses light particles having low settling velocity, not allowing them to settle in the tank. The main advantage of this process is that light algae particles are harvested in short duration (Bosma et al. 2003; Grima et al. 2003).

d. **Ultrasound technique:**
Ultrasound is used for harvesting algae but results show that its efficacy is less because of small size and low particle density of algae. This technique is useful in removing *M. aeruginosa*-a toxic algae species (as it causes the formation of harmful algal blooms)-by its coagulation (Bosma et al. 2003; Grima et al. 2003).

iii. **Dewatering:**
Dewatering involves the removal of water from the algae to the maximum extent (Bosma et al. 2003; Grima et al. 2003). This is done by various methods; some are described below:

a. **Filtration:**
Filtration is done by forcing algae material to flow across filter medium and water is drained out with the help of suction pump and algae mass is retained. The main use of the process is that algae biomass of low density can be harvested. Filtration includes backwashing as a routine practice to avoid clogging and fouling (Bosma et al. 2003; Grima et al. 2003).

b. **Centrifugation:**
Centrifuges are analogous to sedimentation tanks, the one difference being the separation by centrifugal force (higher than gravity), thus causing acceleration of suspended particles. The higher the rotation speed of centrifuge, the quicker the solid will spin out of the suspension. The supernatant is removed using skimming tube. This method is efficient and reliable but expensive (Bosma et al. 2003; Grima et al. 2003).

iv. **Drying:**
The last step of harvesting is the drying of algae, which is really challenging, as algae are really delicate and can degrade reducing the quality of production. Rotary dryers have sloped cylinders which dry by moving content from one side and gravity to the other. Spray drying is an effective way of drying algae used

as food. Solar heat drying is another technique used for this purpose (Bosma et al. 2003; Grima et al. 2003).

## *5.3 Energy from Algae*

Microalgae are used in the production of various number of biofuels by using several methods such as thermochemical, biochemical and chemical for the manufacture of bio oil, biogas, biodiesel, bio ethanol, bio methane and bio hydrogen (Deirue et al. 2012).

### 5.3.1 Biodiesel Production

There are four main methods for biodiesel production which are briefly described below but the main process used by microalgae is trans-esterification:

i. **Direct use and blending:**
This involves the use of plant oil directly as bio diesel without any changes in the engine. The primary concern of vegetable oil as a fuel is high viscosity, its least ability to atomize, leading to problems in the long run such as carbon deposits, coking, thickening and gelling of fuel, oil ring sticking, lack of atomization etc. (Gupta 2004).

ii. **Micro- emulsions for bio diesel production:**
Micro-emulsion is a colloidal dispersion of fluid micro structures in solvent forming two immiscible phases. They are of low viscosity hence their atomization is easy. The solvents used are usually methanol and ethanol (Gupta 2004).

iii. **Pyrolysis for bio diesel production:**
Pyrolysis is the transformation of one element into another by the use of heat energy. Catalysts are used for this process to speed up the reaction. Different products are obtained using this process. The vegetable oils of low hydrocarbons are produced using this method which are used as fuels (Gupta 2004).

iv. **Trans esterification for bio diesel production using micro algae:**
The microalgae produce bio-diesel using lipids or oil present in it. Biodiesel is typically produced using plant oils, thus called green diesel. The reason why micro algae are considered for the production of biodiesel is because these produce additional oil, require minimum space and can be developed in areas inappropriate for agriculture, but they do require more fertilizers (Deirue et al. 2012). Biodiesel production via microalgae using trans esterification involves a production unit to grow microalgae cells, trailed by separation of the cells from growing medium and lipid mining.

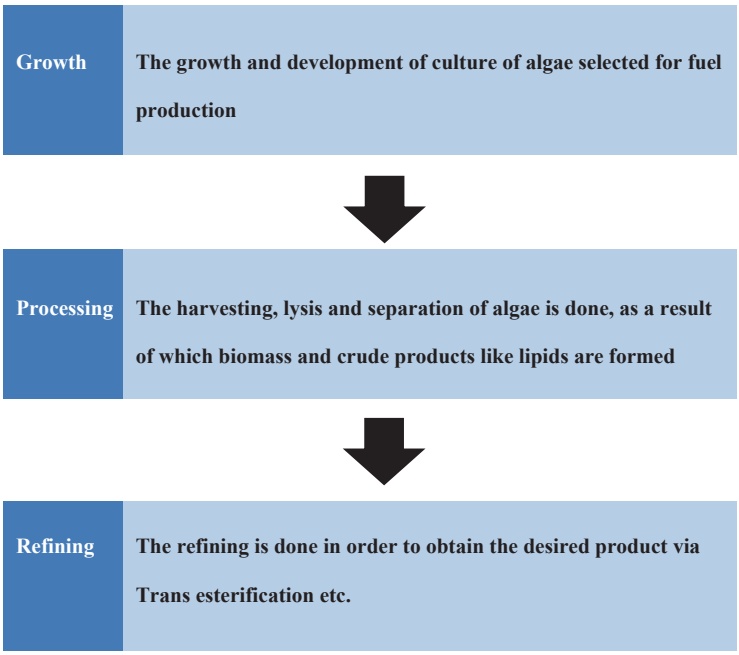


**Fig. 2.5** The steps involved in bio diesel production along with other useful by products using micro algae

The basic steps for biodiesel production are:

a. **Growth of algal culture:**
First of all, an algal strain is selected for the maximum production of biodiesel. Usually a strain with high lipid content is selected for this purpose. The lipid content can also be enhanced by medium modification which usually involves low nitrogen concentrations. In addition to nutrient concentration, the culture conditions too should be specific for algal growth, like pH, temperature, $CO_2$ etc. depending on the strain used (Gupta 2004).

b. **Harvesting and trans esterification of algal culture for bio diesel:**
The processing of algal culture is the harvesting of algal biomass for biodiesel manufacture. The chief process used for biodiesel making is called trans esterification. In this process exchanging of alkoxy group of an ester compound occurs by an alcohol. It involves multi-step in which fat or oil reacts with alcohol in order to produce ester and glycerol. Thus trans-esterification transforms algal oil into biodiesel as algal oil is assorted with alcohol and with an acid or a base catalyst in order to yield fatty acids methyl esters that mainly form biodiesel (Deirue et al. 2012). The whole process of biodiesel production by trans-esterification and various other steps is explained in the schematic Fig. 2.5.

### 5.3.2 Bio-hydrogen Production from Microalgae

The starches are fermented using a group of bacteria for the production of hydrogen and $CO_2$ in this method. The organic waste water has a really high amount of biomass for hydrogen production using microbial organisms (Beal et al. 2011). Hydrogen gas produced by autotrophs is a popular way of using renewable energy. The hydrogen gas is formed by microalgae using different methods, for example, direct and indirect photolysis and ATP driven hydrogen development etc. Main factors which affect the price of bio hydrogen produced by algae are the expenditure on enormous photo bioreactor and hydrogen storage equipment that allows continuous supply of hydrogen (Deirue et al. 2012).

Main methods for bio hydrogen production are:

i. **Direct bio photolysis**:
Photosynthetic hydrogen is produced from water as shown below (Kotay and Das 2008):

$$2H_2O \rightarrow 2H_2 + O_2$$

In algal photosynthesis water is oxidized and oxygen is evolved. The photosystem II generates electrons with the help of light energy and transfers it to ferredoxin, via energy captivated by photosystem I. The electrons are accepted by reversible hydrogenase from reduced ferredoxin for $H_2$ production (Nath and Das 2003). Hydrogenase is responsible for hydrogen gas and oxygen production and thus is extremely delicate. The photosynthetic production of molecular hydrogen along with oxygen should be separated, hence it is a process with two phases. $CO_2$ is fixed into hydrogen abundant substrates in the photosynthesis, which is trailed by molecular hydrogen gas formation via light mediated reaction when the microalga is incubated for anaerobic conditions. This can be attained by incubating microalgae in a phase that is sulfur free, then oxygen synthesis and $CO_2$ fixation rate declines and hydrogen gas synthesis rate increases (Kotay and Das 2008).

ii. **Indirect bio photolysis:**
Indirect bio photolysis can harvest hydrogen gas using water and has the capability to fix nitrogen gas from surroundings. The main reaction is given below (Kotay and Das 2008):

$$12H_2O + 6CO_2 \rightarrow C_6H_{12}O_6 + 6O_2$$
$$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6CO_2$$

Many species of blue green algae also called cyanobacteria possess the enzymes which are involved in the synthesis and metabolism of molecular hydrogen. The important enzyme used is nitrogenase, which helps in the oxidation of hydrogen gas as a byproduct of nitrogen reduction to ammonia. The bidirectional hydrogenases oxidize and synthesize $H_2$ (Kotay and Das 2008).

iii. **Photo fermentation:**
The molecular hydrogen is evolved by purple non sulfur bacteria using nitrogenase, in nitrogen deficient conditions, in the presence of energy and reduced compounds. The main reaction is as follows (Kotay and Das 2008):

$$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6CO_2$$

Photoheterotrophic bacteria have the potential of converting light energy to hydrogen gas by using waste organic compounds in batch processes, continuous cultures or cultures of bacteria immobilized on several materials but the hydrogen manufacture using these bacteria is advanced when cells are restrained in or on a solid matrix related to the cells that are free-living (Nath and Das 2003).

**iv. Hydrogen production by water gas shift process:**
Some photoheterotrophic bacteria have the ability to grow in dark by using CO as a source of carbon in order to generate ATP releasing hydrogen gas and $CO_2$. The process of oxidation of CO to $CO_2$ with the release of hydrogen gas takes place by water gas shift reaction, which occurs at low pressure and temperature. The process is explained below via equation (Nath and Das 2003).

$$CO(g) + H_2O(l) \rightarrow CO_2(g) + H_2(g)$$

**v. Dark fermentation:**
Dark fermentation produces hydrogen gas with no light energy requirements. Variation of carbon sources are used for this purpose. This is an anaerobic procedure as there is no oxygen limitation problem. The hydrogen producing anaerobic bacteria are developed in dark on high sugar substrates. The process of fermentation can be done at different temperature ranges (Kotay and Das 2008).

In the above methods pure hydrogen gas is produced but biogas containing mainly hydrogen gas and $CO_2$ in this process but less amount of methane, CO and $H_2S$ are present. The bacteria used for the production of hydrogen gas via this process include *Enterobacter, Bacillus, Clostridium* etc (Kotay and Das 2008).

Different amount of hydrogen gas can be produced liable on the fermentation pathway used and products formed (Azbar in press). For example when acetic acid is the main product four moles of hydrogen gas are produced. The reaction is given below (Kotay and Das 2008):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$$

vi. **Two-stage hybrid system**
The two stage hybrid system uses the combination of photo and dark fermentation for better production of hydrogen gas. This process utilizes the substrate to the maximum limit which else fails to reach complete transformation due to thermodynamic restriction (Nath and Das 2003).

**Table 2.6** The representative organisms and hydrogen production by each process. (Kotay and Das 2008)

| Process | Representative organism | Maximum reported rate (mmol $H_2$/Lh) |
|---|---|---|
| *Direct biophotolysis* | Chlamy domonas reinhardii | 0.07 |
| *Indirect biophotolysis* | Anabaena variabilis | 0.36 |
| *Photo fermentation* | Rhodobacter spheroides | 0.16 |
| *Dark fermentation* | Enterobacter cloacae DM 11 | 75.60 |
| | Clostridium sp. Strain No.2 | 64.50 |
| *Two-stage fermentation* | Enterobacter cloace DM 11+Rhodobacter sphaeroides OU 001 | 51.20 |
| | Mixed microbial flora + Rhodobacter sphaeroides OU 001 | 47.20 |

The first step of this system involves fermentation of biomass to acetate, hydrogen and $CO_2$ using a thermophilic dark reactor. In the second step the acetate is used in an isolated photo bioreactor for conversion into $CO_2$ and $H_2$. This arrangement can result in reaching the worth nearer to the theoretical determined manufacture of about 12 moles of hydrogen gas (Nath and Das 2003). The reactions are:

(a) Step I—dark fermentation

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \,[123]$$

(b) Step II—Photo fermentation

$$2CH_3COOH + 4H_2O \rightarrow 8H_2 + 4CO_2 \,[123]$$

Table 2.6 shows all the processes along with organisms used and the amount of hydrogen gas produced by each process. The table clearly shows that two stage methods produce the maximum amount of hydrogen while direct photolysis process produces the least amount of bio hydrogen (Kotay and Das 2008).

## 6 Economic Aspects of Biofuels Around the Globe

There is a huge market of motor vehicles and fuel industry. The current scenario of fossil fuel availability and prices is not very encouraging to completely depend on it, so the possible and reliable alternative is biofuels and they are massively growing in the market day by day. In developed countries like USA there is already a saturation of vehicle ownership which is expected to increase more at current rate. In developing countries the vehicle ownership is growing very rapidly and is expected to surpass the total number of vehicles by 2030. There is a worldwide estimation of about 982 million vehicles which is expected to rise to 2.6 million by 2050. Once people purchase a vehicle they use it for convenience in travel which means that

with the growing market of vehicle there is an obvious increase in fuel demand, fossil fuel alone cannot meet this demand both in terms of availability and price factor (Junginger et al. 2010)

While biodiesel and wood pellets are almost exclusively traded as an energy carrier, bioethanol may also be used in other end-uses. Approximately 75 % of the traded bioethanol is used as transport fuel. As far as global trade and market of biofuels is concerned it is growing at very rapid rate especially the biomass from feedstock, due to their easy availability like vegetable oils and agricultural remains. Popular forms of fuels are mostly bioethanol and biodiesel. The current estimate of the market trends of biofuels is not very impactful which is around 1EJ that make up a total of 2 % of current use of bioenergy based fuels. It is assumed that large quantities of biofuels will be traded internationally with Latin American and African countries as the potential exporters and Asia, Europe and North America as the potential importers (Heinimo 2011). Although small scale research and production is being carried out in almost all parts of the world, the sustainability and growth of bioenergy is largely based on trade.

## *6.1 Leading ethanol Producers 2008*

The two prominent ethanol producers of 2008 are Brazil and USA with USA producing 26.8 million t and Brazil 2.6 million t. These combined form 91 % of the ethanol global production. In terms of consumption USA is the largest consumer of bioethanol with the consumption of 28.4 million t in 2008. Out of the net amount 4.6 % is imported. Next is Brazil with consumption quantity reaching up to 16.5 million t. The net utilization of bioethanol in EU is 2.6 million t for transportation and Sweden, France, Germany and Netherlands top the list of consumers (Spelter et al. 2009). There is an unavailability of proper code systems to keep a track over the production and consumption activities of biofuels, so the data is insufficient and incomplete especially in terms of ethanol consumption which is also used as beverage, industry and fuel. It is difficult to quantify the data on production and consumption of ethanol as a source of biofuels only.

## *6.2 Wood Pellets Role and Trade*

Wood pellet provides a potential raw material source for the production of biofuels after several processing steps. Wood pellet production in USA and Canada has been around 680,000–690,000 t.

Last decade brought great deal of trade and consumption of wood pellets and it has grown on mass scale. Its production is mainly centered in North America (1.4 million t) and Europe (8–10 million t). The overall consumption of wood pellet is higher in European countries as compared to USA (Spelter et al. 2009).

**Table 2.7** Percentage of crops used as feedstock for production of biofuels

| Country | Crop used as feed stock for fuel production | Percentage used for fuel production (%) |
|---|---|---|
| USA | Corn | 40 |
| EU | Vegetable oil | 65 |
| Brazil | Sugarcane | 50 |

## *6.3 Ethanol Economics Scenario in Brazil*

In countries with warmer climate like Brazil and India sugar cane is one of the basic raw material source for ethanol production and is produced in massive quantities. Brazil is producing low cost ethanol and the prices have dropped considerably in 2002–2003. It is also used as an additive with gasoline as vehicle fuel. In 2003, many motor vehicles gained prominent position in market which had flexibility in operation with fuel additives like E20–E25.

## *6.4 Biofuels Scenario from Present to 2021*

Biofuels have a deep association and at the same time strong impact on agricultural practices and market trends. The use of different crops; used as feed stock; for biofuel production as well as strong players of the production, USA, EU and Brazil, are listed in Table 2.7 below which is the percentage of crops devoted for the purpose of fuel production in 2008. It is very clear that decision making and investment in agriculture cannot be made without taking into account the share of biofuels in it.

Ethanol price dropped down in 2008 and rose to higher prices in 2011 again. The reason for the increase in the price of ethanol was dwindling ethanol production in USA and decrease in the production of sugar cane in Brazil, which affected the overall increase in the cost. Apart from ethanol, the overall biofuel prices went up in 2011 (Zurbier and Van De Vooren 2008).

Ethanol prices rose beyond the previous records of 2007 and 2008 in 2011 and decreased slightly in 2012. The drop or rise in the price of ethanol is dependent on the prices of ethanol manufacturing raw material. There are various methods of ethanol production but it is mostly produced from feed stock, so production method and cost to the end user depends on the price and availability of the feedstock or any other raw material being used. In the case of other well-known biofuel i.e. biodiesel the prices declined in 2011 (Demirbas 2009a, b).

## *6.5 Development in Global Ethanol Market*

Ethanol production has almost doubled over a span of a stretch of 3–4 years and by 2021 it will reach a total of 180 Bnl and the three major players in ethanol produc-

**Table 2.8** Biofuel produced in different countries and used as transport fuel. (Source: OECD and FAO secretariats)

| | 2009–2011 | | | 2021 | | |
|---|---|---|---|---|---|---|
| Country | Total | Biofuels | Share of biofuels (%) | Total | Biofuels | Share of bioafuels (%) |
| *Argentina* | | | | | | |
| Gasoline type | 3.5 | 0.1 | 2.7 | 4.1 | 0.1 | 3.4 |
| Diesel type | 9 | 0.3 | 3.2 | 11 | 0.4 | 4.0 |
| *Australia* | | | | | | |
| Gasoline type | 15 | 0.2 | 1.3 | 947 | 0.3 | 1.5 |
| Diesel type | 16 | 0.5 | 3.1 | 18 | 0.5 | 3.1 |
| *Brazil* | | | | | | |
| Gasoline type | 23 | 11.0 | 47.0 | 29 | 18.9 | 64.2 |
| Diesel type | 40 | 1.6 | 4.0 | 34 | 2.4 | 4.6 |
| *Canada* | | | | | | |
| Gasoline type | 30 | 0.8 | 2.6 | 32 | 1.1 | 3.4 |
| Diesel type | 26 | 0.1 | 0.7 | 28 | 0.4 | 1.6 |
| *China* | | | | | | |
| Gasoline type | 61 | 1.1 | 1.8 | 104 | 1.4 | 1.3 |
| *EU* | | | | | | |
| Gasoline type | 103 | 2.8 | 2.7 | 103 | 8.6 | 8.3 |
| Diesel type | 189 | 9.4 | 5.1 | 200 | 16.7 | 8.5 |
| *USA* | | | | | | |
| Gasoline type | 409 | 21.9 | 5.4 | 412 | 45.0 | 10.9 |
| Diesel type | 215 | 1.9 | 0.9 | 249 | 3.8 | 1.5 |

tion are expected to be USA, EU and Brazil. Flex fuel vehicle industry in Brazil is a major factor for large scale production of ethanol in this country while some constitutional amendments are being made in the USA and EU for the development of biofuel specially ethanol market (Thoma 2012). In case of biodiesel, world's largest producer is EU and by 2021 the global biodiesel production is expected to reach above 42 Bnl. Other major producers of biodiesel are: Argentina, Thailand, USA, Brazil and Indonesia. Table 2.8 presents a very good illustration of biofuel production by various countries, which is potentially used for the purpose of transportation in context of energy.

## *6.6 Different Production Methods for Bioethanol*

Bioethanol production methods differ from place to place; from maize in the USA and Brazil, and from wheat and sugar beet in Europe. Table 2.9 highlights main bioethanol producing countries and their net production. It is evident from the table that the largest bio-ethanol producers of the world are USA and Brazil.

**Table 2.9** Major bioethanol producing countries of the world. (Source: German Advisory Council on Global Change 2008)

| Country/Region | Production | |
|---|---|---|
| | Amount (in Billion Liters) | Production (%) |
| European Union | 2.3 | 4.4 |
| United States | 26.5 | 51.0 |
| Brazil | 1.7 | 16.5 |
| China | 1.3 | 15 |
| India | 0.4 | 0.8 |
| World | 52.0 | 100.0 |

## *6.7 Biodiesel Global Production*

Around 10.2 billion l of biodiesel was produced on global scale in 2007 and production has increased since then and is expected to increase in the coming years as it has increased ten fold since 2000. The world's largest biodiesel producer is Europe where rapeseed is used for this purpose, palm oil is used in Indonesia and Malaysia and soybean in Brazil. A green place analysis showed that 20 % plant biodiesel is produced from soya in Germany as is clear from Table 2.10. Some biofuels are also produced from the material produced after processing of different crops and data is available on these activities. Some of the cultivation areas are not solely dedicated to biofuel production, so it cannot be marked precisely enough on geographical basis. Production of biofuel generating crops on cultivation areas is also not very old process, because of these factors it becomes difficult to establish direct relationship of deforestation and biofuel production (Jank et al. 2007).

## *6.8 Policy Making to Enhance Biofuel Production*

Commercial production of biofuels has been taking place since very long in different regions of the world and special policies have been made in USA and Europe to increase the production of biofuels as per demand, which has increased significantly mostly because of diminishing supply of standard fuel, sky high prices of petrol and diesel, global warming and greenhouse gas emissions. Thus biofuels are considered very appropriate alternative in fuel production especially for the purpose of vehicle engine ignition (Thoma 2012).

## *6.9 Biofuel Controversy*

There are two contrasting schools of thoughts when it comes to production of biofuels.

**Table 2.10** Cost of ethanol in Germany, a comparison between US and EU

| Germany | | | | | | Cost difference |
|---|---|---|---|---|---|---|
| Plant capacity | 50 million l | 200 million l | | 53 million l | | |
| Raw material | Wheat | Beet | Wheat | Beet | Corn | |
| | a | b | c | d | e | Case a minus case e |
| Feed stock cost | $ 0.28 | $ 0.35 | $ 0.28 | $ 0.35 | $ 0.21 | $ 0.07 |
| Co-product credit | −$ 0.07 | −$ 0.07 | −$ 0.07 | −$ 0.07 | −$ 0.07 | $ 0.00 |
| *Net feedstock cost* | $ 0.21 | $ 0.28 | $ 0.21 | $ 0.28 | $ 0.14 | $ 0.07 |
| *Labour cost* | $ 0.04 | $ 0.04 | $ 0.01 | $ 0.01 | $ 0.03 | $ 0.01 |
| *Other operating and energy cost* | $ 0.20 | $ 0.18 | $ 0.20 | $ 0.17 | $ 0.11 | $ 0.09 |
| *Capital cost recovery (net Investment cost)* | $ 0.10 | $ 0.10 | $ 0.06 | $ 0.06 | $ 0.04 | $ 0.06 |
| *Total pre gasoline equivalent* | $ 0.55 | $ 0.059 | $ 0.48 | $ 0.52 | $ 0.32 | $ 0.23 |
| *Total per gasoline equivalent liter* | $ 0.81 | $ 0.088 | $ 0.71 | $ 0.77 | $ 0.48 | $ 0.34 |

### 6.9.1 Negative Impacts

One perspective is that production of biofuels will cause increase in emission of greenhouse gases, deforestation especially in the tropical areas as well as food insecurity and may cause disputes among small scale producers and farmers.

### 6.9.2 Positive Impacts

The other one which is in support of biofuels states that it can develop new employment opportunities if crops are grown on agricultural land, it will not threaten forests and is the clean burning alternative of fossil fuels ( Keyzer et al. 2008).

## 7 Challenges Faced in Determining Direct Relation of Biofuels to Deforestation

Deforestation takes place due to a number of reasons and also not very reliable and sufficient data is available on world deforestation (Gamett 2009).

### *7.1 Deforestation Definition by UNFCCC*

There is well established or accepted definition for the term deforestation as it does not depend on only one or two factors. Deforestation as defined by the United

Nations Framework Convention on Climate Change (UNFCCC) as the threshold in the canopy cover that is selected by each country and if it falls below this threshold it is termed as deforestation.

## *7.2 Deforestation Data by FAO*

FAO has published the deforestation report of different countries which by far is the only dependable source of data in this area. The organizations of researchers that use this data report that this data is taken and defined differently by different countries of the world (Demirbas 2009a, b).

## *7.3 Different Techniques Used by Different Countries*

The developed and underdeveloped countries produce different results with better and more reliable results produced by the developed countries with latest and accurate equipment for the purpose. For example satellites and better survey analysis methods are used. The results of underdeveloped countries are not so reliable because of out-dated and less accurate techniques. The data submitted to FAO by different countries depends upon different methods of data estimation, different techniques and different area coverage per analysis. Also different parameters and definitions are presumed by different countries on deforestation so the overall data and comparison remains distorted and uneven. It is very difficult to verify techniques and double check the data from different countries on deforestation (Demirbas 2009a, b).

## *7.4 Dilemma of Developing Countries*

The scenario of developing countries is even worse when it comes to taking data on deforestation. These countries, due to lack of resources, use outdated means and methods of land classification and are also not very well characterized and dependable. Thus the interpretation on the available data gives relatively false results. Around 11.8 million ha per year was the land subjected to deforestation between 2000–2005 worldwide, 80 % of the deforested area is in tropical areas of America and Africa. Three prominent biofuel producing countries have shown increased rates in deforestation which include Brazil, Indonesia and Argentina.

# 8 Effects of Bio-Fuels on Food Security

## *8.1 Use of Land*

One of the obvious impacts of biofuels on food security is the use of land to cultivate feedstock or raw material for biofuel production, instead of conventional food on that land.

## *8.2 Price and Availability of Food to the Poor*

The other potential impact of biofuels on global food security is the availability of food to poor and also because the division of land resources for food production may hamper adequate availability of food to the poor (DeFries et al. 2007).

## *8.3 FAO 2008 Report*

An estimate provided by the FAO report in 2008 establishes that biofuel production can cause an increase in prices and can also undermine food self-sufficiency at national levels. The fuel can be provided at comparatively low prices but at the same time people would have to face high food prices. It is assumed that the crises in the prices of sugar crop in Brazil and soybean and corn in USA in 2007–2008 has been due to production of biofuels from these crops. According to the emerging trends it is predicted that increasing demand of biofuels will cause an increase in the food prices on global scale.

## *8.4 Use of Marginal Lands*

The unproductive areas also referred to as the marginal areas are not assumed to be imposing any harmful environmental or social impacts but Rossi and Lambrou (2008) pointed out that these areas support income of some people especially those who depend on this area in the time of need. Apart from the issues of land ownership by the local people, the land is used by biofuel producing companies which jeopardize the social, economic and also cultural setup of these areas (Tengnäs 2012).

## *8.5 Water Resources*

One of the other concerns over the use of biofuels is the division of available land for the growth of food crops or biofuel producing crops. The crops mainly used for

biofuels production are palm oil, *Jatropha* and soybean. Palm oil used for biodiesel production grows best in adequate quantity of water. *Jatropha* on the hand can grow in less fertile land and in lesser quantity of water but its yield is less in such areas as compared to that grown in fertile land with heavy water supply. Also the pesticides that are used on biofuel producing crops cause pollution and pose health problems (Robinson and Harris 2012).

## 9 Conclusion

The enthralling increase in the development of the Industrial sector(IT) since the advent of Industrial Revolution has, much to everyone's belief led to the paucity of natural oil reserves. As most biofuel evangelists are in a dedicated endeavor to expunge the dependence of most industrial markets on oil, it needs to know whatsoever elucidation, that the time to permanently switch over to alternative sources of energy has indeed arrived. But when it comes to confronting issues like agricultural crisis and environmental losses that are concomitant with the production of biofuels, all efforts are nullified and the reversion back to step one occurs. It has been estimated that the increase in global biofuel production has led to 12% increase in the global food prices. However since USA accounts roughly for 60% of the total increase in food prices, the total increase is minute, hardly 7%. Thus it can be deduced that the rise in global food prices is due to many factors other than biofuels alone.

Issues like climatic concerns and global food crisis have denigrated the image of biofuels by inveigling the community into conceding that biofuels weigh more in detrimental aspects. It is crucial to address these issues by altering the framework that developed countries implement for the development of biofuels. This framework is beyond faulty. The first step is to be taken to understand that the primary reason for the aggravation of global energy insecurity is because of the monopoly played by the American and European TNCs (transnational corporations) in the energy sector. Currently most biofuel production programs are designed in such a way as to coerce Third World countries into reducing themselves into the hands of capital and technology provided by the First World. This approach has been adopted by the foreign companies to exploit and benefit from the Third World resources, which leads to increased sensitization of domestic food security, interests of the farmers and environment. Even the positive environmental effects contributed by the biofuel production will be ephemeral due to the current framework of monopoly control. As recent studies show its large-scale production is even more deleterious to the environment, hence it can be correctly concluded that for independence of oil, alternative energy resources should be handled and controlled by government bodies and not foreign bodies that have conceited motives. They should be built against neoliberal restructures and imperialist plunder of the Third World in order to restore agrarian reform and food security.

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# Chapter 3
# Engineered Phyto-Covers as Natural Caps for Containment of Hazardous Mine and Municipal Solid Waste Dump Sites–Possible Energy Sources

**M. N. V. Prasad**

**Abstract** In India the estimated mineral deposits are about 20,000 representing about 60 different kinds. At most of these reserves mining activity is in progress. Abandoned mine sites and smelting areas have often been neglected for restoration. Ecorestoration is a necessary step failing which the detrimental effects would be loss of forest cover, leaching of toxic substances, contamination of cultivated land and ultimately posing threat to biodiversity. Establishment of phyto-cover for containment of hazardous waste, and municipal solid waste; phyto-treatment technologies for environmental remediation and possibilities for energy generation are presented in this paper.

**Keywords** Aluminium smelter · Constructed wetland · Engineered vegetative cover · Fly ash · India · Odisha · Phyto-treatment · Spent pot-line

## 1 Introduction

The demand of natural resources by the global population of 7 billion is resulting in technogenic and anthropogenic pollution. Often these pollutants are piled up resulting in hazardous waste. Unless these hazardous waste dumps are handled properly, the toxic spills keep spreading uncontrollably in the environment "a true chemical time bomb". We are currently living in a world of insecurities. Often we refer to environmental security, energy security, food security, nutritional security and water security etc.

India is forging ahead to attain high GDP growth rate to achieve the status of industrialized and developed world 2020. In order to achieve this heavy consumption of natural resources is inevitable. The other side of the coin of this task is enormous waste generation. The waste management summit held in 2012 has estimated

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that the poised industrial growth would generate 100 million tons of non-hazardous solid waste, 6–7 million tons of hazardous waste annually.

Thus, every one of us are being exposed to contamination from past and present industrial practices. The risk to human and environmental health is rising and there is evidence that this cocktail of pollutants is a contributor to the global epidemic of cancers, and other degenerative diseases. These pollutants belong to two main classes: inorganic and organic. The challenge is to develop innovative and cost-effective solutions to decontaminate polluted environments. Nature's cure using plant resources (=Phytoremediation) is a sustainable solution for environmental decontamination. As of now about 1,8000 articles have been published on various aspects of using biological resources for environmental cleanup starting with only 11 in 1989 (Prasad 2011; Prasad and Prasad 2012).

In natural capping technologies (=engineered vegetative capping), natural resources offer great potential to contain and even decompose hazardous chemical waste. For e.g. the remediation work carried out at Volgermeerpolder, Netherlands is a classic example wherein the local peat lands exhibited excellent containment capabilities. Using peat instead of expensive construction materials in the remediation design resulted in significant reductions in public expenditure. The concept of the natural cap means that the pollutants are being contained by natural vegetation and associated processes by exploiting native biodiversity (Prasad 2001). In the case of the Volgermeerpolder, Netherlands chemical hazardous waste dump site, this has been accomplished by creating a living peat land (moors and bogs) on top of the plastic foil that is used to cover the dumpsite. By the time the foil deteriorates, the newly formed peat bog will assume phytostabilization function. In natural capping technology hydrology (infiltration control), biogeochemistry (isolation and immobilization of hazardous waste) and ecology (venting explosive and combustible toxic gases, controlling decomposition of organic waste) are integrated/monitored with engineered construction and maintenance

A sustainable and ecofriendly approach for redevelopment of polluted areas is in increasing demand. Land reclamation by the removal of contaminated soil is often not a desirable option, particularly for large polluted areas. Conventional remediation by excavation and land filling or ex situ treatment of polluted soil of such a large site is very expensive and would have high impact local community and environment. Mine industry (including abandoned mines) causes environmental pollution and degradation causing loss to biodiversity and national heritage, produces acid mine drainage (Pelo et al. 2009; Elshorbagy and Mohamed 2000).

Air pollution, ecotoxicology and environmental safety are other mine industry issues of human health concern. In recent years, the potential of plants for environmental cleanup has been widely recognized all over the world. The biodiversity and naturally operating principles of bio-geo-chemical cycles have unequivocally demonstrated the role in cleanup of the environment (Bell 2001; Bengson 1995; Bergholm and Steen 1989; Tordoff et al. 2000).

## 2 Engineered Phyto-Covers

Engineered phyto-covers or natural cap means the pollutants (toxic industrial waste) are contained by vegetation integrated with engineering principles and associated processes. Establishment of phytocovers on contaminated sites is a possible solution to reduce the pressure on land resources. Thus, land resources on a global perspective are under immense pressure. The pressure on available land resources is gradually increasing because of land degradation, the growing world population, global economic development, urbanization, industrialization and scarce resources increase the price of available resources. Therefore, contaminated/polluted lands must be put to use.

The Vegetative caps (phyto-cover technology) help in containment of hazardous wastes because of the expense and risk associated with treating or removing large volumes of hazardous wastes. Both regulators and the public usually accept phyto-covers as part of remediation (Bolshakov and Chibrik 2007; Bradshaw 2000) Therefore, phyto-covers enhance *in-situ* remediation. Phyto-covers protect the public health and the environment. In this case the hazardous waste is isolated from receptors and contained in the landfill with the help of phyto-cover. Prior to the establishment of vegetative covers, hazardous wastes are stored in warehouses which has its own disadvantage.

### *2.1 a) Hazardous Waste—Aluminium Smelter Pot Lining Material*

The National Aluminium Company Limited (NALCO) is Asia's largest integrated aluminium production complex is located at Angul in Odisha State of India. NALCO is involved in bauxite mining, alumina refining, aluminium smelting, casting, and production of aluminum ingots. The aluminium smelter plant and Captive Power Plant (CPP) are situated in Angul. Odisha. The Aluminium smelter located at Angul had 2,30,000 tons per anum (tpa) capacity and is being expanded to 3,45,000 tpa.

Aluminium Smelter at Angul also established a Captive Power Plant of 720 MW capacity, comprising 6 × 120 MW for firm supply of power to the Smelter. Presently, the capacity is being expanded to 960 MW. The water for the plant is drawn from River Brahmani through a 7 km long double circuit pipeline. The coal demand is met from a mine of 3.5 million tpa capacity opened up for NALCO at Bharatpur in Talcher by Mahanadi Coalfields Limited. Several hundreds of tons of spent pot line (hazardous waste containing fluoride and cyanide) are produced by the smelter (Fig. 3.1). The problem of piledup hazardous waste has become a serious environmental problem which was tackled by different phytoremediation strategies.

Figure 3.1 Spent –pot line (Hazardous waste containing fluoride and cyanide) packed and stored in several barracks.

Typically engineered cross section of vegetative cap (natural cap) is shown in Figs. 3.2 and 3.3


**Fig. 3.1** **a** Hazardous waste in store house. Several such store houses have been established in the absence of proper disposal **b** Naturally growing trees near hazardous waste

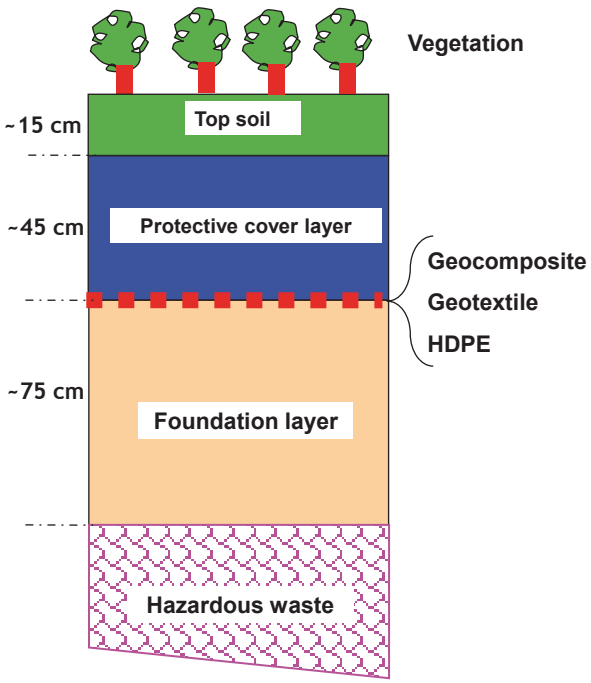


**Fig. 3.2** Schematic view of typical phyto-cover system encompassing vegetative cover, topsoil, protective cover layer, geocomposite (geotextile) and high density polyethylene barrier encapsulating azardous waste

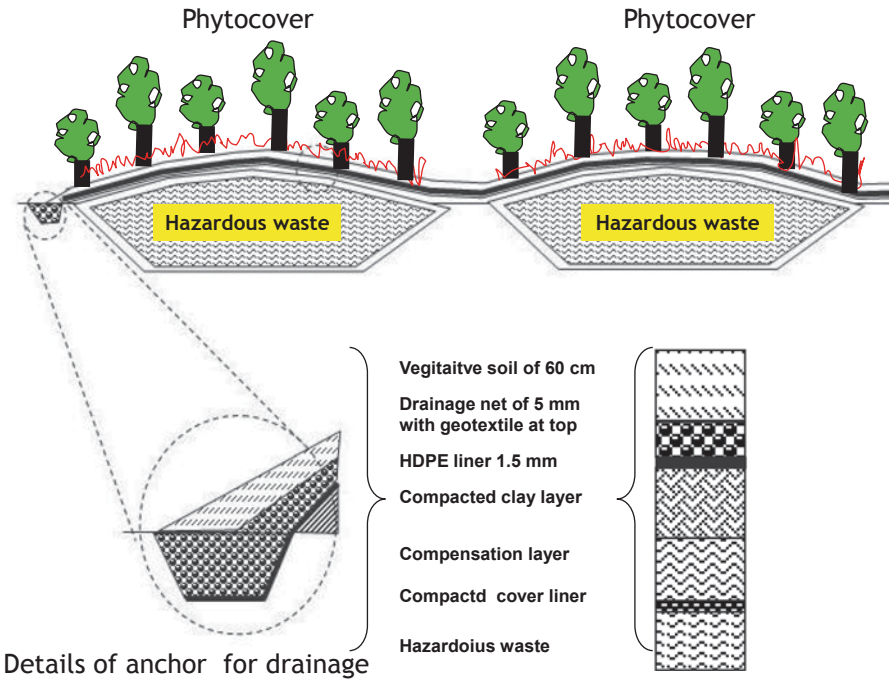


**Fig. 3.3** Sectional views of phytocover and anchoring drainage to contain the hazardous waste from aluminum smelter pot line comprising fluoride and cyanide

The trees normally selected for establishment of a phyto-cover are *Dalbergia sisso*, *Eucalyptus* sp., *Cassia siamea, Acacia auriculiformis, Leucaena leucocephala*, and *Tectona grandis*. In addition to trees, grasses such as *Vetiveria zizanioides and* Industrial crops like *Jatropha curcas*, *Ricinus communis and* Ornamentals like *Canna* sp. *Nerium* sp.are also planted in rows (Figs. 3.4 and 3.5)

## *2.2 b) Stabilization of Nyveli Lignite Corporation (NLC) Open Cast Mines with Energy Plantation Species (Legumes and Non-Legumes)*

Tree crops: *Dalbergia sisso, Eucalyptus sp., Cassia siamea, Acacia auriculiformis, Leucaena leucocephala, and Tectona grandis*

Grasses: *Vetiveria zizanioides*

Sedge: *Carex*

Industrial crop: *Jatropha curcas*, *Ricinus communis*

Ornamental: *Canna* sp. *Nerium* sp.

Hydrophytes: *Typha latifolia*, *Eichhornia crassipes*, *Ipomea* sp. *Lemna minor*, *Polygonum* sp, *Alternanthera philoxeroides*, *Phragmites sp.* (Figs. 3.6 and 3.7)

**Fig. 3.4** Vegetative covers comprising legume and non-legume trees (*Tectona grandis* and *Leucaena leucocephala)*

**Fig. 3.5** Vegetative covers comprising legume and non-legume trees

## *2.3 c) Manganese Mine waste*

In Gumgaon, Manganese ore mine residues have been stabilized by engineered vegetative capping using legume and non-legume tress (Fig. 3.8).

Reference may be made to Prasad (2007a, b) for plant species suitable for revegetation of different mine spoils (Cheung et al. 2000).

**Fig. 3.6** Nyveli lignite mine tailings have been stabilized by legume, non-legume tree sincluding biodiesel producing plants

## 3 Municipal Solid Waste Landfills

There are thousands of landfills exist all over the world. Most of these are municipal solid waste landfills (rubbish pits) (Fig. 3.9). Modern landfills (sanitary and mine waste) are carefully engineered, structure and designed to isolate waste from nearby natural resources, wildlife and people. These landfills are excavated/ dug out of the ground and then the walls are sealed with layers of clay/ HDPE (for e.g. bentonite/zeolites etc. which are by-products of mine waste) and coated with high density plastic sheet or geotextiles as barrier prevent groundwater contamination from wastewater that is locked up in the pit that was dug to bury the waste. These landfills are designed and engineered to stay dry inside, except for liquids that ooze from small decomposable fraction of debris buried inside, and rainwater that trickles through. As water trickles through a landfill, it dissolves chemicals and other particles, creating a liquid called "leachate". The landfills are designed and engineered in such a way that the capping technology provides vents so as to release gas. The design and engineering of natural caps include landfill gas detection meters which are monitored on regular basis. Monitoring wells around the site are also dug for monitoring groundwater quality using lysimeters (Meißner et al. 2010). Landfills are provided with leachate collection system (Fig. 3.10)

**Fig. 3.7** Stabilization of mine tailings with grasses viz. miracle grass (*Vetiveria zizanoides*) lemon grass (*Cymbopogon citratus*) and other non-edible tree crops

Monitoring parameters for landfills are: (a) Water contamination from leachate (b) air emissions (methane and other gases), (c) immobilized hazardous waste.

Landfill Gas (LFG) is generated when organic materials in landfills are decomposed by bacteria. LFG is roughly 50% methane with carbon dioxide being the second most prevalent gas. All solid waste landfills emit this gas in amounts that depend on a variety of factors, such as waste composition and landfill size.

Gases are formed in a landfill when buried wastes decompose (breakdown by bacteria) or volatize (change from a liquid or solid to a vapor). These bacterial and chemical processes produce gases that are unpleasant. The most common type of landfill is the municipal solid waste facility, which accepts household and non-hazardous commercial and industrial waste. It typically contains 60% organic material, such as food and paper. Because organic material tends to produce a great deal of gas, municipal solid waste landfills have the potential to produce odors. At extremely high concentrations, humans may experience eye irritation; headaches, nausea, Sulfides and ammonia are the most common sources of odor in landfill gas. Sulfides produce a strong, rotten-egg smell that humans can detect even at very low concentrations. Ammonia produces a pungent odor that many people are familiar with because it is often used in household cleaning products. Both are normally present in the air, regardless of the presence of a landfill. There is another group

**Fig. 3.8** Gumgaon Manganese mine residue in Central India is stabilized by engineered vegetative caps of Bamboos, nitrogen and non-nitrogen fixing indigenous trees


**Fig. 3.9** Typical example of mixed municipal solid waste dumpsite

**Fig. 3.10** MSW dump site and its cross section

of chemicals, called non-methane organic compounds (NMOCs), which may be present in the air near a landfill, though they are not likely to reach harmful levels.

## 4 Phyto-Treatment of CPP Fly Ash Slurry and Landfill Leachate

In order to meet the power demand, NALCO had its own captive power plant (CPP). Fly ash slurry is collected in wetlands which are treated effectively and the treated water is recirculated for use in the smelter (Kadlec and Knight 1996; Prasad et al. 2006). Aquatic macrophytes e.g. *Typha latifolia*, *Eichhornia crassipes*, *Ipomea* sp. *Lemna minor*, *Polygonum* sp., *Alternanthera philoxeroides*, *Phragmites sp.*are the key players in the wetland (Figs. 3.11 and 3.12)

Macrophytes possess extraordinary ability to survive the adverse conditions of pollution and possess high colonization rate that are virtual tools of excellence for phytoremediation. Both submerged and emergent macrophytes play an important role in metal bioavailability from sediments through rhizosphere exchanges and other carrier chelates. These phenomena facilitates metal uptake by other floating and emergent forms of macrophytes (Prasad et al. 2006). Several of the macrophytes bioconcentrate reduced form of the metals from sediments, making them immobile (Okurt et al. 1999).

*Salix, Sorghum* and Water hyacinth (*Eichhornia crassipes*) contain β—cyanoalanine synthase (CAS) which catalyzes the conversion of free cyanide and cysteine to is β cyanoalanine. The final metabolite is asparagine, a non-toxic essential amino acid (Korte et al. 2000; Ebel et al. 2007; Fig. 3.13).

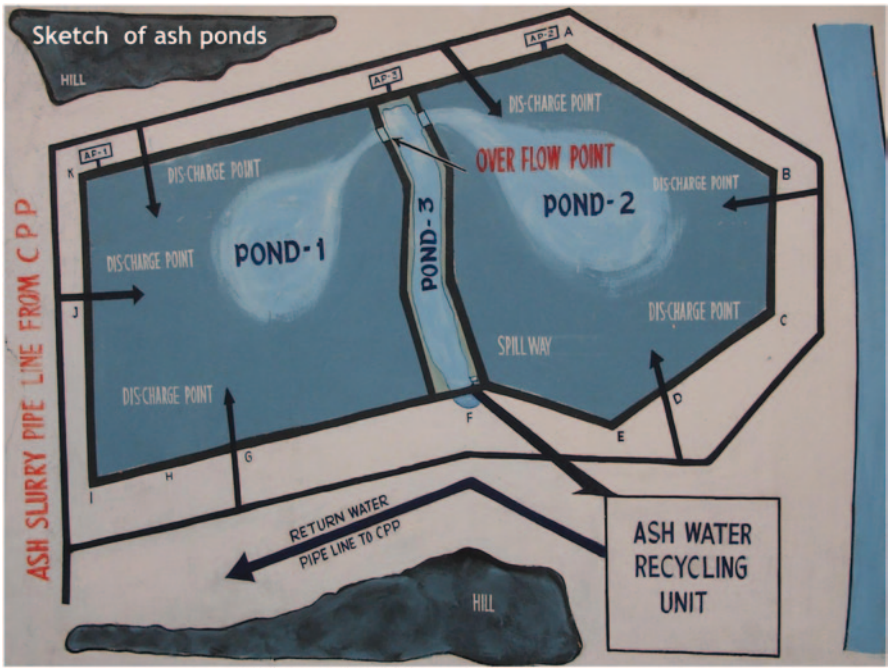


**Fig. 3.11** Schematic diagram of coal fly ash pond assembly (I-III) showing ash slurry supply pipe from from CPP discharge points and recycling of treated water back to CPP for reuse

## 5 Landfil Leachate and Hazardous Wastewater Utilization for Establishment of High Rate Algal Ponds for Biodiesel Production

Algae grown in wastewater (=high rate algal ponds, HRAPs) assimilate nutrients and thus subsequent harvest of the algal biomass recovers the nutrients from the wastewater. Leachate from hazardous waste dump sites are being put to use to establish HRAPs for production of biodiesel (Fig. 3.14; Park et al. 2011). The harvested phytomass of aquatic plants used in treatment wetlands serves as a valuable feedstock for biogas production (Abbassi et al. 1991).

## 6 Conclusions

The engineered phyto-cover functions as a sponge and pump system, with the root zone acting as the sponge, and trees acting as the solar-driven pumps. In contrast to restrictive permeability barrier design, the engineered phyto-cover design involves the storage of free water in soil pores and the extraction of stored water by the tree roots. Use of constructed wetlands is spreading rapidly in developed


**Fig. 3.12** **a** and **b** Granite lined canal discharging ash slurry water to large lagoons. **c** and **d** Constructed wetland with its plant assemblage involved in treatment of the contaminants. Cleaned water is discharged into storage pond for reuse

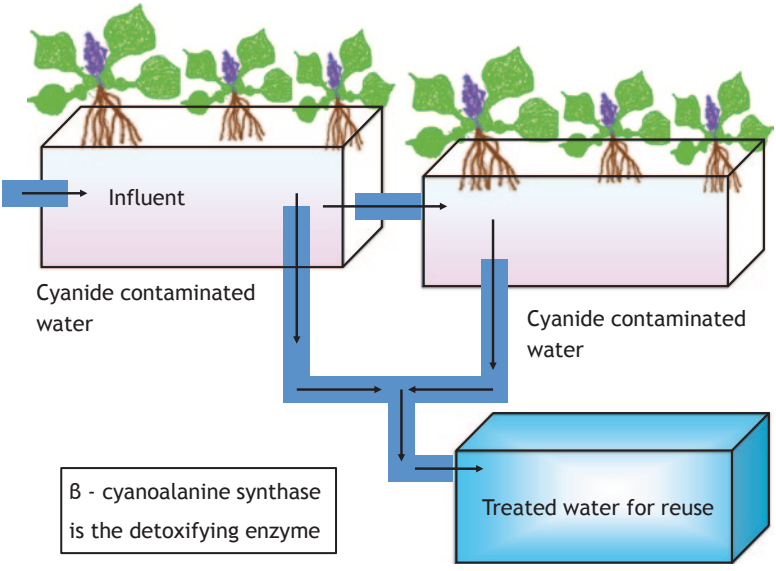


**Fig. 3.13** Cascade of wetland system for cyanide degradation using *E. crassipes* (Water hyacinth). The detoxifying enzyme is β-cyanoalanine synthase (CAS)

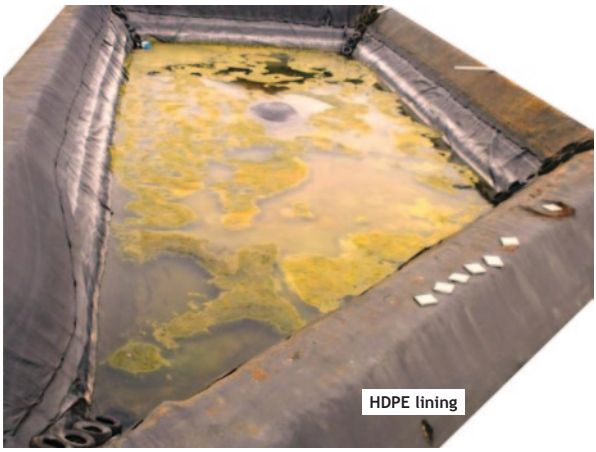


**Fig. 3.14** Use of leachate and hazrdous waste containing water for development of high rate algal ponds [HRAP] (HDPE liner is the barrier). Please note the profuse growth of algae

nations however, in tropical nations due to water scarcity and high surface evapotranspitration the constructed wetlands for treatment of waste waters is not gaining significance. "Green India Mission" launched by Government of India (GOI) has an ambitious plan of 10 year afforestation plan at a cost of ` 46,000 crores (one core = 10 million) to add 10 million ha for forest cover to the nation's 40–42 million ha of good quality forests. This is expected to increase forest cover substantially. To reduce pressure on land and to increase forest cover, it also anticipated to use contaminated land to fulfil this mission. Upon successful completion of this mission, the established green cover is expected to sequester 50–60 million t of carbon dioxide per year by 2020 as per the estimates of the Ministry of Environment and Forests, GOI.

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# Chapter 4
# Chromium and Nickel Phytotoxicity and Genotoxicity

**Agáta Fargašová, Bernd Markert and Karol Mičieta**

**Abstract** The growth inhibition, biomass production and water translocation in roots and shoots of mustard (*Sinapis alba* L.) was evaluated in experiments with Cr(III), Cr(VI) and Ni. On the basis of IC ($IC_{25}$, $IC_{50}$, $IC_{75}$) values for growth inhibition the following rank orders were arranged: for roots: Cr(VI)≥Ni(II)>>Cr(III); for shoots: Ni(II)>Cr(VI)>>Cr(III). All metals tested reduced more root than shoot growth. When the relationship between dry (DM) and fresh mass (FM) was determined, FM production was reduced more than that of DM, and root FM was reduced more strongly than that of shoots. This indicates a reduction in water uptake and problems with water translocation through the plant. For genotoxicity study, simultaneous phytotoxicity and mutagenicity assay with *Vicia sativa* L. var. Klára was used. For phytotoxicity, the following rank orders of growth inhibition could be arranged: for roots: Ni(II)>Cr(VI)>Cr(III); for shoots: Ni(II)>Cr(VI)≥Cr (III). For mutagenicity assay root tips of *V. sativa* were used and chromosome aberrations were determined at least in 500-anatelophases. All tested metals exerted in *V. sativa* a significant increase of chromosomal aberration rate in applied concentrations. Maximum of aberrations invoked Cr(VI) and the rank order of aberrations fall was: Cr(VI)>Ni(II)>Cr(III). Genotoxic effects of metals were also determined by analysis of micronuclei frequency in the pollen tetrads of *Tradescantia* plants. None of the tested metals significantly stimulated micronuclei frequency. Genotoxic effect decreased in order: Cr(VI)≥Ni(II)>Cr(III).

**Keywords** Chromium · Nickel · Phytotoxicity · Genotoxicity · Plants

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## 1 Introduction

Over the last few years, heavy metals had received considerable attention as a consequence of increased environmental pollution from industrial, agricultural, energetic and municipal sources. They function in the soil as a stress factor causing in plants physiological disorders after having been absorbed by the root system, which results in decreased vigor of a plant and retardation of its growth. Physiological responses of plants to a toxic metal treatment are not only growth inhibition, but also changes in various biochemical and physiological characteristics (Vassilev et al. 1998). Many metals have the ability to deteriorate genetic information and chromosome structure. The results of such genotoxic effects can be lethality, generation of mutations, chromosomal aberrations and carcinogenesis (Mišík et al. 2006, 2007). Vascular plants have been found to be highly effective for recognizing and predicting metal stress in the environment (growth inhibition, reduction of biomass production, changes in water absorption and translocation (Chatterjee and Chatterjee 2000; Prasad et al. 2001; Shanker et al. 2005; Szárazová et al. 2008). For genotoxicity studies, plants are highly responsible and sensitive. Their beneficial interest is that seeds and pollen grains can be easily stored and they offer cheap, and relatively easy and accurate toxicological assessment (Kristen 1997). By their ability to accumulate toxic substances, they indicate metal presence in the environment even in very low concentration (Chandra et al. 2004).

## 2 Material and Methods

Mustard (*Sinapis alba* L.) seeds were germinated in Petri dishes (17 cm diameter, filter paper with plastic net on the bottom). Tested samples were exposed to 10 varying concentrations (Ni(II) from 1 to 50 mg/L, Cr(III) and Cr(VI) from 5 to 225 mg/L) and tap water (80 mg/L Ca, 27 mg/L Mg; pH = 7.3 ± 0.05) was used for their dilution. In each Petri dish, 50 healthy seeds of similar size were spread on the filter paper covered with a plastic net, and overflowed with 50 mL of treatment solutions or normal tap water as control. The covered Petri dishes were placed in a thermostat (darkness, t = 25 °C; air humidity 80%). After 72 h, root and shoot lengths were measured.

Basically, the same procedure utilized for growth inhibition was used to determine the dry mass (DM) and water content (WC). After 72 h, Petri dishes with germinated seeds were transferred from the thermostat into a laboratory box with a day-light cycle of 16/8 h and a constant temperature of 23 ± 1 °C. The dishes were shielded from direct sunlight, and cultivation lasted for 7 days. The shoots were not in direct contact with tested solutions of metals. After 10 days growth (3 + 7), the plants were divided into roots and shoots, and fresh mass (FM) was immediately weighed. The plant material was then oven-dried (t = 80 °C) to constant weight. The

water content of the plants was determined on the basis of fresh and dry mass by using Drazic and Mihailovic's equation (Drazic and Mihailovic 2005).

$$WC = (FM - DM) / DM \tag{4.1}$$

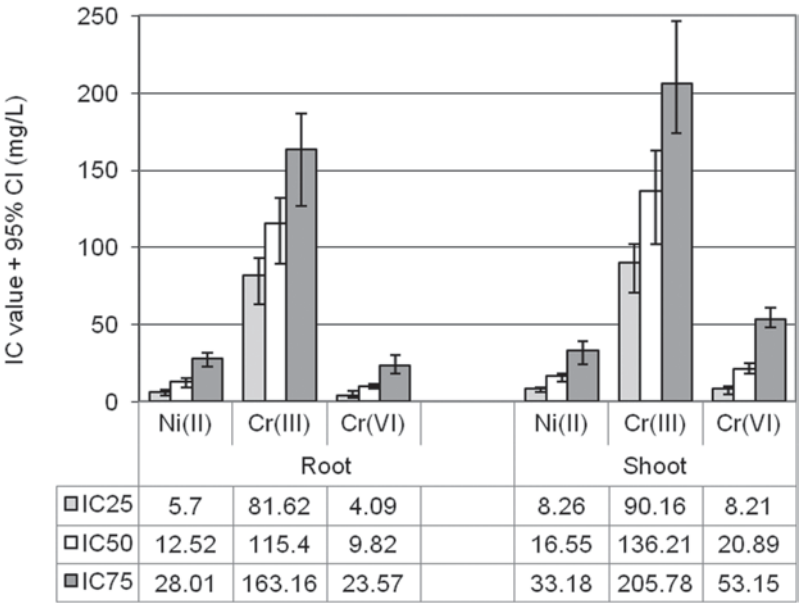
(WC—water content; FM—fresh mass, DM—dry mass; in g/g DM).

Simultaneous phytotoxicity and mutagenicity assay was carried out on plant species *Vicia sativa* L. var. Klára according to Miadoková et al. (2001, 2005). After 24 h of soaking at 25 °C in distilled water or solution with metal concentration equal to $IC_{50}$ value, the seeds of *V. sativa* were allowed to germinate in Petri dishes (diameter = 18.5 cm) with filter paper soaked with the same concentration of tested metal as that used for soaking. Phytotoxicity was assayed after 72 h of the dark cultivation in the thermostat at 25 °C by the same way as for *S. alba*. The seedling roots and shoots of *V. sativa* were measured and percent growth inhibition was assessed. The seedling roots used for chromosome and genome mutability evaluation were fixed and permanent slides were prepared by the Feulgen method. Chromosome aberrations were determined at least in 500-anatelophases. For statistic analysis the Student's t-test was used.

The procedures for maintaining the *Tradescantia* plants and for analyzing micronuclei frequency in the tetrads have been described by Mišík et al. (2006, 2007). *Tradescantia paludosa* clone 03 was cultivated at the Department of Botany, Faculty of Natural Sciences, Comenius University in Bratislava, Slovakia. Inflorescences were harvested at the 8–10-bud stage and immersed into 500 mL of tested metal solutions (100 mg/L $CrO_3$ and $NiCl_2$, 1,000 mg/L $Cr(NO_3)_3$) for 12 h. As control tap-water was used. The 24 h reconvalescence, during which inflorescence peduncles were dipped in 500 mL of tap-water, succeeded to 12 h exposure. Then the buds were fixed for 24 h in ethanol: acetic acid (3:1). The fixed material was stored in 70 % ethanol. Slides were prepared from the fixed material using the aceto-carmine squash technique. Micronuclei were scored in the early tetrad stages of pollen mother cells. In the present study, 15–20 inflorescences were in a sample. Three hundred tetrads were scored from each of five slides prepared from a treatment sample for a total of 1,500 tetrads per plot. Data were recorded as the number of micronuclei (MCN) per 100 tetrads. A change of frequency of MCN/100 tetrads was considered statistically significant (at $P < 0.05$) if the difference between the mean of the control population and the mean of the treated population was at least twice as large as the standard error of the difference between the two means (Mišík et al. 2007; Ma et al. 1994).

The salts of tested metals, $NiCl_2.6H_2O$, $Cr(NO_3)_3.9H_2O$ and $CrO_3$ of analytical grade p.a., were obtained from Lachema, Brno, Czech Republic.

All experiments were set up in a completely randomized design with three replicates. Chronic toxicity was assessed as inhibition of root and shoot growth and the results were evaluated by the Gryck-Haustein method and $IC_{25}$, $IC_{50}$ and $IC_{75}$ concentrations were determined. The results were statistically evaluated by using the Toxicity program. For statistical evaluation of biomass production, statistical program STATISTICA 8.1 was used.




| | Root | | | | Shoot | | |
|---|---|---|---|---|---|---|---|
| IC25 | 5.7 | 81.62 | 4.09 | | 8.26 | 90.16 | 8.21 |
| IC50 | 12.52 | 115.4 | 9.82 | | 16.55 | 136.21 | 20.89 |
| IC75 | 28.01 | 163.16 | 23.57 | | 33.18 | 205.78 | 53.15 |

**Fig. 4.1** $IC_{25}$, $IC_{50}$ and $IC_{75}$ values and their 95% confidence intervals (CI) (mg/L) for *Sinapis alba* L. after 72 h application; mean of three determinations with a standard deviations

## 3 Results and Discussion

### *3.1 Determination of Phytotoxicity*

The first part of the study was carried out to determine the adverse effects of chromium and nickel on *S. alba* seedlings. The deleterious effect was expressed as root and shoot growth inhibition using regression analysis which yielded $IC_{25}$, $IC_{50}$ and $IC_{75}$ values (Fig. 4.1). On the basis of these values, and their statistical evaluation, metals can be arranged in the following rank orders of inhibition: for roots: $Cr(VI) \geq Ni(II) >> Cr(III)$; for shoots: $Ni(II) > Cr(VI) >> Cr(III)$. Both root and shoot prolongation was most inhibited by Cr(VI) and Ni(II). All metals tested reduced more root than shoot growth.

The presence of Cr and Ni in the external environment leads to changes in the growth and development pattern of plants, and both these metals are reported to be very toxic for root and shoot growth (Fargašová 1994, 1998). Ni in the presence of 0.1 μM $NiCl_2$ inhibited root and shoot elongation of canola and tomato seedlings, and the roots appeared more sensitive than the shoots (Burd et al. 1998). Prasad et al. (2001) reported that the root length in *Salix viminalis* was affected more by Cr than by Cd and Pb; and Fargašová (1994) stated that the adverse effect of Cr on *S. alba* root growth was equal to that of Hg, and stronger than that of Cd and Pb, while Ni reduced root length less than that by Cr (Fargašová 1998). In accordance with

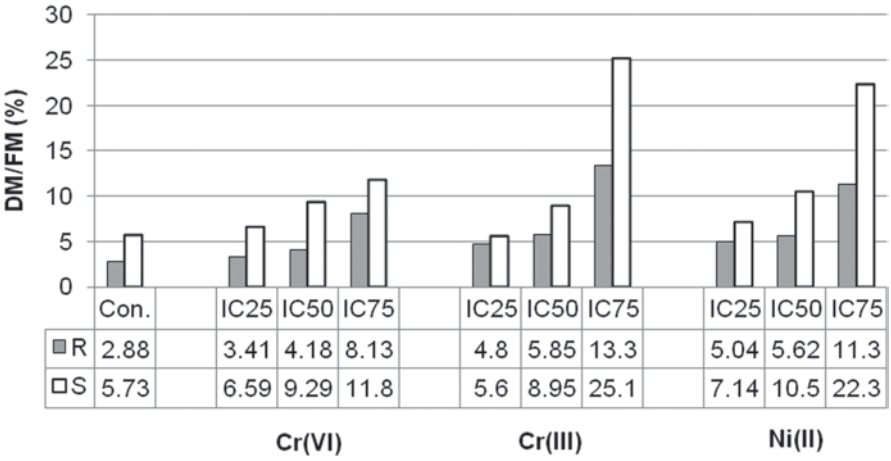



**Fig. 4.2** Relationship between dry (DM) and fresh mass (FM) (%) after 10 days growth of *S. alba* L. in the presence of tested metals; mean of three determinations, standard deviation 6% or less (*R*—root; *S*—shoot; *Con.*—control)

our results, Burd et al. (1998) and Chatterjee and Chaterjee (2000) also reported that root growth was as a more sensitive indicator of metal toxicity than shoot growth. Here-in, this was significantly confirmed mainly for chromium. The general response of decreased root growth due to Cr and other metal toxicity may be evoked by the inhibition of root cell division/root elongation or by the extension of the cell cycle in the roots. Under high concentrations of Cr, Ni and many other heavy metals, the reduction of root growth may be due to the direct contact of seedling roots with a metal present in the medium, causing collapse, and subsequently inability of roots to absorb water from the medium (Barcelo et al. 1986). Adverse effects of Cr, Ni and other metals on plant height and shoot growth were reported by Rout et al. (2000) and Barton et al. (2000). The significant reduction in plant height of *S. alba* observed in this present study was also reported for this plant by Hanus and Tomas (1993) in soil with Cr concentrations of 200 or 400 mg/kg. The reduction in plant height may be due mainly to the reduced root growth and consequent lesser nutrient and water transport to the shoots. Additionally, Cr and Ni transport to the aerial parts of the plant can have a direct impact on the cellular metabolism of shoots, thus contributing to the reduction in plant height (Shanker et al. 2005).

The overall adverse effects of Cr and Ni on growth and development of plants may be a serious impairment of mineral nutrients and water uptake, which leads to deficiency in the shoots. Wilting of various crops and plant species due to Cr toxicity has been reported (Turner and Rust 1971), but little information is available on the exact effects of Cr and Ni on water relations in higher plants. When the relationship between dry (DM) and fresh mass (FM) was determined herein (Fig. 4.2), the DM fraction was increased parallel to increased Cr and Ni concentrations, while for FM fraction the growth trend was opposite. The effect of tested metals was stronger on FM than on DM production, and root FM was reduced more strongly than that

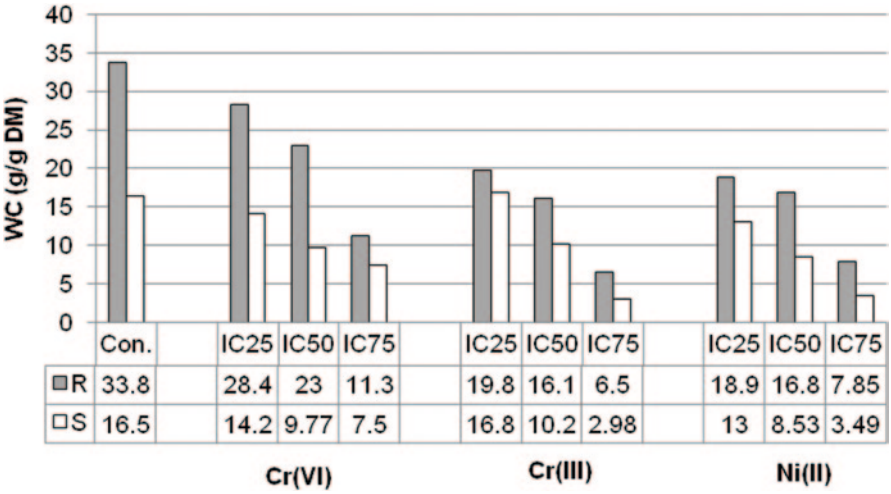
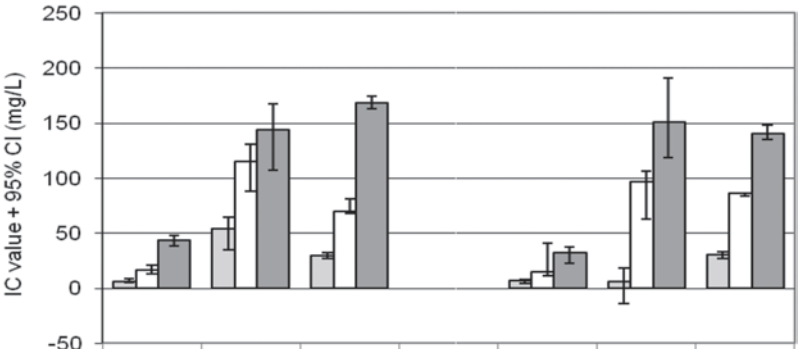



**Fig. 4.3** Water content (g/g DM) in roots and shoots of *S. alba* L. seedlings after 10 days growth in the presence of tested metals; mean of three determinations, standard deviation 6 % or less (*R*—root; *S*—shoot; *Con.*—control)

of shoots. This indicates a reduction in water uptake (Fig. 4.3). Water content in both plant parts was reduced very rapidly in comparison to that in control seedlings, and varied significantly with the tested concentrations. Because the water content in the shoots was significantly reduced in the presence of tested metals, it can be concluded that Cr and Ni inhibited not only water absorption by the roots, but also water tranport into the upper seedling parts. These results disagree with Chatterjee and Chatterjee (2000) conclusion, that excess Cr decreases the water potential and transpiration rates and increases diffusive resistance and relative water content in the leaves of cauliflower. However, Barcelo et al. (1986) observed a decrease in leaf water potential in a Cr-treated bean plant. Decreased turgor and plasmolysis was also observed in the epidermal and cortical cells of bush bean plants exposed to Cr, because toxic levels of Cr decreased tracheary diameter in vessel-bearing plants, thereby reducing longitudinal water movement (Vazquez et al. 1987).

## *3.2 Genotoxicity Study*

Toxic effects of heavy metals, mainly during chronic exposure, are not visible immediately. Hence, eco-toxicological studies suggest the assessment of genotoxicity. Genotoxicity effect is developed as early as the concentration is lower than that for phytotoxicity effect (Mičieta and Murín 1998). For phytotoxicity and clastogenicity study, *V. sativa* seedlings were used. Phytotoxicity was determined through $IC_{25}$, $IC_{50}$ and $IC_{75}$ values and for roots and shoots the strongest inhibitory effect, pursuant to *S. alba*, had Ni(II) (Fig. 4.4). However, Cr(VI) inhibited *V. sativa* root growth less




| | Root | | | Shoot | | |
|---|---|---|---|---|---|---|
| | Ni(II) | Cr(III) | Cr(VI) | Ni(II) | Cr(III) | Cr(VI) |
| IC25 | 6.21 | 53.78 | 28.94 | 6.77 | 6.19 | 30.58 |
| IC50 | 16.46 | 114.41 | 69.69 | 14.62 | 96.49 | 85.96 |
| IC75 | 43.61 | 143.38 | 167.79 | 31.59 | 150.44 | 140.11 |

**Fig. 4.4** $IC_{25}$, $IC_{50}$ and $IC_{75}$ values and their 95 % confidence intervals (CI) (mg/L) for *Vicia sativa* L. after 72 h application; the mean of three determinations with standard deviation

than that of *S. alba*. No significant differences were confirmed between the Cr(III) and Cr(VI) adverse effects on *V. sativa* shoot growth. On the basis of these values, and their statistical evaluation, metals can be arranged in the following rank orders of inhibition: for roots: Ni(II) > Cr(VI) > Cr(III); for shoots: Ni(II) > Cr(VI) ≥ Cr (III).

For mutagenicity assay, root tips of *V. sativa* were used and chromosome aberrations were determined at least in 500-anatelophases. All tested metals exerted a significant increase of chromosomal aberration rate in *V. sativa* due to applied concentrations (Table 4.1). All tested metals Cr(VI) invoked maximum of aberrations in anatelophase cells. The rank order of aberrations occurred was: Cr(VI) > Ni(II) > Cr(III). Genetic variation in susceptibility to environmental agents and metals can be considered as differences in metabolism of these agents in various organisms (Omenn 1991). In addition, DNA target size and DNA content are also important in determining genotoxic hazards of metals. According to Kovalchuk et al. (1998) and Chauhan et al. (1998) genotoxicity can be obtained as a result of multipolar anaphase and c-mitosis or damage of protein synthesis in the presence of DNA toxicant. Simultaneous toxicity and clastogenity of wastes with Cr and Ni contents was also confirmed for *V. sati*va by Miadoková et al. (1999) and for *V. faba* and *Allium cepa* by Chandra et al. (2004, 2005). Chromosomal fragments and bridges created in the presence of Cr(VI) indicated that $CrO_3$ affected DNA structure and conformation (Quian 2004).

For determination the genotoxic effects of Cr and Ni, analysis of micronuclei frequency in the pollen tetrads of *Tradescantia* plants was done. As it is evident from Table 4.2, none of tested metals significantly stimulated micronuclei frequency in comparison with the control and genotoxic effect decreased in order: Cr(VI) ≥ Ni(II) > Cr(III). *Tradescantia* micronucleus test (Trad-MCN) in

**Table 4.1** Potential clastogenicity evaluation of Cr and Ni in *Vicia sativa* L. ($n=500$)

| | Metal concentration (mg/L) | | Number of aberrations ± SD | Percentage of aberrations ± SD |
|---|---|---|---|---|
| | Ni | Cr | | |
| Control | <0.07 | <0.01 | 7 ± 0.69 | 2.33 ± 0.23 |
| Ni(II) | 16.46 ± 2.16 | | 10 ± 0.75** | 3.33 ± 0.25** |
| Cr(III | | 114.41 ± 13.36 | 8 ± 0.75* | 2.67 ± 0.25* |
| Cr(VI) | | 69.69 ± 8.66 | 13 ± 0.69** | 4.33 ± 0.23** |

Control—sterile distilled water
*SD* standard deviation
**Significant differences in comparison with control at $P<0.01$
*Significant differences in comparison with control at $P<0.05$

**Table 4.2** Micronuclei frequency in the *Tradescantia* pollen tetrads after treatment with Cr and Ni solutions ($n=1500$)

| | Metal concentration (mg/L) | | Number of micronuclei ± SD | Percentage of micronuclei ± SD |
|---|---|---|---|---|
| | Ni | Cr | | |
| Control | <0.07 | <0.01 | 43 ± 13.74 | 2.89 ± 0.92 |
| Ni(II) | 24.71 ± 0.25 | | 59 ± 17.48 | 3.93 ± 1.17 |
| Cr(III | | 130.00 ± 1.30 | 47 ± 16.61 | 3.13 ± 1.11 |
| Cr(VI) | | 52.00 ± 0.52 | 60 ± 15.98 | 4.00 ± 1.07 |

*SD* standard deviation

combination with *Allium cepa* L. and *Vicia faba* L. root tips tests are most frequently used genotoxicity tests in plants (Majer et al. 2005) and it is very popular now for *in situ* bio-monitoring of air pollution (Mišík et al. 2006, 2007). The results obtained during our genotoxicity tests are in good agreement with those reported by Knasmüller et al. (1998) when $CrO_3$, $CrCl_3$ and $NiCl_2$ up to concentration 10 mM did not evoke genotoxic effects. The same conclusion was also drawn by Majer et al. (2005) for Cr(III). Higher genotoxicity of Cr(VI) than Cr(III) determined during our experiments also is in agreement with Němeček et al. (2002). Rossman (1995) who reported that molecular mechanism of DNA damage by Cr(VI) involves induction of DNA-DNA and DNA-protein cross-links and genotoxic effect can be also increased by reactive oxygen species produced during intracellular reduction. For Ni(II), no genotoxic effects were confirmed for bacteria. Rossman (1995) and Patierno and Costa (1987) reported that mutations after Ni applications are also the result of DNA damage and DNA-protein cross-links formation.

## 4 Conclusion

Obtained results confirmed chromium and nickel adverse effects on terrestrial plants. Both metals reduced plants' growth and impaired their genetic material. Routinely phytotoxicity used test with *S. alba* and *V. sativa* seedlings confirmed for root and shoot growth the highest toxicity of Ni and Cr(VI) toxicity was several

times higher than that of Cr(III). Cr and Ni also reduced dry (DM) and fresh (FM) mass production of *S. alba* and their adverse effect was stronger on FM than on DM production. The root FM was reduced, similar as growth, more strongly than that of shoots. This indicates a reduction in water uptake. Because tested metals significantly reduced mainly water content in the shoots, it can be concluded that Cr and Ni inhibited not only water absorption by the roots but also water transport into the upper seedlings parts. Genotoxicity of Cr and Ni was determined as number of chromosomal aberrations in *V. sativa* root tips and micronuclei frequency in the pollen tetrades of *Tradescantia paludosa*. All tested metals activated a significant increase in chromosomal aberration rate at applied $IC_{50}$ concentrations. The maximum of aberrations in anatelophase cells invoked Cr(VI). None of tested metals significantly stimulated, in comparison with the control, micronuclei frequency in pollen tetrades of *T. paludosa* and genotoxicity effect decreased in the same order as for majority of observed parameters: $Cr(VI) \geq Ni(II) > Cr(III)$.

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# Chapter 5
# Physio-Anatomical Responses of Plants to Heavy Metals

**Riffat Batool, Mansoor Hameed, Muhammad Ashraf, Muhammad Sajid Aqeel Ahmad and Sana Fatima**

**Abstract** Environmental pollution caused by heavy metals is a global issue, which seriously affects growth and development of agricultural crops, as well as the native flora. It is known that metal toxicity can significantly alter soil physico-chemical properties of the soil, mainly organic matter, pH and cation exchange capacity. The devastating impact of heavy metals may be related to retarded growth and development, ionic imbalance, metal toxicity, reduced photosynthetic rate, degradation of chloroplast and photosynthetic pigments, and more importantly disturbed plant water relation. Heavy metal in the soil can also induce alterations in anatomical parameters. Among anatomical changes, disintegration and reduced size of parenchymatous tissue, reduced size of xylem vessels, degraded and smaller mesophyll tissue, and as a whole reduced root and stem diameter and leaf growth. Moreover, there is a spatial accumulation of heavy metals in different organs, more commonly in dermal, parenchymatous and phloem tissues. However, tolerance to heavy metals varies among different species, and even within populations of a same species. Overall this chapter describes how far heavy metal toxicity can promote the development of specific structural and functional modifications in plants exposed to metal-enriched environment and how these features could help plants to thrive well under such harsh conditions.

**Keywords** Accumulation · Metal toxicity · Plant anatomy · Photosynthesis · Tolerance

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## 1 Introduction

The major sources of heavy metal contamination are industrial effluents, burning, transport, power generation and organic wastes (Lone et al. 2008). Particularly, in Pakistan, industrial sewage water, unprocessed city effluents, and domestic wastes are major sources to pollute the environment, which eventually enters in the food chain thereby negatively affecting the environmental health (Qadir and Ghafoor 1998; Stagnitti 1999).

Metals are among the hazardous environmental pollutants which severely affect growth and development of plants, in addition to causing toxic effects in plants and animals (More et al. 2003). In plants, tolerance mechanism of metal toxicity has been reviewed largely (Clemens 2006; Hossain et al. 2012). It is vital to investigate the relationships between plants and heavy metals for the safety of our environment (Benavides et al. 2005).

The occurrence of heavy metals like Ni, Cd, Cu, Pb and Zn is widespread these days (Yadav 2010). In higher plants, most widely studied metals are cadmium and nickel, which tend to be commonly occurring toxic soil pollutants (Sanitá di Toppi and Gabrielli 1999; Madejón et al. 2003). Many metals are proved to be toxic at elevated levels, which have been studied in several plant species, e.g., *Salix acmophylla* (Ali et al. 2003), *Pisum sativum* (Belimov et al. 2003), *Azolla* sp. (Anju et al. 2004), *Chlorella* (Rehman and Shakoori 2004), *Corchorus olitorius* (Mazen 2004), *Brassica juncea* (Sridhar et al. 2005), *Hordeum vulgare* (Sridhar et al. 2007) and *Medicago sativa* (Jadia and Fulekar 2009). However, specific metals pose specific effects on different species. The metals can cause alterations in a variety of physio-biochemicals and structural attributes. Thus, the present chapter discusses how far metals can alter various physio-anatomical features in different plant species and how these altered attributes could help plants to survive under metal-enriched environment.

## 2 Soils Impregnated with Heavy Metals

Industrial effluents consistently degrading water resources and agricultural lands in Pakistan. Ineffective industrial pollution control policies in addition to lack of implementation of such activities are the major reasons. Only about 1 % industries in the Pakistan are equipped with operating wastewater treatment plants (Bhatti 1996), while the other are causing a major threat to the environments by disposing off untreated solid as well as liquid wastes to soil and water resources (Shah 1987). These effluents enter in the irrigation water systems and ultimately pollute our agricultural lands. A huge amount of contaminated waste-water is discharged daily from the industries and in the absence of proper treatment plants, pollutes the water-bodies (Saleemi 1993). Toxic metals from industrial effluents ultimately accumulate in the soil, causing toxicity in plants, and hence affecting the productivity. Crop plants can uptake heavy metals entering into the food chain and as a result of utilization they cause metal toxicity to humans and livestock (Albering et al. 1999; Jarup 2003).

Industries dealing with tannins, paper, vegetable oil/ghee, cosmetics and pharmaceuticals continuously discharge heavy metal-containing effluents in irrigation system (Nabi et al. 2001). In Pakistan, heavy metal concentrations in soil and water are currently within the maximum permissible limits (Ghafoor 2000; Nabi et al. 2001), and cause no visible toxicity in agricultural crops (Alloway 1995). However, continuous use of these metals-polluted will be the major hazard that will limit crop growth and productivity in the near future.

## 3 Metal-Induced Regulation of Growth and Physio-Anatomical Attributes

### *3.1 Growth Attributes*

Heavy metals severely affect growth and development of plants, which is mainly due to their effect on plant biomass production, photosynthetic pigment concentration, gas exchange characteristics and uptake of micro- and macro-nutrients (Burzynski and Klobus 2004).

Root growth is generally more affected due to metal stress as compared to the shoot growth (Souza et al. 2005); however, reduction in biomass has earlier been reported by several authors, e.g., Jadia and Fulekar (2008) in *Medicago sativa,* Aziz et al. (2007) in *Hibiscus sabdariffam* Chatterjee and Chatterjee (2000) in *Brassica oleracea* var. *botrytis* cv. Maghi, Ali et al. (2003) in *Salix acmophylla,* Hussain et al. (2006) in *Azolla pinnata* and Mukhtar et al. (2010) in *Heianthus annuus*.

### *3.2 Physiological Attributes*

#### 3.2.1 Gas Exchange Characteristics

Heavy metals directly affect photosynthesis and other gas exchange attributes in plants (Pang et al. 2003; Jing et al. 2005; Kaznina et al. 2005; Mukhtar et al. 2010). A reduction in net photosynthetic rate (Bishnoi et al. 1993; Krupa and Baszynski 1995), stomatal conductance (Bethkey and Drew 1992), transpiration rate (Chatterjee and Chatterjee 2000; Pandey and Sharma 2002) and water use efficiency (Bishnoi et al. 1993) has earlier been reported in plants under heavy metal stress. However, an increase in intercellular $CO_2$ concentration has been reported by Seregin and Ivanov (2001).

Stomatal regulation may be one of the major factors affecting photosynthetic rate, which may be mainly due to direct effect on stomatal regulation that may be due to amendment in K fluxes inside the guard cells. This may result in stomatal closure that reduces exchange of gasses and ultimately the photosynthetic rate (Bishnoi et al. 1993; Vernay et al. 2007). Another factor affecting photosynthetic

rate may be due to the noxious effects of nickel on other metabolic processes rather than the regulation of stomata (Papazogloua et al. 2007). For example, increased nickel concentration resulted in photosynthetic reduction that ultimately damaged the chloroplast structure (Bethkey and Drew 1992), reduced functioning of chloroplast (Molas 2002; Boisvert et al. 2007) or, caused breakdown of photosynthetic pigments, reduction in the synthesis of chlorophyll (Seregin and Kozhevnikova 2006), induced changes in electron transport system (Singh et al. 1989) and inhibited enzymes activities (Seregin and Ivanov 2001). Chloroplast size may also reduce significantly along with, in addition to granum and thylakoid membranes disorganization and deformation and also changes in the lipid composition present in chloroplast membranes (Molas 1997).

A mechanism that could also be involved in inhibition of photosynthesis could be reduced formation of components of light and dark reactions. During light reaction, nickel is known to impair the electron transport system (Krupa and Baszynski 1995). In many plants, PSII is the key site of Ni-induced ETC disruption (Mohanty et al. 1989; Krupa and Baszynski 1995; Maksymiec 1997). Veeranjaneyulu and Das (1982) showed that the dominant sites of nickel deposition are lamella regions of chloroplast. Furthermore, Cyt. $b_6f$ and $b_{559}$, ferredoxin as well as plastocyanin in thylakoid membranes also reduce by the increased Ni concentration. Due to these changes, efficiency of electron transport may be decreased (Veeranjaneyulu and Das 1982). Secondly, photosynthesis may be decreased by metal-induced changes in the activities of key enzymes involved in the regulation of Calvin cycle (dark reaction) (Sheoran et al. 1990; Krupa and Baszynski 1995). The deleterious effects of toxic metals on metabolic processes may also result in direct inhibition of photosynthesis.

### 3.2.2 Chlorophyll Contents

Exposure to heavy metals can significantly affect the concentrations of photosynthetic pigments as has been reported in a number of plant species (McIlveen and Negusanti 1994; Balaguer et al. 1998; Gajewska et al. 2006; Seregin and Kozhevnikova 2006). A decrease in concentration of photosynthetic pigments mainly chlorophyll (*a*, *b*, total), xanthophylls and carotenoids have been reported by several authors, such as Krupa et al. (1993), Pandey and Sharma (2002), Gajewska et al. (2006) and Ahmad et al. (2007).

Heavy metals may also hamper the uptake of other essential nutrients like Fe, Mg, Mn, Zn and Cu, and causing their deficiency in a plant body (Krupa and Baszynski 1995; Maksymiec 1997). Deficiency of essential nutrients, in particular Mg, Mn and Fe, can directly lead to reduced chlorophyll synthesis (Gajewska et al. 2006; Shukla and Gopal 2009). In addition, extreme toxicity of heavy metals can lead to the breakdown of existing chlorophyll in chloroplast (Checkai et al. 1986; Voss 1993; Bennett 1993). Thus, metal-induced decrease in photosynthetic pigments and/or deterioration of ultra-structure of pigments may result in decreased photosynthetic rate.

### 3.2.3 Organic Osmolytes

Soluble Proteins

Regulation of the metabolism of biomolecules is a reliable selection criterion for the tolerance of a particular plant species to withstand any type of environmental stress. Synthesis, concentration and activities of proteins (and nucleic acids) along with the activities of their metabolizing enzymes are of special interest for plant physiologists (Syros et al. 2005). Any limitation in the activity of enzymes like RNAase and protease may affect protein synthesis, which is very critical for germinating seeds that can significantly affect seed germination (Booker 2004; Maheshwari and Dubey 2008).

Toxic effects of heavy metals, were reported on protein contents in *Brassica oleracea* (Chatterjee and Chatterjee 2000), *Azolla pinnata* (Masood and Abraham 2006), *Brassica juncea* (John et al. 2009). In contrary, Parys et al. (1998) in *Pisum sativum* and (Bhattacharya et al. (2010) in *Paspalum distichum* reported no impact of heavy metals on protein contents. However, Bhardwaj et al. (2009) in *Phaseolus vulgaris* and Muneer et al. (2011) in *Vigna radiata* reported a substantial decrease.

Free Amino Acids

An increased level of free amino acids is an important criterion to assess stress tolerance/resistance in plants, specifically under drought (Mapelli et al. 2001; Santos and Pimentel 2009), salinity (Dubey and Rani 1989; Hartzendorf and Rolletschek 2001), and heavy metal toxicity (Shah and Dubey 1998). Metal-induced accumulation of free amino acids, for example alanine, asparagine and proline, is the indication of disruption of metabolic activities in plants, which is crucially important to cope with stressful conditions (El-Shintinawy and El-Ansary 2000). Moreover, accumulation of cysteine under heavy metal stress has been reported by El-Shintinawy and El-Ansary (2000) in *Glycine max* seedlings, which constitute a major portion of total free amino acids. Similarly, Maheshwari and Dubey (2008) reported the increased level of free amino acids under heat stress, which is essentially required for the maintenance of developmental processes under stressful environments (Alia and Saradhi 1991; Chen et al. 2001; Sengar et al. 2008). An increased accumulation of free amino acids as a result of heavy metal stress has also been reported in *Corchorus olitorius* (Mazen 2004) and *Phaseolus vulgaris* (Bhardwaj et al. 2009).

Proline

A plethora of literature is available on the accumulation of proline as an osmoprotectant under abiotic stresses, like salinity, low temperature, nutrient deficiencies, drought and heavy metal toxicity (Stefl et al. 1978; Goring 1979; Carceller and Fraschina 1980; Aspinall and Paleg 1981; Naidu et al. 1991; Dubey and Pessarakli

2002; Ashraf and Harris 2004; Munns 2005). This osmolyte is also important for other metabolic functions like regulation of osmo- and redox-systems (Sharma and Dietz 2006), stabilization of biological membranes (Matysik et al. 2002), chelation of metals (Cobbett 2000; Sharma and Dietz 2006), scavenging of reactive oxygen species (Alia et al. 2001), and protection of enzymes (Öztürk and Demir 2002).

A significant increase in the accumulation of proline has been reported by several researchers in a number of plant species under metal stress, which is comparable to other abiotic stresses. Schat et al. (1997) reported a significant increase in the proline content in *Silene vulgaris,* which is a metal-sensitive plant. However, metal-tolerant plants may accumulate high quantities of proline under heavy metal stress, which is critical in the protection of a plant species from oxidative damage of biological membranes (Dubey and Pessarakli 2002; Gratao et al. 2008). Schat et al. (1997) observed that Cu concentration can induce proline accumulation much higher than Cd and Zn. Alia and Saradhi (1991) and Bassi and Sharma (1993) rated Cu and Cd as strong inducers of proline in many plant species. Accumulation of proline under toxic levels of Cd has also been reported by several authors in different plant species, e.g., *Vetiveria zizanioides* (Pang et al. 2003), transgenic plants and algae (Sharma and Dietz 2006), *Oryza sativa* (Shah and Dubey 1998) *Vigna radiata* (Muneer et al. 2011) and *Brassica juncea* (John et al. 2009). Overall, like for other streses, praline accumulation is an important indicator of metal tolerance in most plant species.

### 3.2.4 Inorganic Ions

Inorganic ion uptake and translocation, particularly during germination and early seedling stages, has been reported to be sensitive to high concentration of heavy metals (Gabbrielli et al. 1990; Kovačević et al. 1999; Pandey and Sharma 2003; Seregin and Kozhevnikova 2005; Hasinur et al. 2005). There are several reports on metal toxicity that affect concentrations of Ca, Mg, Zn, Fe, Mn and many other essential nutrients (Heale and Ormrod 1982; Marschner 1995; Nieminen and Helmisaari 1996; Küpper et al. 1996). Aziz et al. (2007) and Ali et al. (2009) reported a reduction in N, P, K and S contents under nickel toxicity, whereas Palacios et al. (1998) reported a significant reduction in the absorption and translocation of $Na^+$. Nickel (Ni) can also competitively remove $Ca^{2+}$ ions from its binding site (Boisvert et al. 2007) by replacing $Mg^{2+}$, which inhibits the reaction of electron transport during photosynthesis (Küpper et al. 1998; Souza and Rauser 2003; Solymosi et al. 2004). This ultimately causes nutrient deficiencies, which can severely affect growth and development of a plant species (Liu 2008; Gonçalves et al. 2009).

Metals like Fe, Cu, Zn and Mn are the primary components of many metabolically active enzymes (superoxide dismutase, catalase, etc.). Heavy metal toxicity can lead to reduced biosynthesis of these enzymes (Molas 2002; Gajewska et al. 2006). This can significantly suppress vegetative growth, and ultimately the poor biomass production and yield reduction (Schützendübel and Polle 2002; Gajewska and Skłodowska 2007).

### 3.2.5 Water Relations

Maintenance of plant water relations is critical under environmental stresses such as drought and salinity (Krämer and Boyer 1995). However, the impact of heavy metal stress is still unclear, as there are controversial reports available in the literature (Barceló and Poschenrieder 1990; Menon et al. 2005; Vernay et al. 2007). Still there are many reports available in the support of negative impact of heavy metal stress on imbalances in plant-water relations (Sharma and Sharma 1987; Barceló and Poschenrieder 1990; Chatterjee and Chatterjee 2000).

Plant water relations may undergo changes under heavy metal stress, which may be due to the accumulation of compatible solutes/osmotica. For example, proline accumulates in plants under metal stress (El-Enany and Issa 2001; Lin and Kao 2006). Soluble sugars and other free amino acids also accumulate in metal-stressed plants (Baccouch et al. 1998). It is now well established that organic osmotica can significantly reduce water and solute potentials in metal-stressed plants which help plants to maintain cell turgor.

## *3.3 Anatomical Characteristics*

### 3.3.1 Root Anatomy

The uptake of heavy metals by a plant may trigger a series of anatomical alterations with potential functional consequences in the plant in addition to other morpho-physiological changes (Fig. 5.1). One of the most prominent among these is a marked decrease in cell size of root tissues. This may be due to a decrease in the elasticity of cell walls of the root as was previously reported by Barceló et al. (1986). Heavy metal-induced reduction in the cell size includes all root tissue, whether these are parenchymatous tissues in the root cortex and/or pith or sclerified tissues in sclerenchyma or xylem vessels, thereby resulting in a shrinkage of root diameter (Kasim 2006).

A significant decrease in xylem vessels, in particular, metaxylem vessels may significantly limit the movement of water and mineral nutrients from root to aerial parts of the plant. Consequently, the plant might need to decrease water loss through transpiration as was indicated by Greger and Johansson (1992) in sugar beet. This is certainly of great ecological significance, particularly under a variety of abiotic stresses. Gowayed and Almaghrabi (2013) also reported a reduction as a result of heavy metal stress in root anatomical traits such as root diameter, central cylinder diameter, cortex thickness, cross section area of root and cross section area of central cylinder.

In view of a number of studies it is evident that reduction in root growth may be due to a decrease in cell division that leads to increase the thickness of cell wall, and/or a disorder in the activity and contents of phytohormones like auxins in the roots exposed to heavy metals (Sharma and Dietz 2006; Farzadfar and Zarinkamar

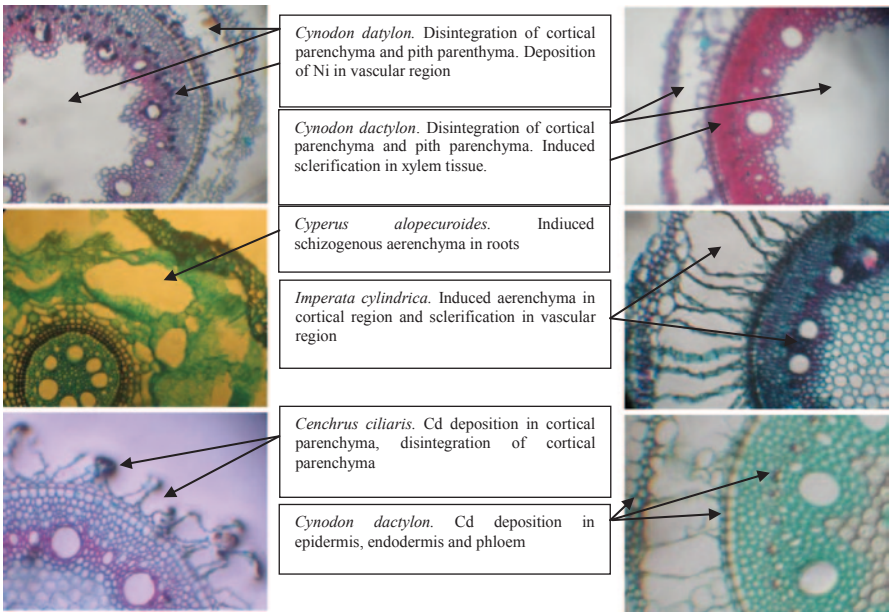


**Fig. 5.1** Influence of heavy metal stress on root anatomy of some selected plant species

2012). High concentrations of heavy metals in root rhizosphere can considerably alter root anatomical parameters (Seregin and Kozhevnikova 2008), the most important among them are the dermal tissues, i.e., endodermis and exodermis (Lux et al. 2011).

Several studies have showed that the heavy metal stress had an adverse effect on root anatomical structures, (e.g. Kovačević et al. 1999; Shalini et al. 1999; Khudsar et al. 2001; Papadakis et al. 2004; Kasim 2006). Qaisar et al. (2005) reported some specific anatomical modifications like reduced epidermis cell size, development of large air spaces, and consequently, a reduced vascular region area, and as a result significant reduction in the root diameter.

Llamas et al. (2008) reported the symptoms of heavy metal toxicity in rice (*Oryza sativa*) and reported no change in root anatomical characteristics when the roots were exposed to heavy metals for a short-term, however, a long-term exposure resulted in a significant affected root structure, which affected membrane permeability.

Heavy metals are more commonly accumulated in the parenchymatous tissues, epidermis and phloem in root and stem, but less frequently these can accumulate in other tissues also (Fig. 5.1). Rabier et al. (2008) reported a significant accumulation of Ni in epidermis and phloem of basal stem and roots in Australian native *Grevillea exul*. Nickel stress also causes reduction in the number of xylem vessels, and consequently, the function of the vascular tissues (Kovačević et al. 1999).

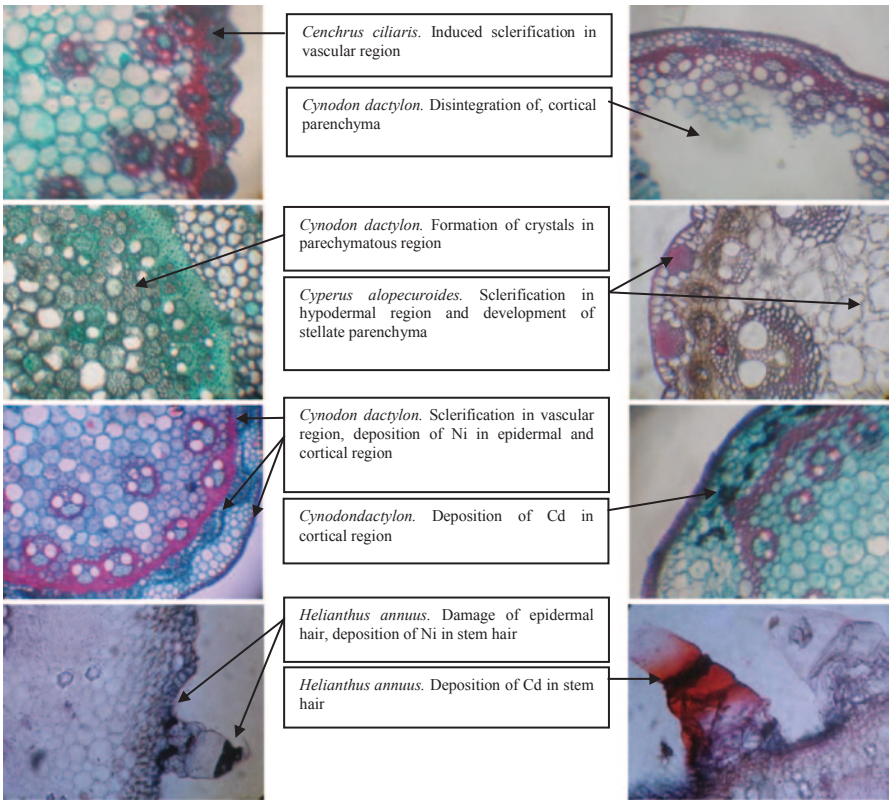


**Fig. 5.2** Influence of heavy metal stress on stem anatomy of some selected plant species

### 3.3.2 Stem Anatomy

Stem tissue organization can be severely affected due to heavy metal toxicity (Fig. 5.2). Setia and Bala (1994) reported disorganization of epidermal cells, disintegration of root cortical cells and a reduction in cell wall thicknesses of epidermis and hypodermis as a result of Ni toxicity. Sresty and Rao (1999) observed a reduction in stem diameter, vascular bundle number and cell size of storage regions under Ni toxicity. In contrast, Kasim (2006) reported a significant reduction in root diameter, but not in stem diameter when plants were exposed to Cu and Cd stress.

de Silva et al. (2012) studied the effects of heavy metal stress on xylem characteristics in *Acer rubrum* and reported a reduction in the proportion of xylem tissue. Diameter and density of xylkem vessels also reduced significantly. The reduction in vessel size may result in reduction in hydraulic conductance in both root and shoot.

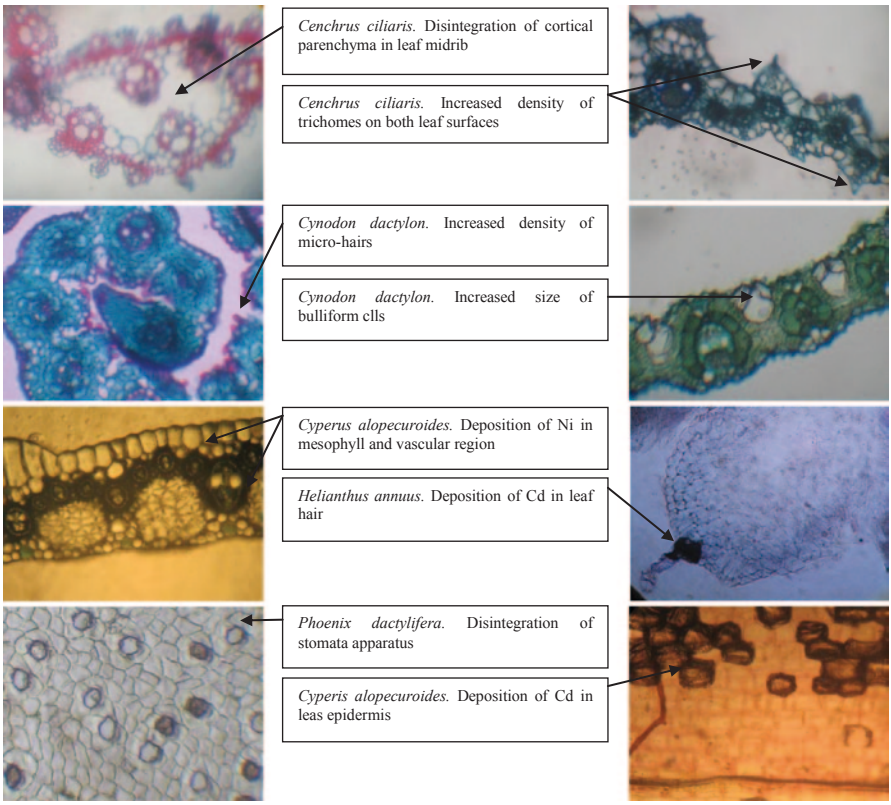


**Fig. 5.3** Influence of heavy metal stress on leaf anatomy of some selected plant species

### 3.3.3 Leaf Anatomy

Toxic effects of heavy metals may alter the plant anatomy (Fig. 5.3). The devastating effects of heavy metals on leaf anatomy are decrease in size of mesophyll parenchyma, size of vascular bundles, diameter of the xylem vessels, and size of epidermal cells (Kovačević et al. 1999). Decrease in the volumes of intercellular spaces and size of palisade and sponge mesophyll has been reported in the leaves of *Brassica oleracea* (Molas 1997). In another study, Kravkina (2000) reported the formation of large inclusions in the leaves of *Dianthus repens* plants growing under excess nickel concentration. Leaf size of sea grass (*Halophila ovalis*) showed a significant reduction when its plants were treated with increased concentrations of heavy metals (Ambo-Rappe et al. 2011).

Mikus et al. (2008) reported a spatial accumulation of heavy metals in different leaf tissues of *Thlaspi praecox*. Metals generally accumulated in epidermal region, S and Ca in palisade mesophyll, and K and P in vascular bundles. However, the accumulation of Cl was the maximum in vascular bundles and spongy mesophyll,

whereas Pb, Cd, K and Cl were accumulated in vascular bundles and collenchymas, Pb and Cd in mesophyll and Zn in epidermis. Kachenko et al. (2008) reported a significant accumulation of Ni in the epidermis of hyperaccumulating shrub *Hybanthus floribundus*.

Stomatal density, size and orientation are sensitive to heavy metal toxicity. Kastori et al. (1992) showed a significant increase in stomatal density on both adaxial and abaxial leaf surfaces under heavy metal toxicity, in particular Cd, Cu and Zn. Stomatal density was more responsive to Cd stress, followed by that of Cu and Zn, while the least by Pb stress. Additionally, the stomatal size also decreased as a result of heavy metal stress. Molas (1997) reported a significant reduction in the stomatal density, as well as density of open stomata in *Brassica oleracea*. In addition, deformation of stomatal complexes has also been reported under nickel stress.

Anjana et al. (2006) and Gostin (2009) were of the strong view, that cadmium causes reduction in the size of stomata and their frequency on the adaxial and abaxial sides of leaves. However, according to de Silva et al. (2012), the only plant response specific to metal stress was decreasing trends of stomatal density and chlorophyll content. Reduction of the size of stomata and their frequency can lead to more negative impact on transpiration, photosynthesis and gas exchange, because in the presence of metal the most of the stomata are closed.

## 4 Mechanism of Heavy Metal Tolerance

Physiological and biochemical mechanisms are of heavy metal tolerance has gained considerable insight during the last few decades. Plants employ specific strategies to tolerate noxious levels of heavy metals in the soil (Kochian et al. 2002). Most of the plants have developed complicated strategies for the acquirement of relatively unavailable micronutrients like Zn, Mn, Cu, Fe and Ni from soil.

The mechanisms of tolerance to metal stress in plants may range from exclusion of toxic metals, and inclusion and accumulation at inert places, which may vary from species to species (Raskin and Ensley 2000). Prasad (1995) discussed five possible mechanisms that enable plants to tolerate heavy metals: binding of metals to cell wall, reduced transport, active efflux, compartmentalization, and chelation.

Metal tolerance or accumulation can be enhanced by production of binding proteins and peptides in plants. High specificity of these peptides or proteins for more toxic metals like Cd, Hg and Pb instead of less toxic Zn and Cu will be of great significance in plants (Ryu et al. 2003).

On the basis of accumulation of heavy metals, plants can be broadly divided into three categories, first that can accumulate Cu or Co, second Zn, Cd or Pb and the third Ni accumulators (Raskin et al. 1994). However, tolerance level in plants can be grouped into sensitive, resistant excluder, tolerant non-hyperaccumulator, and hypertolerant hyperaccumulator species, each with specific physio-anatomical and molecular mechanisms for their resistance/tolerance to metal toxicity. Plant responses against toxic effects of heavy metals are regulated in a process called metal

homeostasis, which also includes regulation of the metal-induced reactive oxygen species (ROS) signaling pathway. Generation and signaling of ROS plays an important role in heavy metal detoxification and tolerance.

## 5 Conclusion

Heavy metal toxicity can severely hamper growth and development of a plants species. This generally occurs due to with a number of physiological and anatomical changes in the plant body. The devastating impact of heavy metals can be related to ionic imbalance, metal toxicity, reduced gas exchange parameters (especially photosynthetic rate), degradation of chloroplast and photosynthetic pigments, and changes in plant water relations. Among anatomical changes, disintegration of parenchymatous tissue, reduced size of cells in different tissues, reduced root and stem diameters, reduced leaf growth, and narrower xylem vessels are more important. Moreover, there is a spatial accumulation of heavy metal in different organs, more commonly in epidermal, parenchymatous and phloem tissues. However, tolerance to heavy metals stress varies from species to species, and even within a same population.

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# Chapter 6
# Integration of Different Bioindication Methods for Chemical Elements: The Multi-Markered-Bioindication-Concept (MMBC)

**Bernd Markert, Agáta Fargašová, Stefan Fraenzle and Simone Wuenschmann**

**Abstract** Before entering the field of integrating different bioindication methods, clear-cut definitions of the terms bioindication, biomonitoring and others are given. For purposes of bioindication and biomonitoring of chemical elements obviously, both a highly specific approach concerning each single chemical species of an element and a comprehensive treatment of general features are required. The latter is given in the Biological System of Elements. To observe the quality of our environment the use of living organisms in biotests, bioindication and biomonitoring activities is an established method of determining inorganic and organic contaminants. To achieve a more public-related prophylactic healthcare feature derived from these biotechniques in the future, all existing tools of analytical and biological investigations of the past must be concentrated on a common focus. A first approach, including an example for transferring trace elements from food into children via the nursering mother, is given in a so called Multi-Markered-Bioindication-Concept (MMBC). Further on, the collaboration between analytical scientists,

Definitions, strategies and scientific results of this review article correspond to Markert, (1996); Markert et al. (2003a); Markert (2007); Wünschmann (2007) and Wuenschmann et al. (2008) of the reference list

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ecotoxicologists and especially medical people is of elementary importance. For reaching this communication and exchange of essential information, different forms of education and teaching of students on an international level combined with common research projects are key functions for a global success.

**Keywords** Biomonitoring · Bioindication · Biotests · The Biological System of Elements (BSE) · The Multi-Markered-Bioindication Concept (MMBC)

## 1 Introduction

Previous thought on the distribution of elements in an ecosystem has been based on the fact that the information relative to content and concentration is valid for the specific plant species with more or less broad limits (Markert 1996). At the same time one should note that there can be natural fluctuations in the element contents among individuals of a plant species growing on similar soil types (Marschner 1983).

The position and classification of the chemical elements in the classical Periodic System of the Elements (PSE) does not permit any statements to be made about their functional essentiality or their acute chronic toxicity for living organisms. This is related to the fact that the PSE is based on purely physiochemical aspects. In the past years, a so-called Biological System of Elements (BSE) has been established (Fig. 6.1), which primarily considers aspects of basic analytical, biochemical and physiological research (Duvigneaud and Denayer-De Smet 1973; Keith 1988; Adriano 1992; Farago 1994; Saiki et al. 1997; Breulmann et al. 1998; Bargagli 1998; Lieth 1998; Djingova and Kuleff 2000; Wolterbeek 2002; Golan-Goldhirsh et al. 2004; Lux et al. 2004; Freitas et al. 2006; Mench et al. 2006; Fraenzle et al. 2007; França et al. 2007; Broadley et al. 2007; Lepp and Madejon 2007; Cakmak 2008; Chaney et al. 2008; Fraenzle et al. 2008; Greger 2008; Irtelli and Navari-Izzo 2008; Marmiroli and Maestri 2008; Prasad 2008; Quevauviller et al. 2008; Schroeder P et al. 2008; Schwitzguébel et al. 2008; Trapp et al. 2008; Verbruggen et al. 2008; Verkleij 2008; Pla et al. 2000; Smodis 2003; Suchara et al. 2007; Zechmeister et al. 2007). This includes:

- the inter-elemental relations of single elements within an individual organisms expressed as a linear correlation coefficient,
- the physiological function of single elements paying attention to evolutionary—development during the emergence of organic life from the inorganic environment, and
- the uptake form of individual elements and their compounds by the living organisms.

For purposes of bioindication and biomonitoring of chemical elements, obviously both a highly specific approach concerning each single chemical species of an element and a comprehensive treatment of general features are required (Markert 1996; Bargagli 1998; Carreras et al. 1998; Garty 1998; Herpin et al. 2001;

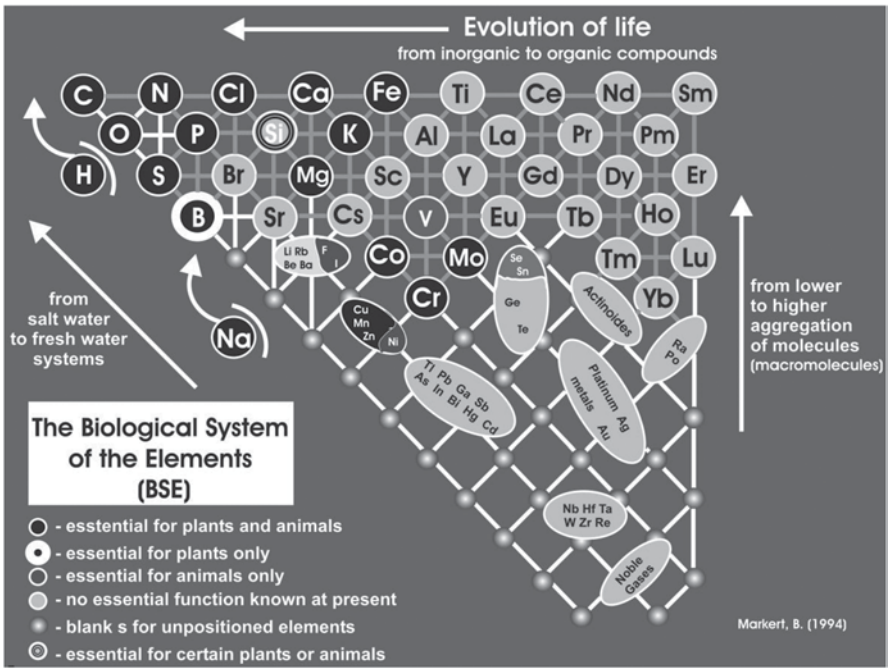


**Fig. 6.1** The biological system of the elements (*BSE*) compiled from data on correlation analysis, physiological functions of the individual elements in the living organisms, development out of the inorganic environment, and with respect to their uptake form by the plant organism as a neutral molecule or charged ion. The elements H and Na exercise various functions in the biological system so that they are not conclusively fixed. The ringed elements can at present only be summarized as groups of elements with a similar physiological function since there is a lack of correlation data or else these data are too imprecise (Markert 1996)

Herzig et al. 1990; IAEA 2001; Jeran et al. 1993; Loppi and Bonini 2000; Fomin et al. 2003; Markert et al. 2003a; Markert 2007; Wünschmann 2007; Figueiredo et al. 2007; Mohr 2007; Wuenschmann et al. 2008; De Bruyn et al. 2009).

## 2 Definitions of the Terms Bioindicatiors and Biomonitors

The use of living or formerly living organisms in biotests, bioindication and biomonitoring is an established method of determining inorganic and organic contaminants.

In the following section some definitions summarized in (Markert et al. 1997, 2003a) are given:

A bioindicator is an organism (or part of an organism or a community of organisms) that contains information on the quality of the environment (or a part of

the environment). A biomonitor, on the other hand, is an organism (or part of an organism or a community of organisms) that contains information on the quantitative aspects of the quality of the environment. The clear differentiation between bioindication and biomonitoring using the qualitative/quantitative approach makes it comparable to instrumental measuring systems. Such effects (information bits) of bioindicators (biomonitors) may include changes in their morphological, histological or cellular structure, their metabolic-biochemical processes (including accumulation rates), their behaviour or their population structure. Accumulation indicators/monitors are organisms that accumulate one or more elements and/or compounds from their environment. Effect or impact indicators/monitors are organisms that demonstrate specific or unspecific effects in response to exposure to a certain element or compound or a number of substances. According to the paths by which organisms take up elements or compounds, various mechanisms contribute to overall accumulation (bioaccumulation), depending on the species-related interactions between the indicators/monitors and their biotic and abiotic environment. Biomagnification is the term used for absorption of the substances from nutrients via the epithelia of the intestines. It is therefore limited to heterotrophic organisms and is the most significant contamination pathway for many land animals except in the case of metals that form highly volatile compounds (e.g. Hg, As) and are taken up through the respiratory organs (e.g. trachea, lungs). Bioconcentration means the direct uptake of the substances concerned from the surrounding media, e.g. the physical environment, through tissues or organs (including the respiratory organs). Besides plants, that can only take up substances in this way (mainly through roots or leaves), bioconcentration plays a major role in aquatic animals. The same may also apply to soil invertebrates with a low degree of solarisation when they come into contact with water in the soil.

*Active* bioindication (biomonitoring) means when bioindicators (biomonitors) bred in laboratories are exposed in a standardised form in the field for a defined period of time. At the end of this exposure time, the reactions provoked are recorded or the xenobiotics taken up by the organism are analyzed. In the case of *passive* bioindication (biomonitoring) organisms already occurring naturally in the ecosystem are examined for their actions.

Various newer methods (biomarkers, biosensors, biotests in general) have been introduced into the application field of bioindication, besides the classical floristic, faunal and biocoenotic investigations that primarily record unspecific reactions to pollutant exposure at higher organismical levels of bioindication.

*Biomarkers* are measurable biological parameters at the suborganismic (genetic, enzymatic, physiological. morphological) level in which structural or functional changes indicate environmental influences in general and the action of particular in qualitative and sometimes also in quantitative terms. Examples are enzyme or substrate induction of cytochrome P-450 and other Phase I enzymes by various halogenated hydrocarbons; the incidence of forms of industrial melanism as markers for air pollution; tanning of the human skin caused by UV radiation; changes in the morphological, histological or ultrastructure of organisms or monitor organs (e.g. liver, thymus, testicles) following exposure to pollutants. A *biosensor* is a measuring

device that produces a signal in proportion to the concentration to a defined group of substances through a suitable combination of a selective biological system, e.g. enzyme, antibody, membrane, organelle, cell or tissue, and a physical transmission device (e.g. potentiometric or amperometric electrode, optical or optoelectronic receiver).

Biomarkers and Biosensors can be used as *biotest* (bioassay) which describes a routine toxicological-pharmacological procedure for testing the effects of agents (environmental chemicals, pharmaceuticals) on organisms, usually in the laboratory, but occasionally in the field under standardized conditions (with respect to biotic and abiotic factors). In the broader sense, the definition covers cell and tissue cultures when used for testing purposes, enzyme tests or tests using microorganisms, plants and animals in the form of single-species (Fargašová 1994) or multispecies procedures in model ecological systems (e.g. microcosms and mesocosms). In the narrower sense, the term only covers single-species and model system tests, while the other procedures may be called suborganismic tests. Bioassays use certain biomarkers or—less often—specific biosensors and can be used in bioindication or biomonitoring.

The term tolerance can be described as desired resistance of an organism or community by unfavourable abiotic (climate, radiation, pollution) or biotic factors (parasites, pathogens), where adaptive physiological changes (e.g. enzyme induction, immune response) can be observed (Oehlmann and Markert 1997). Unlike tolerance, *resistance* is a genetically derived ability to withstand stress (Oehlmann and Markert 1997). This means that all tolerant organisms are resistant, but not all resistant organisms are tolerant. *Sensitivity* of an organism or community means its suspectibility to biotic or abiotic changes. Sensitivity is low if the tolerance or resistance to an environmental stressor is high, and sensitivity is high if the tolerance or resistance is low.

## 3 The MMBC-Concept

Wünschmann (2007) and Wuenschmann et al. (2008) have reported the results from single-species tests will give only limited information on effects of chemical substances in higher biological integration levels (populations, biocoenoses or ecosystems). Accordingly, problems even arise when just extrapolating data obtained in one species of plant or animal to another one (even if it belongs to the same genus); the same holds for transfer of (laboratory) test results to the freeland situation which is distinguished by more complex structures and corresponding timescales (Fraenzle 1993).

Thus ecotoxicology, focussed on identifying and evaluating effects of hazardous compounds on ecosystems, can only partly meet the precautionary ends of toxicology (concerned with human health). This necessitates working on human-based samples in the framework of a broad-viewing investigation focussed on such effects which possibly affect humans (Markert et al. 2008).There is a chance to

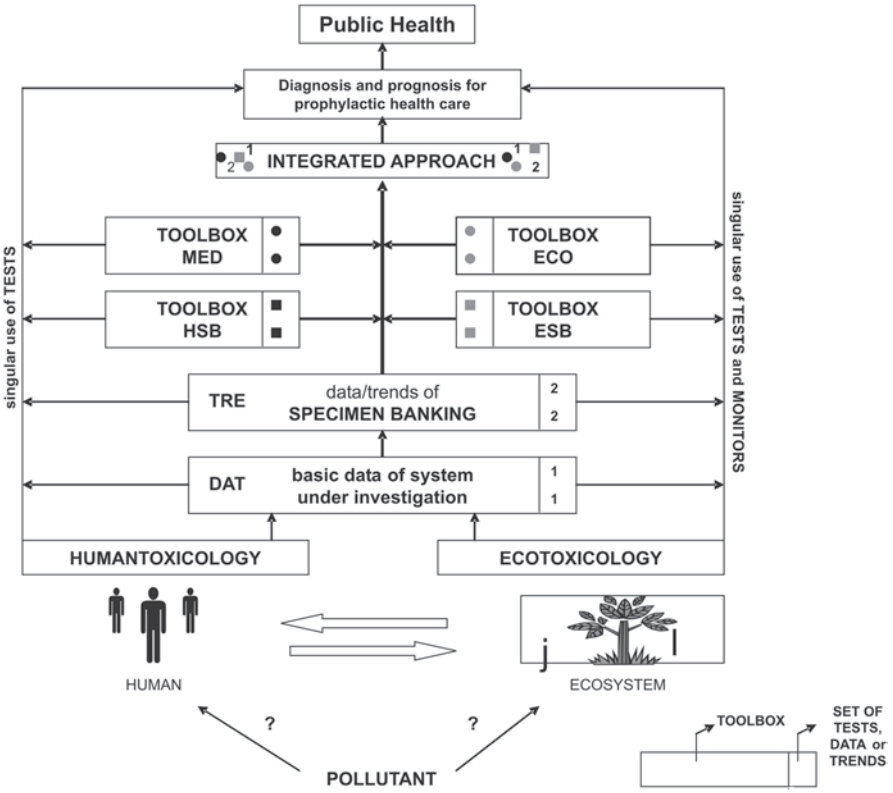


**Fig. 6.2** Possible hierarchical structure of a bioindicative toolbox model for integrative approaches in human- and eco-toxicology. The toolboxes MED and ECO contain single sets of tests that can be combined functionally to allow an integrated approach to the particular frame of reference or a specific scientific problem. The toolboxes *HSB* (human specimen banking) and *ESB* (environmental specimen banking) represent years of results from international environmental sample banks specializing in environmental and human toxicology; in addition to MED and ECO, they provide an important information on the ecotoxicological and human-toxicological behavior of environmental chemicals. In the integrated approach, all the results obtained singly are substantiated by existing basic data available from (eco-) systems research, toxicology and environmental sample banks. The parameter constellations necessary for this are taken from the toolboxes TRE and DAT (Markert et al. 2003b)

meet the comprehensive precautionary expectations of toxicology only if investigations are combined into a biointegrative approach in a systematic manner. Thus both temporal trends of environmental burdening and newly developing centres of pollution can be identified. For this purpose, (Markert et al. 2003b) designed the Multi-Markered-Bioindication-Concept (MMBC; Fig. 6.2); This approach depends on some combinations of ecotoxicological data-sets with those from human medicine (especially toxicology). This method which is based on “tool boxes” (cp. the explanations for Fig. 6.2) thus implies an approach integrating different instrumental and bioindicative methods.

As presented already by (Markert et al. 2008) Fig. 6.2 represents one proposal of a complete dynamics of an environmental monitoring system supported by bioindication to integrate human- and ecotoxicological approaches. It can recombine its measurement parameters according to the particular system to be monitored or the scientific frame of reference. The two main subjects of investigation—man and the environment—and the disciplines human toxicology and ecotoxicology derived from them are associated with various "toolboxes" and sets of tests ("tools", e.g. bioassays) for integrated environmental monitoring (Markert 2003b). The system shown in Fig. 6.2 consists of six toolboxes. The first two are derived mainly from environmental research: DAT (for data) and TRE (for trend). DAT contains, as a set, all the data available from the (eco-)system under investigation, i.e. including data acquired by purely instrumental means, e.g. from meteorological devices. DAT also contains maximum permissible concentrations of substances in drinking water, food or air at the workplace and the data for the relevant ADI ("acceptable daily intake") and NO(A)EL ("no observed (adverse) effect level"). The toolbox TRE contains data on trends; these have been compiled mainly from years of investigations by national environmental sample banks, or information available from long-term national and international studies (e.g. Ellenberg et al. 1986). Specific conclusions and trend forecasts can then be prepared using the subsequent toolboxes HSB (human specimen banking) and ESB (environmental specimen banking). The toolbox MED (medicine) contains all methods usually employed in haematological and chemical clinical investigations of subchronic and chronic toxicity, whereas ECO is largely made up of all the bioindicative testing systems and monitors relevant to ecosystems which may be combined to suit a particular situation to be monitored (Markert et al. 2003b).

By relating data from all the toolboxes with some network, it must be achieved to assess average health risks to certain parts of the population or at least upper limits of future risks posed by pollutants (Ellenberg et al. 1986). For this kind of risk assessment, all the information on kinds of effects, dose-effect relationships, and toxicological limits derived there from by present level of scientific knowledge are combined and used (WHO 1996). Although toxicological experiments on humans would be unethical, corresponding data pertinent to toxic risks can be obtained from workplace experiences and cases of accidental, homicidal or suicidal poisoning. For both statistical reasons and evaluation of sub-acute-dose effects which might yet bring about diseases, results of epidemiological surveys which compare exposed to control groups must be added. Recent information technology allows for development and use of simulation models which integrate all these data, integrating a large number of parameters which are not directly linked to each other.

As the way how the MMBC combines functional and integrated windows for prophylactic healthcare was outlined in more detail before, we refer to the corresponding literature rather than repeating matters here (Markert et al. 2003b). An integration of data on ecological quality and human health takes knowledge of the sites, methods and locations where and how the data were obtained, producing metadata upon superposition of these pieces of information. By combination of such metadata with geostatistical information including and using GIS techniques, some

integration of environmental monitoring data concerning exposure to and effects brought about by contaminants was achieved in Germany. This metadata system contains some 800 items producing, some picture of environmental quality and human health beyond a detailed consideration of "classical" topics of environmental monitoring (water, air, soils, plants, animals, landscapes (Schroeder W et al. 2003)).

Analysis of biogenic samples in either biomonitoring or bioindication will produce data; these, however, must not be taken as pieces of information on the "state of the environment" directly, except for measurements of atmospheric deposition (by means of mosses, Tillandsiae, etc). Even then, no organism might enrich all the elements from the environment by some identical bioconcentration factor BCF, but there will always be selectivity with drawbacks in biomonitoring (Fraenzle and Markert 2007)

## 4 Physiological and Dynamic Features of Chemical Elements in the Food/Milk System

Mother's milk has a peculiar significance for being the best-quality and physiologically best-suited source of food for nutrition of small children. For that reason, there is a history of several decades of extensive research on quantification of hazardous substances in mother's milk. As important as these works were, they could not attribute detected burdens to any path of uptake, nor could they attribute them to that via maternal food. Acknowledging the human organs system to be highly complex in all biological, physiological and biochemical aspects, the development of such methods is required which allow for pinpointing the origins and behaviour of invading materials, giving clues to certain environmental influences on mother's milk quality and composition.

Living beings are exposed to chemicals in the environment throughout. Elements may be resorbed on each dermal, inhalative or oral pathways depending on their physicochemical properties, getting into cells of whatever kind/differentiation, causing, given corresponding properties, an active exposition of the target organ to an element just resorbed. Limited by their active or passive elimination in different ways (urine, feces, other body liquids, etc.) they are often capable of accumulating in organ systems and tissues, sometimes augmented by a high affinity towards the corresponding target organ or tissue. Taking histological and biochemical effects into consideration (Fraenzle 2009) derived typical accumulation and resorption properties by correlating the electrochemical ligand parameter and the corresponding complex dissociation constants. Like this approach covers ligand properties of biological matter throughout a species (Boese-O'Reilly et al. 1999; Muckle et al. 2001; Fraenzle et al. 2004; Czub 2004) also transfer/relocation processes within some organisms will be understood better when considering rules of coordination chemistry.

Even without discussing this reasoning in much detail, it is feasible to describe the pathway of chemical elements by a so-called transfer factor. This also gives information on exposition of the breast-fed children.

Transfer factor (TF) is a measure for transfer of chemical elements from maternal food into mother's milk. TF values are used to identify aspects of coordination behaviour of specific elements including possible mechanisms of protection and regulation in the maternal organism. Thus, an attempt is made to understand why there are no dangerously elevated levels of elements such as Cd and Pb, but also why other elements which are more abundant in a polluted area do not get into milk up to a proportional scale with maternal intake.

Though these results are hard to explain even when taking interactions between metals into account, they do imply an advantage of breast-feeding just in polluted regions, except if there are high levels of organic contaminants such as PCBs or DDT due to special nutritional habits. Up to now, the latter was observed only with indigenous peoples of the Upper Arctic, like Inuits and Chukhots (Boese-O'Reilly et al. 1999; Muckle et al. 2001; Czub 2004) except for sites of accidents with corresponding chemicals (e.g. at Seveso in 1976). For Germany, levels of organics like PCBs or DDT in mother's milk are constantly decreasing for many years, being below acutely toxic threshold levels (Boese-O'Reilly et al. 1999).

Now an attempt is made to interpret these data and the fact that different levels of regional pollution are not represented in/by milk composition, given variations of TF. There are two topics of interest:

- How does the milk level of an element and thus its TF respond to an increase or decrease in maternal supply? Is there regulation or (even) homoeostasis?
- Do elements that are chemically similar or dissimilar influence each other on their biochemical transportation ways until they get into milk, be it synergetistic or antagonistic?

TF values determined in Euroregion Neisse (Wappelhorst et al. 2002), a triangle in the south east part of Germany, imply that just 0.1 and 0.5 %, respectively, of Mn and Mo are partitioned into milk ($TF_{Mn} = 0.001$ d/kg; $TF_{Mo} = 0.005$ d/kg). Their TF values thus are far lower than those of the other elements. On the other hand, both essential I (highest extent of transfer—about 50 % or $TF_I = 0.56$ d/kg) and physiologically less significant elements Rb and Cs display the highest TF values ($TF_{Rb} = 0.20$; $TF_{Cs} = 0.27$ d/kg). This large extent of elimination via milk might have been anticipated, given the similarity of these heavy alkali ions with the colloquial electrolyte ions Na and K, with Rb likely undergoing electrolyte fractionation at membrane interfaces in the same way and manner as K (Anke and Angelov 2004). Cesium (Cs) presumably will distribute among the sinks without any preceding interaction with other binding partner because it hardly forms complexes (Kaim and Schwederski 1993) With mother's milk representing about one-fourth of secreted liquid volumes, it can be expected that one-fourth of Cs resorbed in the gastrointestine will show up in milk eventually. Given the simplicity of this model, the agreement with the actual extent of partitioning of Cs (23 %) is outright excellent.

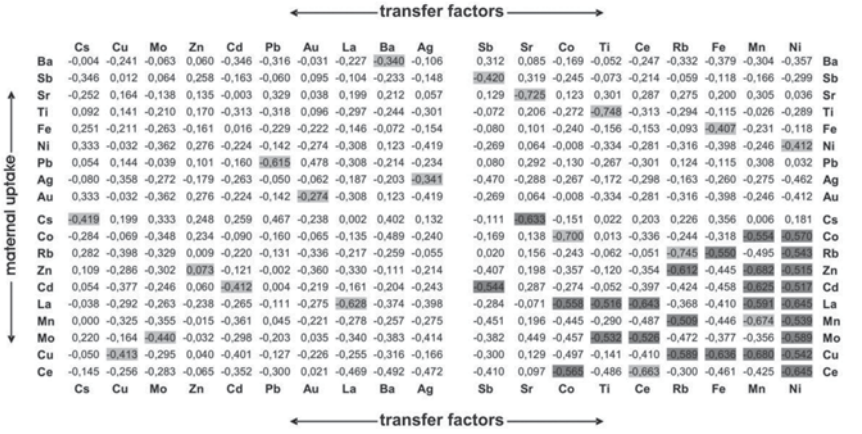


**Fig. 6.3** Analyzes of correlation between element-specific transfer factors ((d/kg), vertical columns) vs. element-specific maternal uptake amounts (µg/d) via food to determine synergistic or antagonistic effects. *Dark grey* shadowed values: significant correlations ($r \geq 0.5$), *light-grey*: self-correlation effects (Wünschmann 2007; Wuenschmann et al. 2008)

A more general problem is why TF value and partition for, for example, Zn are that much higher than for those for Mn. Possible reasons include interactions of the elements with one another but also effects on the transport of other elements, e.g., if they compete for carrier binding sites. Given the latter, TF values should be a complex function of different chemical factors. The only practical way to address this problem is to correlate (nonaveraged, individual) TF values of one element with the amounts consumed of another (or this very) element consumed by the mother. If so, the TF of one element significantly may responds to changing uptake amounts of another one, which causes an influence—either synergistic or antagonistic—which could be detected in TF variations. Correlation diagrams for the corresponding data are summarized in Fig. 6.3. There are no significant influences of maternal intake amounts of other elements to TF values of Pb or Cd.

The relatively low TF of Cd (0.014 d/kg) is interpreted as a result of its considerably lower stability of complexes as compared to those of for example Ni, Zn or Cu (0.054, 0.077 and 0.156 d/kg) (cf. data in (Irving and Williams 1953)) therefore biochemical transport of Cd and some other metals during production of milk will take place mainly as simple aquaions, much like with alkali metals and alkaline earths (Neville 1991; Wuenschmann et al. 2004b). Accordingly, Cd (or Sr or Ba) cannot effectively compete with other metals—neither such supplied in far larger amounts like Fe, Zn, Mn or Cu nor those which are similarly rare (10–20 µg/d ≈ 100–200 nmol/d like Y, Cs, Co or Zr)—for carriers. The latter carrier may be both transport proteins and ligands, the amounts of which in milk for more than outweigh the metal contents (chloride, citrate, oxalate, some amino acids like glutamate (Wuenschmann et al. 2004a) Thus, there is no effect of other elements on the TF of Cd and its partition into milk. Although there is no corresponding effects for Pb in these data, the fragmentary data on complex stability between $Pb^{2+}$ and "milk ligands" preclude similar statements like those referring to Cd.

Likewise, TF values of essential elements Cu, Mo, Zn, and nonessential elements Ag, Au, La, Ba and Cs are not influenced by supplies of other elements. Transportation of Cu cannot be compared to those of other elements (DaSilva and Williams 2001), because it usually involves special, Cu-specific proteins (metallochaperons) in order to avoid toxic effects from the very strong binding of $Cu^{2+}$ to a multitude of ligands. This strong coordination is the probable reason for the highest TF of any di- or higher valent ion observed with Cu ($TF_{Cu}$ = 0.156 d/kg). Molybdenum (Mo) is taken up and resorbed as molybdate ion by means of the sulfate carrier (Anke and Angelov 2004); hence Mo is unlikely to compete with "genuine" cations for their carriers.

The interaction of an element with itself is particularly interesting because it bears some information on both homoeostasis and deposition within in-body depots (e.g., bones). Figure 6.3 shows that several elements absorbed in larger amounts (Rb, Sr, Ti), which are neither toxicologically relevant nor essential, display a pronounced negative self-correlation (light grey coloured), while, though other authors postulate homoeostatic regulation for this element, there is no relationship between supply and TF for example for zinc. Up to now, this can only be noted as a kind of phenomenon which warrants further explanation, as do the many different interelemental interactions which influence TFs of Ni and Mn. TF values of Ni and Mn depend on the uptake rates of following elements (cp. the last two columns of Fig. 6.3):

$TF_{Mn}$ depends on supply of elements: Cu Zn Co Cd La

$TF_{Ni}$ depends on supply of elements: Cu Zn Co Cd La Ce Mn Mo Rb

Mn is affected by increasing supplies of Zn, Cu or Cd (pronounced negative correlation in each case); possibly these metals which are known to induce oxidative stress by radical chain reactions (Huang et al. 1994) induce oxidation of manganese to Mn(III), causing stronger retention to transferrin. Cobalt (Co), more than 90 % of its total not being absorbed as cobalamine (vitamine $B_{12}$) but as $Co^{2+}$ aquaion or some complexes far less stable than cobalamine, does correlate negatively with uptakes of La and Ce. La and Ce, in turn, are coordinated to transferrine, like some heavier REEs (Nd, Sm, Yb) Hirano and Suzuki, 1996), yet not as strongly as trivalent Fe, Mn or Y do. This strongly suggests a competition for (protein) carrier binding sites. Figure 6.4 symbolizes the modes of interaction for the two metals distinguished by lowest TF values, namely, Mn and Mo. Whereas Mn responds to increased allowances of many other elements with a decreased TF (see above), Mo does not do because it is resorbed as an oxoanion by means of the sulfate carrier (Williams and Da Silva 1996) and thus is not perturbed by cation carrier competition.

Mother's milk contains low amounts of Mn and Mo. As both are essential, one is required for depots built up in the foetus before delivery (Rossipal et al. 2000) analyzed blood samples from both arteries and vein of the umbilical cord for contents of Zn, Mo, Mn, Sn, Ca, Mg, Co, Se, and Cu. Taking differences of concentrations of Mn in arteries and vein, the irreversible part of Mn transfer from maternal blood into the fetus-placenta amounted to 0.4 mg/l blood serum (16.2 % of a primary 2.4 µg/l content); the corresponding value for Mo is 0.12 µg/l (16.7 % of 0.7 µg/l). Considering the fact that these values were measured at the very end of

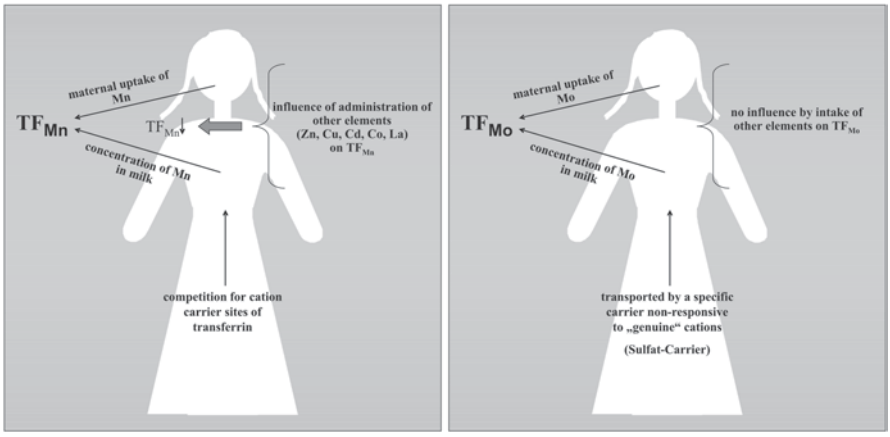


**Fig. 6.4** Influences of administration of other elements on TF values of Mn and Mo which have the lowest TF values of all the metals investigated. The reduction of $TF_{Mn}$ may be due to competition for cation carrier sites also employed by Zn, Cu, Cd, Co or La. $TF_{Mo}$, on the other hand, is not influenced by intakes of other elements; presumably because it is transported by a specific carrier non-responsive to "genuine" cations (Wünschmann 2007; Wuenschmann et al. 2008)

pregnancy, that period of time when weight gain of the fetus per day is largest, one would anticipate the largest possible uptake of metals into whatever the depot is. Moreover, metabolic activity of the going-to-be mother is increased by some 30 % as compared to a non-pregnant woman of similar activity patterns. At low physical activity levels, some 8–10 l of blood per minute are circulated through the body; accordingly, the state of pregnancy will add some 2.5–3 l/min of blood circulation, that is, about 4.000 l/day. Because Mn is not given away from the placenta by the umbilical cord vein at all, some 1.6 mg/day[1] of manganese will remain in the fetus. For Mo, (Rossipal et al. 2000) detected a chance of back-transfer, rendering the calculated uptake of some 0.4–0.5 mg/d just a conservative upper limit. Given the very small demand even of adults for this ultratrace element (≤0.1 mg/d), this value would be an extremely high uptake. This is to say that depots may be constructed prenatally for both Mn and Mo. Given that such depots exist indeed, a supply of either element via milk thereafter would not be required by physiology.

Unlike many other mammals (e.g. ungulates), humans are physiologically very premature when just born and thus depends on milk nutrition entirely for a longer period of time. On the other hand, birth weight just about doubles in humans until breast-feeding is terminated. One can compare this situation with that of marsupial mammals or monotremes (kangaroo, platypus etc.), whose youngsters are very small when born or hatching at less than 1 g of weight (Grzimek 1965) thus they cannot have acquired any relevant amounts of depots before delivery. When milk-nutrition ends in these mammals, they would grow to (at least) several hundred

[1] 4.000 l/day additional blood circulation times a carryover of 0.4 mg/l correspond to 1.6 mg/day, the transfer being irreversible (see above).

times their weight at birth or hatching. Compared to both human and cow's milk, milks of both platypus (Griffiths 1988) and some non-specified marsupial (most likely grey giant kangaroo) contain 50–100 times as much iron (20–30 mg/l). Obviously, milk has to cover all the metal demands of young platypuses directly from delivery onward. Data for Mn, Zn, Mo or other metals are lacking. While the body weights of humans and giant kangaroos (grey or red) are similar when exclusive milk nutrition comes to an end (of order 5 kg), the Fe depot in a human newborn of about 1 g is identical to the entire weight of a newborn kangaroo, material balances being correspondingly much different. African summon-primates other than humans can also be raised/nursed with infant milk formulae based on cow's milk, implying the situation (existence of metal depots) is similar in the great apes—even including gorillas which feed on plants only. This agrees with the DGE stating there is no Fe demand whatsoever for the "pure" (human) phase of breast-feeding (<4 months).

Nickel (Ni) also interacts with numerous other elements; though it is coordinated e.g. to albumin, this complex is so labile in both kinetic and thermodynamic terms that $Ni^{2+}$ will be transferred to the latter already upon addition of simple amino acids (Tabata and Sarkar 1992), suggesting intense interactions. Unlike Mn, Ni will not undergo redox reactions in aerobic organisms, rendering effects by other heavy metals by generation of oxidizing radicals unlikely. Nevertheless, increased maternal administrations of Cu, Cd, Co and Zn, here lower $TF_{Ni}$ once again. Mangenese (Mn) exerts an unsymmetrical effect: Manganese (Mn) lowers transfer of Ni but not the reverse. Such kind of asymmetry is to be anticipated in exactly those cases where transport of some pair of elements is accomplished using different pathways and/or carriers. There are similar asymmetries with obviously the same reasons in the element couple Cu/Fe.

With respect to their effect on the flow of matter and of energy in the food chain, plants represent an important link between the atmosphere and the soil on the one hand and between consumers from the first to the highest order (animals and humans) on the other. Frequently, pollutants are introduced into the food chain via plants which have taken them up from the soil or the atmosphere, and these pollutants often cause irreversible damage to individual organisms or to entire communities as a result of accumulation and exclusion processes (Fargašová and Beinrohr 1998; Szárazová et al. 2008). Therefore highest quality on the control of the influence on soil chemistry and microbial activities has to be given in the future.

Quantitatively, the uptake of substances is adequately characterized by the intensity and scale of the uptake up to a particular point in time. For a defined nutrient, the uptake by the plant is dependent on the amount of the nutrient in the medium taken up and its availability. As a rule, the plant has no positive influence on the supply, but it does have an effect on the material and spatial availability of the nutrients. For example, from a material aspect, the nutrient availability can be changed by modifying the pH of the soil solution.

## 5 Conclusion

As could be stated in this chapter, it seems to be of highly important to harmonize definitions and practical meanings of international used terms as bioindication and biomonitoring. We are preferring it in the sense of a qualitative and quantitative approach i.e. means relative and absolute existence of trace metals via the bioindicator or around a biomonitor. This will have, for sure, influence on practical experimental procedures as sampling, washing, and measurement. In the same way, we prefer additional to the single element approach a stronger multielement approach, e.g. given by the Biological System of the Elements (BSE). Clear-cut experimental investigations, as here given for a performed food and mothermilk transfer study, are necessary to fill the gap in between eco- and human toxicology. A well defined system for reaching these common goals are to work internationally on integrative models, as the Multi-Markered-Bioindication-Concept (MMBC) tries to stimulate. For coming ahead with such a multidisciplinary approach it is of strongest need to educate students worldwide transdisciplinary to go into this highly motivating field of scientific research.

**Acknowledgement** A very strong "Thank you" is given to a lot of students, co-workers and international colleagues supporting us intensively with newest interdisciplinary ideas, innovations, collaboration and assistance during a long time dealing with trace metal and ecotoxicological research.

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# Chapter 7
# Interaction Between Plants and Biosurfactant Producing Microorganisms in Petroleum Contaminated Absheron Soils

**Elmira Akhundova and Yamen Atakishiyeva**

**Abstract** The chapter focuses on the roles that plants, hydrocarbon degrading microorganisms and biosurfactants produced by them play in petroleum contaminated soils. Consortia of hydrocarbon degrading bacteria including *Rhodobacter fascians* AZCC 1501, *Alcaligenes feacalis* AZCC 1164, *A. feacalis* AZCC 1165, *A. eutrophus* AZCC 1171, *Bacillus subtilis* AZCC 1288 and *B. subtilis* AZCC 1289 were used as high producers of biosurfactants. Enhanced degradation of added Absheron oil occurred in the rhizosphere of alfalfa, *Artemisia fragrans* and perennial ryegrass, and a significant decrease in oil concentration was detected in the presence of microbial consortia and liquid biosurfactant. It was noted that biosurfactant facilitated *Artemisia rhizodegradation* had a higher degradative potential than that of alfalfa and ryegrass plants.

**Keywords** Phytoremediation · Biosurfactant · Petroleum contamination · Hydrocarbon degrading microorganisms

## 1 Introduction

Contamination of the soil environment with hazardous wastes has brought about new applications and technologies to address this growing concern. Many of these contaminants being organic compounds, and petroleum hydrocarbons, as probably one of the most widespread classes of this type, are of great concern because of the risk of exposure and toxicity to humans and ecosystem. In addition, petroleum hydrocarbons can move from the source of contamination to soil, air and water. They can also pose a fire hazards as well as interfere with normal soil processes because of their effect on nutrient cycling and water relations (CCME 2000).

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Although traditional chemical and physical means of treating contaminated soil have had been in use, biodegradation technology is currently considered as one of the most effective ways of rendering polluted soil fit for use. Recent attention has been given to the use of vegetation as a means of remediating contaminated soil systems (Davis et al. 2002). Evidence that plant roots and rhyzosphere associated microbial community are capable of enhancing the degradation of petroleum chemicals in soils provides a potentially important approach for the *in situ* treatment of contaminated sites. Vegetation may act to immobilize water soluble contaminants, increase their stability in soil structure, and create a favorable environment for degradative microorganisms. Before phytoremediation can be efficiently employed, more basic research is needed to reveal basic mechanisms involved.

The combined impact of plants and microbes on soil pollutants is numerous, and many attempts have been made to manipulate enhanced contaminant degradation. Our approach refers to rhizoaugmentation which is the addition of hydrocarbon degrading microorganisms to soil with the objective to get them associated with rhizospheres of plants involved in remediation (Miller and Dyer 2002; Shaw and Burns 2007). However, we have enhanced the aims of such rhizoaugmentation by using not only simple hydrocarbon degrading microorganisms but that those which demonstrated an ability to produce biosurfactants.

Biosurfactants have been reported to be effective for environmental bioremediation (Shin et al. 2006; Juwarkar et al. 2007; Das et al. 2009). Biosurfactants can enhance bioremediation via two processes, i.e. by solubilization and by increasing desorption rate constants. The rate of soil bio- and phyto-remediation may be limited when a spatial separation between contaminants, microbial population and/or plant roots is present. For example, the contaminants such as polycyclic aromatic hydrocarbons must be transported to the bacteria, and rhamnolipid has the greatest effect on the highly hydrophobic contaminants (Rosario et al. 2007).

In this study, we examined the role of three different plants, a microbial inoculant containing the association of hydrocarbon degrading and biosurfactant producing bacteria and pure biosurfactant on a degradation of 10,000 ppm petroleum spiked into sterilized Absheron soil. It was hypothesized that the addition of plants with microbial inoculants to contaminated soils would increase petroleum degradation, and addition of biofurfactant would further increase efficacy of the phytoremediation system.

## 2 Materials and Methods

### *2.1 Soils*

Grayish-brown Absheron non-contaminated soil was removed from the surface horizon (5–20 cm depth) and sieved through a 2-mm screen. Then it was autoclaved at 121 °C for 1 h and left covered at the room temperature. This was repeated twice

**Table 7.1** Composition of petroleum received from Bibi-Eybat oil deposit (Absheron)

| Fraction | Mass content in crude oil | Boiling range (°C) |
|---|---|---|
| *Paraffins* | | |
| $C_6$–$C_{12}$ | 15–18 | 69–230 |
| $C_{13}$–$C_{25}$ | 6–7 | 230–450 |
| *Cycloalkane* | | |
| $C_6$–$C_{12}$ | 19–20 | 70–230 |
| $C_{13}$–$C_{23}$ | 16–18 | 230–405 |
| *Aromatic hydrocarbons* | | |
| Mono- and di-cyclic $C_6$–$C_{11}$ | 2–3 | 80–240 |
| Poly-cyclic $C_{12}$–$C_{18}$ | 4 | 240–400 |
| *Naphthene* | | |
| $C_9$–$C_{25}$ | 20–22 | 180–400 |
| S compounds | < 0.06 | |
| Residue | 10–12 | 400 |

to eliminate as much indigenous microorganisms as possible. The soil was stored at 4 °C in the dark until required for planting. Soil and nutrient analyses for plant available phosphorous (colorimetric autoanalyzer), potassium and magnesium (atomic absorption spectrophotometer), total carbon, organic carbon, inorganic carbon, total nitrogen (Leco furnace), $NH_4N$, $NO_3$–N, $NO_2$–N (KCl extractable) and pH (soil slurry method) were conducted by the Soil and Agriculture Institute under the Azerbaijan National Academy of Sciences using standard methods. During the experiment, soil samples were also analyzed according to the Environmental Protection Agency's (EPA) standard methods (2000) using spectrophotometric infrared (method # 418.1) to assess total petroleum hydrocarbon content (TPH) and GC-MS (method # 610) to assess PAH content.

Three hundred gram dry weight of soil were placed in 10-cm diameter ceramic pots with a drainage hole in the bottom, and brought to approximately 9–10 % gravimetric water content with sterile distilled water. Pots were equilibrated in a growth room for 7 days prior to planting. Moist was kept at 9–10 % by addition of sterile distilled water. Introduction of contaminants (Absheron petroleum, 10,000 ppm, Table 7.1), inoculation of hydrocarbon degrading microbial consortia and/or biosurfactant started 2 and 1 day, respectively, before planting. Sampling was done at weekly intervals for 7 weeks following planting.

## *2.2 Plants*

The choice of plant species for remediation is an important consideration that will surely affect the outcome of contaminant degradation. It has to depend on the type of contaminants, their concentrations and distribution of contaminants again. Since contaminants must be in contact with plant roots, so the depth and density of plant roots must be considered (Tsao 2003). Perennial ryegrass (*Lolium perenne*) is often

used subject for phytoremediation experiments due to easy growing and dense root system (Binet et al. 2000), so we chose it as a subject for this study. Some herbaceous species such as clover, alfalfa also have relatively dense root systems, and are often used in phytoremediation studies, so that our second experimental plant pattern became alfalfa (*Medicago caucasica* L.).

In addition, the plant must be suitable for soil and climatic conditions at the site and must be able to tolerate the contaminant. This was the reason for choosing the third object—wormwood (*Artemizia fragrans* L.), which is one of the widespread inhabitants of petroleum contaminated Absheron soils.

Plants were propagated different ways based on their peculiarities. Perennial ryegrass was propagated from seeds germinated in the dark at room temperature on a sterile filter paper soaked in 5 ml of sterilized deionized water. Germination occurred after 4 days.

Alfalfa and wormwood were propagated vegetatively as shoots from a grown plant. Propagation had been carried out via hydroponics until seedlings emerged the roots, and then replanted to experimental pots.

### *2.3 Microorganisms*

The soil microbial inoculum was obtained from the consortia of hydrocarbon-degrading bacteria maintained in Azerbaijan Culture Collection (AZCC). Consortia include *Rhodobacter fascians* AZCC 1501, *Alcaligenes feacalis* AZCC 1164, *A. feacalis* AZCC 1165, *A. eutrophus* AZCC 1171, *Bacillus subtilis* AZCC 1288 and *B. subtilis* AZCC 1289. All these strains were selected as high hydrocarbon degrading strains with an ability to synthesize biosurfactants. Biosurfactants were isolated by acid precipitation following Das et al. (2008)

## 3 Results and Discussion

The Absheron oil composition is specific compard to other types of petroleum. The C/H ratio is 84/12 which means a large amount of non-saturated hydrocarbons. It has much of cycloparaphines, and less alkanes and aromatic compounds which might influence on its utilization by microbes and plants. First, we analyzed the fraction content of the petroleum used in our study. The sample we selected was received from Bibi-Eybat terrestrial oil field, which is characterized as light petroleum with priority of light short-chained fractions (Table 7.1).

Selected microorganisms stored in AZCC were initially isolated from the typical Absheron petroleum contaminated soil and proved highly adaptive to such type of petroleum hydrocarbons to utilize.

Three different plants (ryegrass, alfalfa and Artemisia), a microbial inoculant containing the association of hydrocarbon degrading and biosurfactant producing

**Table 7.2** A list of treatments used in the experiment. Those treatments not receiving bacterial inoculum received 75 ml of sterile distilled water

| Treatment | Petroleum conc. (ppm) | *Artemisia* | Alfalfa | Basal salt mixture (BSM)[a] | Bacterial inoculum | Biosurfactant |
|---|---|---|---|---|---|---|
| 1. Plant control | 0 | Yes | Yes | Yes | No | No |
| 2. Petroleum control | 10,000 | Yes | Yes | Yes | No | No |
| 3. Plant only | 10,000 | Yes | Yes | Yes | No | No |
| 4. Bacteria only | 10,000 | Yes | No | No | Yes—75 ml | No |
| 5. Plant + Bacteria | 10,000 | Yes | Yes | Yes | Yes—75 ml | No |
| 6. Plant + Biosurfactant | 10,000 | Yes | Yes | Yes | No | Yes—30 ml |
| 7. Plant + Bacteria + Biosurfactant | 10,000 | Yes | Yes | Yes | Yes—75 ml | Yes—30 ml |

[a] 75 ml per pot

bacteria and pure biosurfactant were tested on a degradation of 10,000 ppm petroleum spiked into sterilized Absheron soil. These factors alone, and in combination, were studied together with petroleum degradation data, plant biomass and microbial analysis with the intention to understand the complex interactions present in the petroleum contaminated soil.

In Table 7.2 different treatments examined are mentioned. Samples from control plant with no petroleum content demonstrated distinguished amount TPH/g dry soil at zero time; taking into account that the chromatogram did not show the characteristic peaks for used petroleum samples, so we considered it as a zero point.

Twenty eight days after the start of the experiments, less than 20 % of TPH remained in all treatments (Fig. 7.1). After 56 days, this value stabilized at about 10 % with no further observable degradation. Although the difference among treatments was not significant, it did appear that variants 6 (with bacterial inoculum) and 7 (bacterial inoculum + biosurfactant) had more rapid degradation of petroleum in the rhizosphere of all plants but more in wormwood. TPH was found to be 15 ± 6 % and 9 ± 5 % after 14 days as compared to about 40 % under treatment with petroleum control. The sample from our petroleum control showed about 14 % decrease in TPH content by the end of the experiment, which could have been due to evaporation of light petroleum fraction (Gremoin et al. 2004).

Another trend revealed in the course of this study was the increase in number of bacteria over 70 days of incubation in treatments 5 and 7 compared to that in 4. Presence of all plants affected the increase of bacterial population by 70th day of incubation with some advantage of wormwood and alfalfa versus ryegrass (Fig. 7.2). Such an effect can be explained that nutrition from root exudates may influence the structure and activity of a soil microbial community (Burgmann et al. 2005). The samples with biosurfactant addition demonstrated a sharp increase in bacterial amount sooner, than without it, and at 28th and 56th day of incubation respectively. It might be due to turning on by facilitation of hydrocarbons by biosurfactant for bio- and phyto-degradation (Huang et al. 2004).

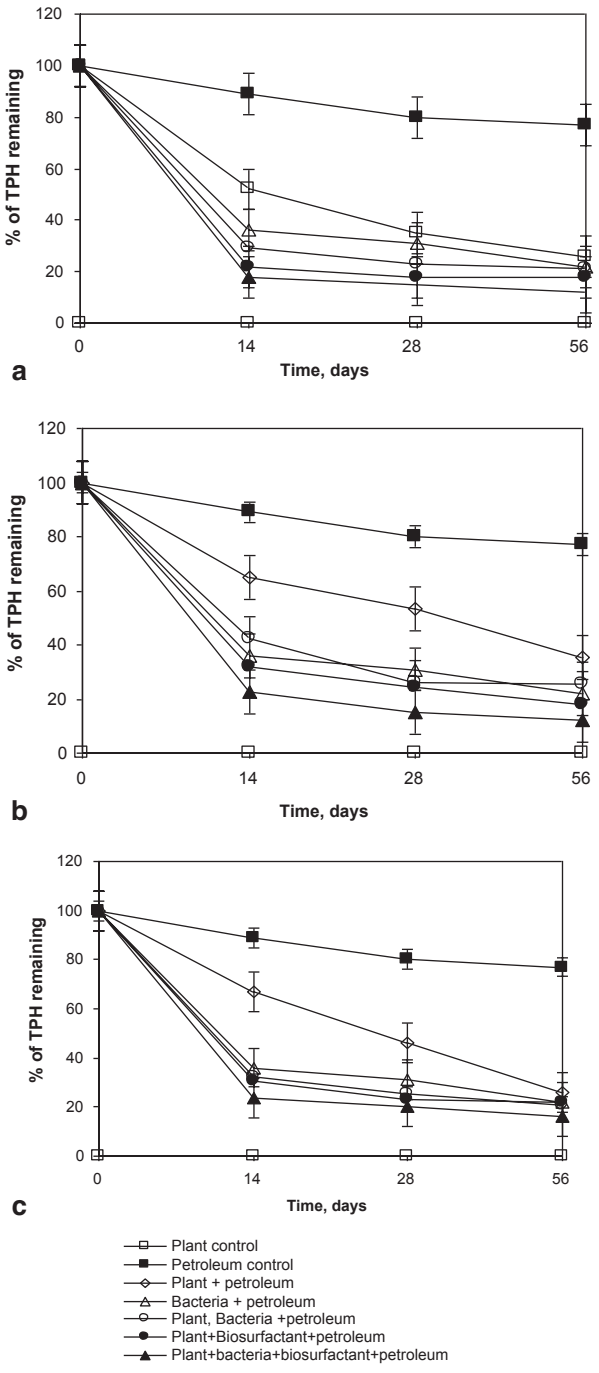


**Fig. 7.1** Percent of total petroleum hydrocarbon content (TPH) remained after 56 days for all treatments with **a** Wormwood, **b** Alfalfa, **c** Ryegrass

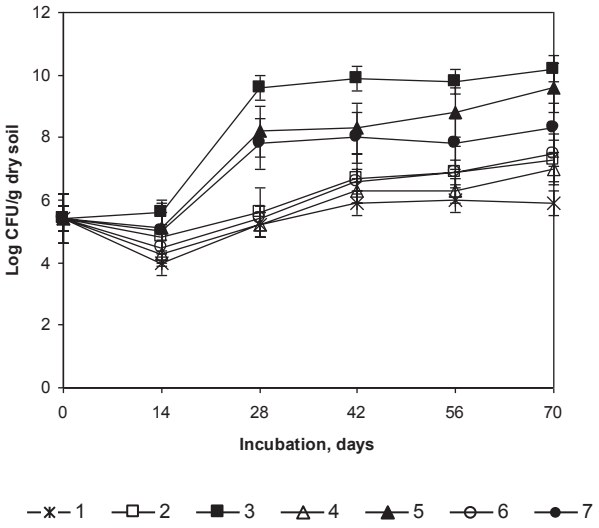


**Fig. 7.2** Culturable heterotrophic hydrocarbon degrading bacteria counts. **Treatment Legend**: *1* Bacteria inoculum only, *2* Bacteria inoculum + wormwood, *3* Bacteria inoculum + wormwood + biosurfactant, *4* Bacteria inoculum + alfalfa, *5* Bacteria inoculum + alfalfa + biosurfactant, *6* Bacteria inoculum + ryegrass, *7* Bacteria inoculum + ryegrass + biosurfactant

There was no statistically significant difference in dry root or shoot biomass for treatments with different plants growing in petroleum contaminated soil. Dry root biomass at 116 days was about 60 ± 26, 54 ± 11 and 14 ± 2 g for wormwood, alfalfa and ryegrass, respectively. The presence of microbial inoculant did not affect plant biomass at any time point during this experiment, whereas biosurfactant caused an augmentation of dry plant biomass about 5–7 % more than that by other treatments.

Despite of the high penetrability of arid soils for hydrocarbons, they are in ecological danger due to poor plant cover, non-diverse microbial pool and low biodegradability.

## 4 Conclusion

Our research indicates the complexity of a phytoremediation system, were present even under controlled laboratory conditions. This complexity of interacting factors which interfere to each other, makes it difficult for researchers to study the role of the irrespective of factors. Although some research has been done on interactions occurring in rhyzosphere of contaminated soils, still the mechanism and effect of biosurfactants facilitated hydrocarbon degradation by complex association of plant and bacteria remain exclusive for us. This obvious increase of phytodegradation can be used to its fullest potential after we completely determine the interactions that occur in the rhyzosphere along with the factors that influence these interactions.

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# Chapter 8
# Phytoremediation of Crude Oil-Contaminated Soil by *Medicago sativa* (Alfalfa) and the Effect of Oil on its Growth

**Saeed Minoui, Dariush Minai-Tehrani and Malak Hossein Shahriari**

**Abstract** In oil producing countries, crude oil is one of the main organic pollutants of soil and water. The use of plants to phytotreatment of crude oil contaminated soil has been a particular interest in environmental cleansing. Some plants such as grasses and legumes have been demonstrated to have better capacity in biodegradation of oil in the soil. In this study, the effect of different concentrations of light crude oil (up to 10%) on the growth and germination of *Medicago sativa* (alfalfa) was studied. Our results showed that the germination number and the number of leaves per plant decreased by increasing light crude oil concentration in the soil. About 75% of germination was observed in control while it was 15% in high concentration of crude oil in soil (10%). Total dry biomass of plant was higher in control (2 g) sample while it was lower in 7 and 10% sample. Number of leaves was higher in control but it was lower in 7 and 10% oil-polluted soil. The presence of high concentration of oil in soil caused chlorosis of leaves and there were no green plants at the end of experiment (120 days). Total colony and oil-degrading colony counts in soil showed that in all vegetated samples, the microbial population was higher than non-vegetated samples. In vegetated samples, the total microbial population in 7% samples was higher than control and also higher than that in low concentrations of crude oil (1 and 3% samples). The effect of plants on reduction of oil in soil was also investigated. In all vegetated samples, the reduction of crude oil was higher than that in non-vegetated samples. The higher reduction occurred in 1% sample (70%), while the lower reduction observed in 10% sample (20%). In conclusion, *Medicago sativa* as a plant in legume family could not tolerate high concentration of crude oil and crude oil could severely affect its growth and germination and cause untimely chlorosis. Our results propose that *Medicago sativa* is not a good option for removal of oil from the soil by the method of phytoremediation.

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**Keywords** Crude oil · Germination · Phytoremediation · Soil

## 1 Introduction

Oil-contaminated soil can damage environment by affecting the plants and microorganisms of the soil. The effect of contaminant on microorganisms and plants depends on the concentration and the kind of contamination (Boethling and Alexander 1979). Heavy crude oil has higher resin and asphaltine than light crude oil. These compounds do not well biodegrade by microorganisms and plants and remain in soil for many years (Walker et al. 1975). On the other hand, some gaseous and volatile hydrocarbons are higher in light crude oil than those in heavy crude oil. These compounds are toxic for biological systems of soil. The effect of crude oil and its components on germination and growth of some plants has been studied (Adam and Duncan 2002).

Some plants are able to remediate the organic pollutant from the soil. Phytoremediation is the use of plants and their associated microorganisms to remediate contaminated soil and water. Among the plants, grasses and legumes have higher potential on removal of oil from contaminated soil (Minai-Tehrani 2008). The plant roots stimulate the bacteria in rhizosphere area, which enhance the biodegradation of petroleum hydrocarbons. Legumes are able to fix nitrogen and do not compete with microorganisms for limited supplies of available nitrogen at oil-contaminated soils. In this study, the effect of different concentrations of light crude oil on growth and germination of a legume, *Medicago sativa* (alfalfa), was studied and the reduction of light crude oil in the soil in the presence of plant was investigated.

## 2 Materials and Methods

**Soil Analysis** The soil and light crude oil (API = 40) were obtained from Sarkan zone, near the oil processing factory of Sarkan in the west of Iran. The soil was dried at room temperature and then sieved through 2 mm mesh. Light crude oil was added to the dry soil with concentrations of 0, 1, 3, 5, 7 and 10% (w/w). The soil and oil were well mixed to make homogenized contaminated soil, and then transferred to pots each of one L size. Each sample consisted of 800 g of dry soil.

Chemical fertilizers were added to the soil before seeding. Each of the soil sample was supplied with 75 mg/kg nitrate ($NH_4NO_3$) and 30 mg/kg of phosphate ($KH_2PO_4$). Twenty seeds of alfalfa were planted in each sample. All vegetated samples were prepared as three replicates. The control samples for each concentration were also prepared as three replicates which were without seeds (non-vegetated).

The number of germinations was counted 30 days after planting and indicated as germination number. The number of leaves was counted 60 days after planting and

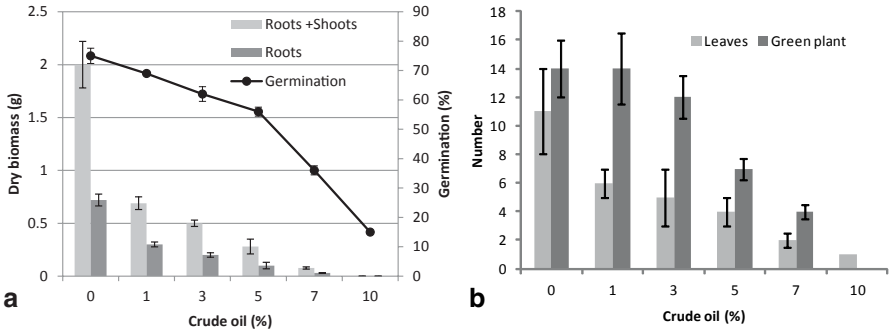


**Fig. 8.1** **a** The number of germinations in different concentrations of crude oil after 30 days of seeding and total dry biomass (shoots + roots) and dry biomass of roots after 120 days of planting. **b** The number of leaves in vegetated samples after 30 days of seeding, and number of green plants after 120 days (end of experiment) of planting. Average values ± standard deviation (± SD), $P < 0.05$

also the number of green plants was counted at the end of the experiment. The plant biomass was collected at the end (120 days) of the experiment, dried at room temperature and reported as total dry weight for roots and shoots. The colony count was done for the determination of total colonies and also for oil-degrading colonies of soil, 60 days after planting. Determination of total colonies in soil was done by the *pure-plate* method with nutrient agar as medium. Determination of oil-degrading colonies was also done by the same method in agar-agar with 1% sterilized light crude oil as sole carbon source. Crude oil extraction was conducted according to the method used by Minai-Tehrani and Herfatmanesh (2007). For 48 h, 1 g of treated soil was dried in 50 °C then crushed well to make a homogenous soil. An aliquot of 10 ml of $CH_2Cl_2$ (Aldrich) was added to the soil and shaken firmly to separate the oil from the soil. The sample was centrifuged (3,000 × *g* for 10 min.) to precipitate the soil, and the solvent phase removed. The solvent extraction was repeated twice. The solvent vaporized during 24 h and the amount of oil was measured by the gravimetric method and its reduction compared with time zero. Two samples from each replicate were taken for crude oil extraction.

## 3 Results

**Germination, Growth and Biomass** The germination number in the vegetated samples was high in 0% sample and it was low in 10% sample (Fig. 8.1). There was a sharp reduction in number of germinations above 5% concentration of crude oil. Dry biomass of roots and shoots were measured 120 days after seeding (Fig. 8.1). The separation of roots from the soil showed that the distribution of roots in the soil decreased by increasing the crude oil concentration. The higher root biomass was observed in 0% sample, in which the roots were well distributed

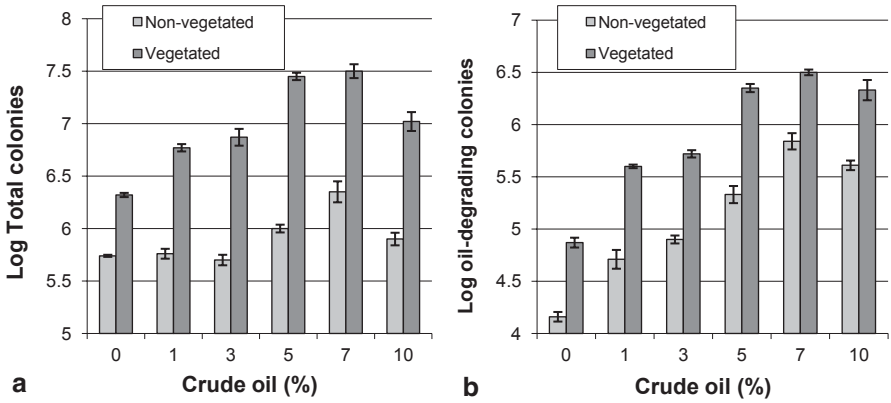


**Fig. 8.2** Total colony count (CFU/g soil) after 2 months of planting (**a**) Oil-degrading colony count (CFU/g soil) after 2 months of planting (**b**) (± SD, $n = 3$, $P < 0.05$)

in the soil. The roots and shoots in 7% sample were very low and there was no root in 10% germinated sample. The total dry biomass (roots + shoots) was also high in 0%, while it was markedly low in 7 and 10% samples. A sudden decrease in total dry biomass was observed in 1% sample in comparison with that in 0% sample. Figure 8.2 shows the relationship between the concentration of crude oil in soil and the number of leaves. The presence of high concentrations of crude oil in soil reduces the number of leaves; the lower numbers were observed in 10% sample. The number of green plants was also shown in Fig. 8.2. There was no green plant in 10% sample at the end of the experiment. The number of green plants decreased in 7% sample.

**Colony Count** Total colony count and oil-degrading colonies were determined in vegetated and non-vegetated soils (Fig. 8.2a). In vegetated samples, the higher microbial population was observed in 5 and 7% samples and the lower in control (0%). Increasing crude oil concentration increased the total microbial population in vegetated samples. In non-vegetated samples, the higher microbial population was also observed in 7% sample, while the lower at 3% sample. In all vegetated samples, the total colonies were higher than their equal concentrations of crude oil in non-vegetated samples.

**Crude Oil Reduction** Fig. 8.3 shows the reduction of crude oil in vegetated and non-vegetated contaminated soil after 120 days. The higher reduction was observed in 1% vegetated sample and the lower in 10% sample. No significant difference was observed in vegetated and non-vegetated samples in 10% sample. Increasing crude oil concentration decreased the reduction of crude oil in both vegetated and non-vegetated samples. In all contaminated vegetated soils, the reduction of crude oil was higher than that in non-vegetated soils, except 10% sample. A significant difference in reduction between vegetated and non-vegetated samples was observed in concentrations up to 5%.

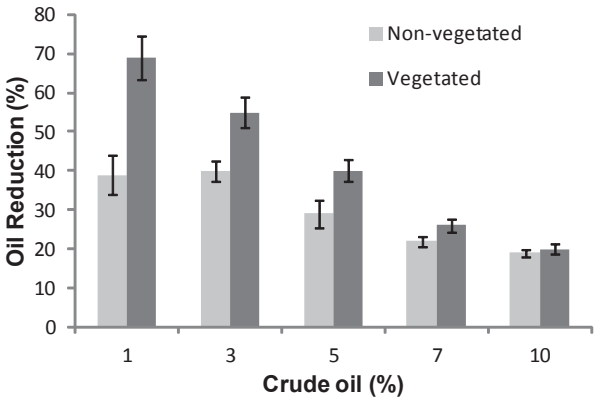


**Fig. 8.3** Reduction of crude oil after 120 days in different concentrations of light crude oil-contaminated soils (± SD, $n = 3$, $P < 0.05$)

## 4 Discussion

This study investigates mainly the effect of light crude oil-contaminated soil on growth and germination of *Medicago sativa* (alfalfa). Low germination of Alfalfa in 10% light crude oil-contaminated soil showed that the toxicity of light crude oil decreased the number of germinations in 10% sample distinctly in comparison with the control, suggesting that the toxic materials of crude oil could inhibit the germination of plant partially. No germination has been reported in maize in 10.6% crude oil (Yang et al. 2009; Agbogidi et al. 2011; Barua et al. 2011; Zhu et al. 2012; Eze et al. 2013). The number of leaves per plant also decreased in high concentrations of crude oil, suggesting that the plant has not grown well in 10 and 7% samples in comparison with control (Langer et al. 2010; Peter and Ayolagha 2012). The sharp reduction in biomass of 1% and other contaminated samples in comparison with control suggests that despite good germinations of the plant in contaminated samples up to 5%, the toxicity of crude oil has prevented the roots and the shoots of vegetated samples to grow well in comparison with control (0% sample). This phenomenon was significant in 7 and 10% samples. The distribution of roots in contaminated soil mainly decreased in comparison with control. No green plant was observed in 10% sample at the end of the experiment, and there was a significant reduction of green plants in 7% sample, suggesting that the toxic effect of crude oil caused early chlorosis in the plant. Exposure of plants to tolerable concentrations of petroleum can cause chlorosis of leaves, plant dehydration, stunted growth and death (Minai-Tehrani and Herfatmanesh 2007; Shahriari et al. 2007; Minai-Tehrani 2008). Total colony count and hydrocarbon degrading colony count showed that despite the high concentrations of crude oil in 5, 7 and 10% samples the population of microorganisms was higher than the control and also higher than that in low concentrations of crude oil (1 and 3% samples). This showed that the microbial population has been increased in higher crude oil concentrations, suggesting that the presence of crude oil in soil could prevent the fast evaporation of water from the soil, so the contaminated soils were nearly always wet in microenvironment of

the soil, while in control and lower concentrations of crude oil, the soil might have lost its moisture due to fast evaporation of water. Soil moisture is one of the most important elements for growth and reproduction of microorganisms (Nicolotti and Egli 1998; Al-Mailem et al. 2010; Ayu et al. 2011). In all vegetated samples the microbial population was higher than that in non-vegetated samples suggesting that in rhizosphere area, the microbial population of the soil was increased. Previous reports also indicated the increase of microbial number in the vegetated contaminated soil (Anderson et al. 1993; Barrutia et al. 2011; Kathi and Khan 2011; Maqbool et al. 2013). The reduction of crude oil in vegetated and non-vegetated soils showed that in all vegetated samples the crude oil reduction was higher than that in the non-vegetated samples suggesting that plant and its root in the soil can enhance biodegradation of oil contamination in soil (Brandt et al. 2002; White Jr et al. 2006; Shahriari et al. 2007).

## 5 Conclusion

In conclusion, light crude oil either in low or high concentration (1–10 %) contains toxic compounds that can prevent normal growth and germination of alfalfa in soil. It can reduce the plant biomass, early chlorosis and decrease the length of root and shoot. These damages were very significant in treated samples in comparison to control. Our results showed that *Medicago sativa* did not biodegrade crude oil from the soil and had not enough potential for phytoremediation of oil-contaminated soil.

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# Chapter 9
# Bioremediation of Petroleum Polluted Soils using *Amaranthus retroflexus* L. and its Rhizospheral Funji

**Fariba Mohsenzadeh and Abdolkarim Chehregani Rad**

**Abstract** Environmental pollution with petroleum is a global disaster. Bioremediation of oil contamination in soils is based on the stimulation of petroleum hydrocarbon-degrading fungal and microbial communities. Prior researches showed that there are some petroleum-resistant plants and their root associated fungal strains which grow in petroleum polluted soils. *Amaranthus retroflexus* L. (Amaranthaceae) is one of these, that was collected from both Kermanshah and Arak refineries polluted sites in Iran. The root associated fungi of the plant were determined and results showed the presence of 6 species which were associated with the roots of the plants growing in the polluted areas but only three of them were found in non-polluted soils. Culturing of fungi in oil-contaminated media showed that all the studied fungi were resistant to low petroleum pollution (1% w/w) and a few species, especially *Fusarium* species, showed higher resistance to petroleum pollution (10% w/w) and it seems that they may be suitable for bioremediation in highly polluted areas. Bioremediation tests with *A. retroflexus*, with and without fungal strains, showed that application of both plant and its root associated fungal strains was more effective than plant and fungi separately. Results indicated that fungal strains had the main role in bioremediation of petroleum polluted soils but plant roots enhance the process.

**Keywords** Petroleum pollution · Rhizosphere fungi · Bioremediation · *Amaranthus retroflexus*

## 1 Introduction

Petroleum pollution of soil is a global problem. It is a common phenomenon the most countries (Merkel et al. 2004a, b). There are several soil cleaning methods including burning, washing, application of chemicals and bioremediation

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(Garcia et al. 2000). Bioremediation is use of plants and microorganisms to remove or detoxify environmental contaminants. It has been intensively studied over the past two decades, driven by the need for a low-cost, *in-situ* alternative to more expensive engineering-based remediation technologies (Merkel et al. 2004a; Chehregani and Malayeri 2007; Chehregani et al. 2008). In petroleum polluted conditions, plants or plant associated microflora can convert hydrocarbons (HCs) to non-toxic forms (Cunningham et al. 1996). Bioremediation has been applied to remove crude oil (Wiltse et al. 1998; Radwan et al. 1998; Merkel et al. 2005), motor oil (Dominguez-Rosado and Pichtel 2004) and diesel fuel (Chaineau et al. 2000) from soil but the removal efficiency is highly variable (Angehrn et al. 1998). Since bioremediation of petroleum-contaminated soils is mainly based on biodegradation by the fungal strains that are present in the rhizosphere of plants (Mohsenzadeh et al. 2009) or are associated and attached with roots (Frick et al. 1999), the root system of the plant is one of the most important factors. Plants can indirectly influence degradation by altering the physical and chemical conditions of the soil (Cunningham et al. 1996). Many organic and inorganic substances are exuding from the plant roots during normal metabolism. These root exudates act as substrates for soil microorganisms, thereby enhancing the degradation of toxic organic chemicals (Anderson et al. 1993).

Merkel et al. (2005) have shown that some tropical grasses and legumes are resistant to petroleum pollution and root surface showed an increase in the graminoids namely *Brachiaria brizantha*, *Cyperus aggregatus* and *Eleusine indica* in petroleum polluted soils.

There are different economically and environmentally important uses of microorganisms, one of these being remediation and rehabilitation of petroleum contaminated soils (Eggen and Majcherczyk 1998; Yateem et al. 1997; Nicolotti and Egli 1998; Obuekwe et al. 2005; Dritsa et al. 2007; Friedrich et al. 2007). Some prior researches have shown that some fungal species are resistant to petroleum and oil derived pollutants and they are capable of cleaning soil pollution. The results of Ulfig et al. (2003) depict that keratinolytic fungi, specially *Trichophyton ajelloi,* are a potential tool for assessment of soil petroleum hydrocarbon contamination and associated bioremediation process. Fungal strains namely *Alternaria alternate*, *Aspergillus flavus*, *Curvularia lunata*, *Fusarium solani*, *Mucor racemosum*, *Penicillium notatum* and *Ulocladium atrum* have been isolated from the soils in the petroleum polluted areas in Saudi Arabia (Hashem 2007). Eggen and Majcherczyk (1998) showed that white rot fungus, *Pleurotus ostreatus* could remove polycyclic aromatic hydrocarbons (PAH) in contaminated soil. According to Seker and Öztürk (2006) white rot fungi can very well be utilized for the decolorization of highly contaminated waste waters. Little attention has been paid to the role of plant root associated fungal species in the environmental biotechnology and bioremediation of petroleum pollution, especially in Middle Eastern region (Yateem et al. 1999; Hashem 2007).

Numerous sites are contaminated globally with crude or refined oil in different countries. Iran as a one of the major oil producing countries faces the same situation. For bioremediation purposes we have to think first about the ecological

features of the area which differ from country to country depending upon the climate and other geographical conditions. Therefore while using plants for bioremediation, we need to apply native plants or microorganisms for each area involved. Our aim here is to evaluate the ability of *A. retroflexus* and its root associated fungal species for remediation of petroleum polluted soils.

## *1.1 Study Site*

Kermanshah oil refinery is located in Kermanshah city (previously named Bakhtaran) in the west of Iran, near the border with Iraq (Mohsenzade et al. 2009). It is an old refinery established in 1933, with a production capacity of 25000 barrel a day. Soils around the refinery are of sandy loam character, containing 85% sand, 8% loam, 6% sludge and 1% organic matter with 5.9 pH. Chemical composition of crude oil used in the refinery is as follows; 13.4% saturated hydrocarbons, 40% aromatic hydrocarbons, 46.6% polar compounds. Regarding the oil refining activities in this region, a high degree of petroleum pollution was observed in some areas. The identification of soil contamination was based on a visual examination of the soil and also experimental assays. *A. retroflexuas* was collected from the petroleum polluted area in the refinery. The taxonomical identification was done at Bu-Ali Sina University.

## *1.2 Isolation of Fungi Associated with the Roots*

Plant root samples with 1 cm length were harvested, washed and dried. The samples were kept in sodium hypochloride (1%−3 min) and then ethanol (70%−3 min) for removing the peripherally attached microorganisms, washed with distilled water, dried and kept in PDA media containing lactic acid. The petri dishes were incubated in 25±2 °C for 4 days. Different fungal colonies were isolated and cultured separately in PDA. Fungal specimens were examined under light microscope and identified using morphological and other taxonomical features (Nelson et al. 1983; Gilman 1998; Watanabe 2002). The root associated fungi for each plant collected from the petroleum polluted area were compared with the non-polluted ones in order to find out oil resistant species.

## *1.3 Determination of the Fungal Growth Ability Under Petroleum Pollution*

Growth assay was used to find out fungal species resistant to soil petroleum contamination. The assays were conducted by comparing the growth rates of fungal strains, as colony diameter, on the oil contaminated and control petri dishes. Test dishes were prepared by adding crude oil to warm PDA solution. In order to have a

uniform concentration of oil in all plates, the solution was thoroughly mixed with a magnetic stirrer, right before it was added to the plates. Three concentrations of Oil/PDA mixture (1, 4, and 10 % w/w) were prepared. Pure PDA was used in control plates. All dishes were inoculated with 2 mm diameter plugs of fungal mycelia taken from agar inoculum plates. The dishes were incubated at 25 ± 2 °C in an incubator. Fungal mycelia extension on the plates (colony diameter) was measured using a measuring tape after 4 days and compared with the control plates.

## *1.4 Evaluation of Petroleum Removal*

*A. retroflexua* is a petroleum resistant plant, common and native in the studied petroleum polluted area. It is a dominating species in the area, especially in the central region of the petroleum polluted sites. Seventy pots were prepared in April 2010 and filled with 2 kg of sterilized soil collected from the *Amaranthus* growing area. These were divided in to 16 groups; each group with 5 pots. The groups were divided as follows; (1) Sterile soil, (2) Sterile soil + Plant, (3) Sterile soil + *Altenaria*, (4) Sterile soil + *Altenaria* + Plant, (5) Sterile soil + *Fusarium acuminatum,* (6) Sterile soil + plant + *F. acuminatum*, (7) Sterile soil + *F. equiseti*., (8) Sterile soil + plant + *F. equiseti*, (9) Sterile soil + *F. reticulatum*, (10) Sterile soil + Plant + *F. reticulatum*, (11) Sterile soil + *Penicillium,* (12) Sterile soil + Plant + *Penicillium*, (13) Sterile soil + *Rhizoctonia*, (14) Sterile soil + Plant + *Rhizoctonia*, (15) Sterile soil + all the fungi, (16) Sterile soil + Plant + all the fungi.

In the groups which contained the plant, each pot had two seedlings of *A. retroflexus*. All pots were polluted with crude oil at a pollution level 5 % w/w. They were left under the same conditions in greenhouse at Bu-Ali Sina University. *A. retroflexus* plants were removed and deposited at the end of the growing period. The soil of experimental and control pots was homogenized separately and kept at 4 °C in a refrigerator until further evaluation. Concentrations of crude oil were determined in the soil of experimental and control pots.

## *1.5 Determination of Total Oil and Grease (TOG)*

Soil samples from the experimental and control pots were collected separately. Each soil sample, without root segments, was homogenized and stored at 4 °C until further processing. TOG was analyzed according to the EPA method 9071 A and EPA Method 3540 B (U.S. EPA 1994). Fifteen gram of the soil in two replicates were acidified with hydrochloric acid to pH = 2 and dehydrated with magnesium sulphate monohydrate. After 15 min, samples were transferred into paper extraction thimbles and placed in a soxhlet apparatus. TOG was extracted with dichloromethane for 8 h. The extract was filtered through filter paper (Whatman No. 4) with 1 g sodium sulphate. The solvent was evaporated with a rotary evaporator and the weight of dry extract was determined. Percentage of TOG was calculated based on soil dry weight and compared in the vegetated and non-vegetated areas.

**Table 9.1** Comparison of fungal species in the roots of *Amaranthus retroflexus* plant in polluted and non-polluted areas

| Fungi in non-polluted area | Fungi in petroleum polluted area |
|---|---|
| *Alternaria, Penicillium, Rhizoctonia* | *Alternaria, Fusarium acuminatum, F. equisetti, F. reticulatum, Penicillium, Rhizoctonia* |

**Table 9.2** Growth ability of rhizospheral fungi in PDA containing crude oil. (Data expressed as diameter of colony-mm)

| Oil treatment Microorganism | Non-contaminated (control) | 1% Oil | 4% Oil | 10% Oil |
|---|---|---|---|---|
| *Alternaria* | 49 ± 6 | 28 ± 8* | 18 ± 6* | 14 ± 4* |
| *Fusarium acuminatum* | 34 ± 5 | 42 ± 10.3 | 50 ± 10 | 48 ± 12 |
| *F. equiseti* | 12 ± 2 | 46 ± 4* | 63 ± 4* | 85 ± 8* |
| *F. reticulatum* | 33 ± 9 | 45 ± 6 | 61 ± 7 | 55 ± 5 |
| *Penicillium* | 40 ± 5 | 30 ± 4 | 24 ± 4 | 11 ± 2 |
| *Rhizoctonia* | 88 ± 1.2 | 67 ± 9* | 42 ± 3* | 18 ± 5* |

Each data represents the mean ± SE of 3–5 samples
*Data significantly different from the control ($p \leq 0.05$)

For statistical evaluation between the experimental groups and control, analysis of variance (ANOVA) followed by the least significant difference test (LSD) were performed between studied groups (Chehregani et al. 2005). Each data was represented as mean ± SD of five samples for experimental groups and also 5 for control.

## *1.6 Evaluation*

The rhizospheric fungi of *A. retroflexus* were collected, isolated and identified by morphological characters and taxonomical keys (Table 9.1). The results of the identification of plants root associated fungi showed the presence of 6 fungal species in the roots of this plant collected from the petroleum polluted soils. These were; *Alternaria sp., Fusarium acuminatum, F. equisetti, F.reticulatum, Penicillinium sp., Rhizoctonia sp.* Only three of these were found to be associated with the roots of the plants in non-polluted soils namely; *Alternaria sp., Penicillinium sp., Rhizoctonia sp.* The studied plants had different fungal population as their root association and only three fungal species were common in the roots of all plants in both polluted and non-polluted areas (Table 9.1).

The growth activity of 6 fungal strains was carried out under different concentrations of crude oil and was expressed as the diameter of the colony (Table 9.2). The results showed that all the studied fungi were more or less resistant to petroleum pollution and they made a sufficient colony in 1% crude oil concentration; but only some of them save their growth activity in 10% petroleum pollution. Among the studied fungi, *Fusarium equiseti, F. reticulatum* and *F.cuminatum* had the highest resistance to petroleum (with 48 and 55 mm diameter of colony) and *Penicillium sp.* was the most sensitive one (with 11 mm diameter of colony) in the 10% petroleum polluted PDA.

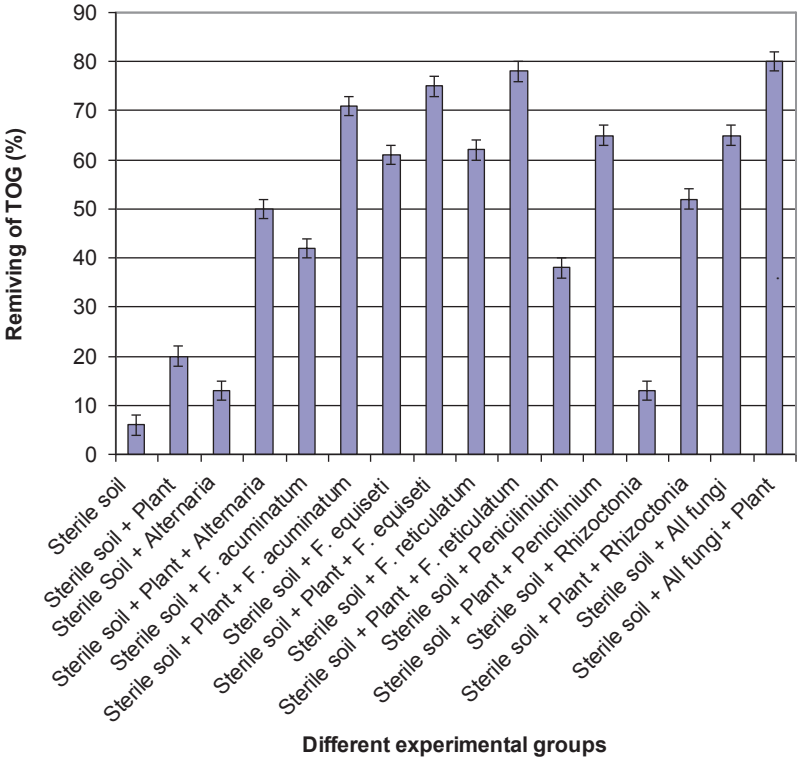


**Fig. 9.1** Decrease of petroleum pollution concentration (%) in the polluted soils after bioremediation by *Amaranthus retroflexus* and its rhizospheric fungal strains. All pots contained 5% w/w petroleum pollution before the beginning of experiment. Data indicates amount of petroleum pollution decreased due to bioremediation. Decrease of petroleum in control pots is the result of evaporation. Decrease of pollution between experimental and control groups are significant ($P < 0.01$). Each data represents the mean ± SE of five samples

## *1.7 Bioremediation by Root Associated Fungi*

The perennial herb *A. retroflexus* is one of the common plants in the polluted areas of Kermanshah Petroleum site and could grow effectively on such soils. It propagates by means of seeds and underground gemma. After 6 months bioremediation using plants and their root associated fungal strains, concentrations of petroleum pollution were determined in the soil of controls and contaminated soils. The data showed that concentration of petroleum pollution decreased considerably in the all pots but was constant in control ones (Fig. 9.1). It also showed that decrease in the experimental pots containing plant together with all fungal strains was more than other groups (up to 80%). Meanwhile, decrease of petroleum pollution was also considerable in the pots containing plant added *Fusarium equiseti* and *F. reticulatum* (up to 74 and 78%). The data showed that all fungal species were capable to decrease petroleum pollution solitary (Fig. 9.1), but they were more effective when

applied with the plant. *Altenaria sp.* singly result in a decrease up to 14% but when applied with plant, decrease was 50% and also *Rhizoctonia* reduced soil pollution up to 14% solely, when applied with plant decrease was raised up to 52%.

Petroleum pollution of soils is a major environmental pollution in many countries (Klokk 1984), Serious risks can occur to the public health and environment when the soil is polluted by crude oil (Nicolotti and Egli 1998). Results of this work shows that crude oil, in the concentrations presented here (up to 10%) did not kill the studied plant species. This is in accordance with the results of an earlier study by Nicolotti and Egli (1998), who showed several legumes and graminoids can flourish on petroleum polluted soils with about 5% pollution. The crude oil indirect effects the soil and results in more or less marked reduction in plant growth and biomass (Merkel et al. 2004). Similar findings have been reported for other plant species: *Festuca rubra* and *Puccinellia maritime* (Baker 1999), *Trifolium rubra* (Klokk 1984) and different legumes and grasses (Merkel et al. 2005).

Study on fungal species showed that *Alternaria, Penicillium* and *Rhizoctonia* were the common fungi that have been observed in the roots of all studied plants both in polluted and non-polluted soils. Based on our data, fungal variation in petroleum polluted area was more than non-polluted one (Table 9.1). This means that roots of the plants had more fungi yielded in polluted areas than non-polluted ones, which is in accordance with the findings of some prior workers (Anderson et al. 1993; Hashem 2007). It seems that the fungal species use oil compounds as nutrients, because petroleum pollution increases fungal growth. Similar results have been reported by some other researchers (Eggen and Majcherczykb 1998; Yateem et al. 1997; Nicolotti and Egli 1998; Obuekwe et al. 2005; Dritsa et al. 2007).

*In vitro* growth test of fungi showed a species-specific response. Most of studied fungal strains were capable of growth in 1% w/w oil pollution and therefore could be useful for the remediation of light soil pollution. Some fungal species were inhibited by high oil concentrations (10% w/w). These species were *Alternaria sp., Penicillium sp.,* and *Rhizoctonia sp.*, while others actually grew well in oil-contaminated media, even at very high concentrations. These are *Fusarium acuminatum, F. equiseti,* and *F. reticulatum*. It seems that crude oil could supply some nutrients for these fungi and they could prove more effective for oil degradation. Our findings are in accordance with those of other researchers about other fungal species (Eggen et al. 1998; Yateem et al. 1997; Nicolotti and Egli 1998; Obuekwe et al. 2005; Dritsa et al. 2007).

Bioremediation of a petroleum-contaminated soil is mainly based on biodegradation in the rhizosphere (Frick et al. 1999), root-associated fungi are one of the most important factors. The results of this study propose that above mentioned fungi can be evaluated for the future remediation tests and this is the first report about their remediation capacity. The data of this study indicates that isolated strains of *Fusarium acuminatum, F. equseti* and *F. reticulatum* may have the potential for bioremediation of crude oil in highly polluted soils especially in semi-dry regions.

*A. retroflexuus* abundantly found in the polluted areas when chosen for bioremediation test together with its root associated fungi show that the concentrations of crude oil decreased in the pots containing plant with all fungal strains added.

The pots containing plant added *Fusarium equiseti, F. acuminatum* and *F. reticulatum* also showed the highest decrease in the petroleum pollution (Fig. 9.1). Results show that although all the subjected fungal strains cause decrease in the petroleum concentration in soils but application of plant together with associated fungal strains proves more effective (Fig. 9.1). It means that plant root exhausts result in an increase of petroleum biodegradation driven by fungal strains as proposed by few prior reports (Garcia et al. 2000; Mohsenzadeh et al. 2009; Mohsenzade et al. 2009). Phytoremediation of petroleum pollution is a cost-effective green technology; there are more advantages, when it comes to the use of native plants and fungi (Schröder et al. 2002). This is the first report on the ability of *A. retroflexus* and its rhizospheric fungi for remediation of petroleum polluted soils.

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# Chapter 10
# Reciprocal Effects of Oil-contaminated Soil and *Festuca* (Tall fescue)

**Dariush Minai-Tehrani, Saeed Minoui and Malak Hossein Shahriari**

**Abstract** Contamination of soil by crude oil can damage ecosystem and environment. The oil can cause damage to the plants, the first element in food cycle. On the other hand, there are some plants that can be used to remediate crude oil-contaminated soil. Some plants such as grasses have been demonstrated to have better capacity in biodegradation of oil in the soil. In this study, the effect of different concentrations of light crude oil (1–10 %) on the growth and germination of *Festuca arundinacea* (Tall fescue) was studied for 120 days. The results showed that percent germination and dry biomass of the plants decreased by increasing light crude oil concentration in the soil. The total biomass (root + shoot) was higher (2.1 g) in 1 % crude oil sample while it was lower (0.06 g) in 10 % crude oil sample. The length of leaves decreased in higher crude oil concentration compared with that in control (27 cm). Total colony and oil-degrading colony count in soil showed that the microbial population in 7 and 10 % oil samples was higher than those in the control and low concentrations of crude oil. On the other hand, the effect of the plant on crude oil reduction was also studied and compared in vegetated and non-vegetated oil-contaminated soil. The crude oil reduction in the vegetated and the non-vegetated samples was higher in 1 % oil sample. All vegetated samples had higher crude oil reduction than the non-vegetated samples. The higher reduction (73 %) occurred at 1 % sample, while the lower reduction (24 %) was seen at 10 % oil sample. In conclusion, *Festuca arundinacea* as a grass could tolerate high concentration of light crude oil in soil and is a suitable plant for phytoremediation of oil-contaminated soil. However high concentration of oil could affect its growth and germination, reducing the root distribution in soil and causing untimely chlorosis.

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## 1 Introduction

Crude oil and its by-products are widely used by human for various reasons, such as home heating and fueling of the vehicles. The leakage of crude oil in to soil damages the biological systems residing in the soil including microorganisms and plants. During the past decades, the use of petroleum products have increased and this has resulted in the contamination of the soil and water (Bauman 1991; Liang et al. 2009; Riser-Roberts 2010; Cioni and Petarca 2011; Liang et al. 2012). Some petroleum components are toxic for living organisms, however, some plants and microorganisms are able to biodegrade the crude oil hydrocarbons into products less toxic than the parent compounds (Eweis et al. 1998; Al-Mailem et al. 2010; Das and Chandran 2010; Thavasi et al. 2010; Tyagi et al. 2011; Thavasi et al. 2011; Speight and Arjoon 2012; Gojgic-Cvijovic et al. 2012). Phytoremediation is on-site use of plants and their associated microorganisms to remediate contaminated soil and water (Cunningham et al. 1996; Euliss et al. 2008; Peng et al. 2009; Infante et al. 2012). Phytoremediation can be applied to terrestrial and aquatic environments. In this process, the plant absorbs and breaks down organic chemicals in contaminated soil through its metabolic processes. Various plants have been identified for their potential to facilitate the phytoremediation of petroleum contaminated soils (Brandt et al. 2006; Minai-Tehrani 2008). In many studies, grasses and legumes have been used for their potential in this regard (Aprill and Sims 1990; Gunther et al. 1996; Soleimani et al. 2010; Njoku et al. 2012; Cook and Hesterberg 2013). Grass roots have the maximum root surface area compared to other plant types and may penetrate the soil to the depth of up to 3 m (Aprill and Sims 1990; Soleimani et al. 2010; Sinha et al. 2013). In this study, the effect of different concentrations of light crude oil on the growth and germination of a in grass, *Festuca arundinacea* (Tall fescue), was studied and the reduction of light crude oil in the soil as a contaminant in the presence of plant was investigated.

## 2 Materials and Method

Light crude oil (API gravity = 40) was obtained from an oil processing factory of Sarkan from west of Iran and added to the dry soil with concentrations of 0, 1, 3, 5, 7 and 10 % (w/w). Each sample consisted of 800 g of dry soil. Chemical fertilizers were added to the soil before seeding. Nitrate ($NH_4NO_3$) @ 75 mg/kg and phosphate ($KH_2PO_4$) @ 30 mg/kg were added to all samples. Thirty seeds of Tall Fescue were planted in each sample. All vegetated samples were prepared as three replicates. The control samples for each concentration were also prepared as three replicates; the control samples did not receive seeds (non-vegetated). Prior to plant-

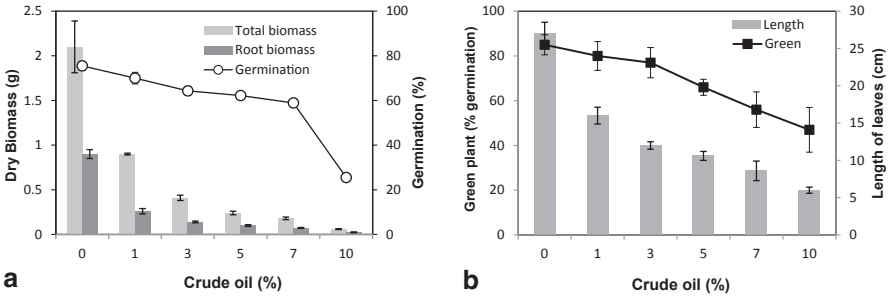


**Fig. 10.1** **a** The number of germinated seeds in different concentrations of crude oil after 30 days of seeding and total dry biomass (shoots + roots) and dry biomass of roots after 120 days of planting. **b** Length of leaves measured 60 days after planting; the green plants were counted at the end of experiment (120 days). Average values ± Standard deviation (±SD), $p<0.05$ are given

ing (T = 0), 10 g of soil were removed from each sample and stored at −20 °C for further preparations. The number of germinated seeds was counted after 30 days and the length of shoots was measured 60 days after planting. The number of green plants was counted at the end of the experiment (120 days). At the end of 120 days, roots and shoots were separated and dried at 50 °C. Plant biomass was reported as total dry weight for roots and shoots. Crude oil extraction was done according to Minai-Tehrani and Herfatmanesh (2007). For 48 h, 2 g of treated soil was dried at 50 °C then crushed well to make a homogenous soil. A total of 10 ml dichlromethane ($CH_2Cl_2$) (Aldrich) was added to the soil and shaken firmly to separate oil from the soil. The sample was centrifuged (3000 × g for 10 min.) to precipitate the soil, and the solvent phase was removed. The solvent extraction was repeated twice. The solvent vaporized during 24 h and the amount of the oil was measured by the gravimetric method and its reduction compared with time zero (T = 0). Two samples from each replicate were taken for crude oil extraction.

## 3 Results

**Germination and Biomass** Figure. 10.1a shows the number of germinated seeds in the vegetated samples 30 days after planting. In the control (0 %) sample, the number of germinated seeds was higher than that in the other samples while it was lower in 10 % oil sample. There was a sudden decrease in the number of germinations in 10 % oil sample in comparison with the other contaminated samples. The dry biomass of roots and shoots was measured at the end of the experiment (Fig. 10.1a). The separation of roots from the soil showed that the distribution of roots in the soil has decreased by increasing the crude oil concentration. The higher root biomass was observed in 0 % oil sample, in which the roots were well distributed in the soil. The lower roots biomass was noted in 10 % oil sample. The root distribution was poor in high crude oil concentrations (7 and 10 %). The total dry

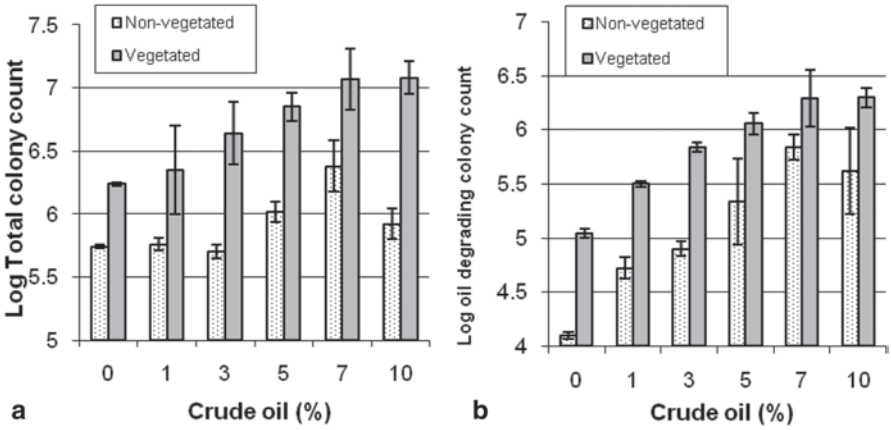


**Fig. 10.2** Total colony count (CFU/g soil) after 2 months of planting (**a**). Oil-degrading colony count (CFU/g soil) after 2 months of planting (**b**). (±SD, $n=3$, $p<0.05$)

biomass (roots + shoots) was also high in 0% oil sample while it was low in 7 and 10% oil samples. A sudden decrease in total dry biomass was observed in 1% oil sample in comparison with the control (0%) sample. The length of leaves decreased by increasing crude oil concentration; the shorter leaves were observed in 10% oil sample, while the tallest were noted in 0% sample (Fig. 10.1b). The number of green plants at the end of experiment was lower in 10% followed by 7% oil samples, while the higher was observed in 0% followed by 1% oil samples (Fig. 10.1b).

**Colony Count** Total colony count was determined in the vegetated and the non-vegetated soils (Fig. 10.2a). In the vegetated samples, the higher microbial population was observed in 7 and 10% oil samples, the lower being in the control (0%) sample. Increasing the crude oil concentration increased total microbial population in the vegetated samples. In the non-vegetated samples, the higher microbial population was observed in 7% oil sample, while the lower was observed at 0% sample. In all the vegetated samples, the total colonies were higher than those in their equal concentration of crude oil in the non-vegetated samples. Counting for the oil-degrading colonies in the vegetated samples showed that the higher microbial population was also observed in 7 and 10% samples and the lower was seen in 0% sample (Fig. 10.2b). In the non-vegetated samples, the higher count for oil-degrading colonies was observed in 7% oil sample, while it was lower in 0% oil sample, In all the vegetated samples the oil degrading colonies were also higher than those in their equal concentrations of crude oil in the non-vegetated samples.

**Crude Oil Reduction** The crude oil reduction in the vegetated and the non-vegetated contaminated soils was measured and compared after 120 days (Fig. 10.3). The higher reduction was observed in 1% vegetated sample and the lower was observed in 10% in both vegetated and non-vegetated samples. Increasing crude oil concentration decreased the reduction of crude oil in both vegetated and non-vegetated samples. In all the contaminated vegetated soils, the reduction in crude oil

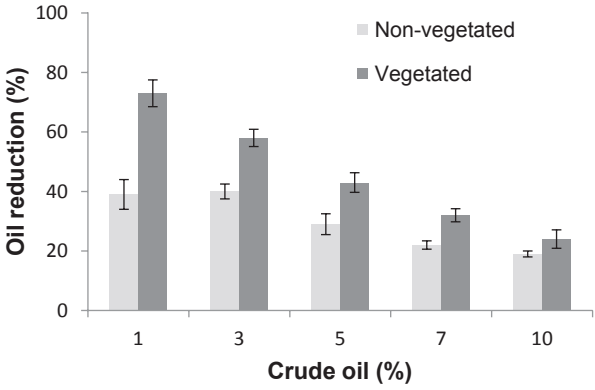


**Fig. 10.3** Reduction of crude oil after 120 days in different concentrations of light crude oil-contaminated soils. (±SD, $n=3$, $p<0.05$)

was higher that in the non-vegetated soils. In the higher concentrations (7 and 10%) the difference in crude oil reduction between the vegetated and the non-vegetated samples was not significant, while the reduction was significant between the vegetated and the non-vegetated samples in concentrations up to 5%.

## 4 Discussion

This study focused on assessing the reciprocal effects of light crude oil-contaminated soil in a potential grass *Festuca arundinacea* (Tall fescue). The reduction in biomass and the length of leaves in high concentration of light crude oil (7 and 10%) suggests that the toxic compounds of crude oil in the soil could reduce the number of germinated seeds and adversely affect the normal growth of roots and shoots in contaminated vegetated samples. The germination of this plant in 10% light crude oil-contaminated soil suggests that the plant could tolerate high concentration of crude oil (10%) in the soil. The distribution of fibrous roots of the plant in contaminated soil decreased significantly in comparison with the control. The germination of Tall fescue in 5% TPH contaminated soil was found to reduce about 70% of control (Huang et al. 2005). Exposure of the plants to tolerable concentrations of petroleum can cause the chlorosis of the leaves, plant dehydration, stunted growth and also death (Udo and Fayemi 1975).

At the end of experiment, the number of green plants decreased in high concentrations of light crude oil (5–10%) which was accompanied by chlorosis and dehydration. This was in accordance with other studies that have reported the effect of oil on growth and germination of plants (Merkl et al. 2005).

Previous reports show that increasing crude oil concentration decreased the microbial population of the soil (Delille and Siron 1993). On the contrary, our results showed that in the presence of high concentration of crude oil (10 and 7%), the microbial population and oil degrading bacteria, increased in comparison with the control and lower concentrations of crude oil (1 and 3%). This phenomenon might

be due to presence of high concentration of crude oil in the soil that prevented the fast evaporation of water from the soil. Thus, the contaminated soils were always wet in the microenvironment area of the soil. Consequently, in the soil with higher concentrations of crude oil, the presence of water in microenvironment could also prevent the diffusion of non-polar toxic materials of crude oil into the microenvironment which could help the bacteria to be vigorous, but the absence of sufficient oxygen reduces the crude oil degradation by the bacteria in the samples with high crude oil concentration. The oily shield may prevent free diffusion of oxygen to microenvironment area in the soil. Oxygen plays an important role in the biodegradation of crude oil and its components (Von Wedel et al. 1998; Ahamed et al. 2010).

Our results also showed that in all the vegetated samples the microbial populations were higher than the non-vegetated samples, suggesting that the presence of roots in the soil could increase the microbial population in comparison with that in non-vegetated samples. The comparison of crude oil reduction in the vegetated and non-vegetated soils showed that in all the vegetated samples the crude oil reduction was higher than that in the non-vegetated samples. It has been shown that the planted contaminated soil had higher efficiency of reducing oil than did the unplanted soil (Pradhan et al. 1998; Minai-Tehrani 2008). The high reduction of crude oil in the 1 and 3 % vegetated samples suggests that the oil reduction has been enhanced in the presence of well distributed plant roots, and the plant roots play an important role in removal of crude oil and its components. Almost equal reduction of crude oil in the vegetated and the non-vegetated of 7 and 10 % samples suggests the importance of the presence of plant roots and their role in reduction of crude oil.

## 5 Conclusion

In conclusion, *Festuca arundinacea* is a tolerant plant for growing in the oil-polluted soil. This plant might be a good choice for phytoremediation with low concentration of crude oil contaminated—soil, while the high concentration of crude oil could be harmful for this plant. A well distributed of root in the soil and microenvironment around the roots is two important factors for better degradation of contamination and increasing bacterial population in the soil.

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# Chapter 11
# Fundamentals of Hydrogen Production via Biotechnology (Bio-H2)

**Nuri Azbar**

**Abstract** Hydrogen is considered to be the fuel of the future due to its promising properties in terms of sustainability. Among common hydrogen production methods processes like thermo-chemical, physico-chemical, electro-chemical and biological have been gaining more and more interest lately. A special term "biohydrogen" has been coined which refers to hydrogen production by living organisms. It is considered to be more environmentally friendly alternative since it neither requires high temperature nor high pressure during the production, moreover it provides eco-friendly solution to organic wastes via conversion of organics into a biofuel which emits only water vapor when combusted. Among different technologies of hydrogen production, bio-hydrogen production perhaps exhibits the greatest potential to replace fossil fuels. In this chapter, various hydrogen production methods such as anaerobic dark fermentation and light-driven photo-biological processes are discussed.

**Keywords** Biohydrogen · Anaerobic dark fermentation · Light · Driven fermentation · Hydrogen economy · Photo · Fermentation

## 1 Introduction

The main drivers of the hydrogen economy are; ever-growing consumption of energy and decarbonisation need due to the negative environmental effects of refinery based energy alternatives, which have negative environmental impacts related to climate change and global warming, both threatening our existence on the planet earth. It is estimated that over 38 Mt (5,000 petajoules), with a market value of $ 60 billion hydrogen is produced worldwide (Levin and Azbar 2012). Hydrogen has been most commonly used for the purpose of processing of oil in refineries and for the production of industrial chemicals such as ammonia and methanol. Other uses of hydrogen are in industrial processes, chemical manufacturing, and food preparation. The current use of hydrogen is expected to increase significantly in

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**Table 11.1** Comparison of various conventional methods of hydrogen production

| Method | Advantages | Disadvantages |
|---|---|---|
| *Reformation of natural gas*<br>$CH_4 + H_2O \rightarrow CO + 3H_2$<br>$CO + H_2O \rightarrow CO_2 + H_2$ | Most common (80 % $H_2$ production)<br>Well understood process<br>Widespread infrastructure | Dependent on non-renewable natural gas<br>High $CO_2$ (GHG) emissions |
| *Gasification of coal*<br>$C + H_2O \rightarrow CO + H_2$<br>$CO + H_2O \rightarrow CO_2 + H_2$ | Coal is abundant and inexpensive | Low yields<br>High $CO_2$ (GHG) emissions<br>High SOx and CO emissions |
| *Electrolysis of water*<br>$H_2O \rightarrow O_2 + 2H_2$ | Second most common method used<br>Well understood<br>Widespread infrastructure<br>Potentially emission free, depending on source of electricity generation | Energy intensive<br>High $CO_2$ (GHG) emissions if fossil fuels (coal, natural gas) used to generate electricity |
| *Biomass reformation*<br>$C_6H_7O_2N + 2H_2O \rightarrow 4CO{+}CH_4 + 3 / 2H_2$<br>$CH_4 + H_2O \rightarrow CO + 3H_2$<br>$CO + H_2O \rightarrow CO_2 + H_2$ | Potentially carbon neutral<br>Inexpensive<br>Can use organic waste streams | Not yet well understood |
| *Biohydrogen production*<br>$H_2O$ + light energy $\rightarrow O_2 + 2H_2$<br>$C_6H_{12}O_6 + H_2O \rightarrow CO_2 + H_2$ + Organic molecules | Carbon neutral<br>Can use light or organic waste streams<br>Low energy input | May have poor yields<br>Not yet well understood |

the near future in order to meet the demand for refining increasingly heavier, higher sulfur crude oils and oil sands and to meet more stringent regulations on the levels of sulfur in gasoline and diesel fuel. Hydrogen use will also increase up to 40 million t of hydrogen per year in order to meet the fuel need of transportation sector for 100 million fuel cell-powered cars after full market penetration.

Thermo-chemical and electro-chemical methods are the common hydrogen production methods using a diverse array of potential feedstock including fossil fuels, water, and organic matter (Table 11.1). Currently, over 80 % of hydrogen production occurs via steam reformation of natural gas during which methane, the primary constituent of natural gas, is combined with high temperature steam (700–1000 °C) in the presence of a catalyst, breaking it apart into $H_2$ and CO. The CO produced further reacts with water at high temperatures to produce $H_2$ and $CO_2$ via a process known as the gas shift reaction. The main drawback of this process is that it is dependent on a limited reserve of natural gas and the carbon dioxide emissions. Similar to natural gas gasification, hydrogen can be produced via coal gasification, however this process produces even more $CO_2$ emissions and is more expensive ($H_2$ to $CO_2$ production ratios: 1:1 for coal gasification and 4:1 for natural gas reformation).

Alternatively; biomass gasification, a potentially carbon neutral method; can be used to break down biomass into $H_2$, $CH_4$, and CO, which can in turn be used for steam reformation and the gas shift reaction. Although net $CO_2$ production is observed during these processes, the overall process of biomass gasification can be considered carbon neutral since new biomass generated during the photosynthesis fixes $CO_2$. With this process, municipal and agricultural wastes could also be turned into valuable commodities. The electrolysis of water, which is one of the most common methods for $H_2$ production, is carried out by an electric current passing through water, splitting it up into $H_2$ and $O_2$. This is a very energy intensive process and could be potentially emission free if only clean, renewable sources such as wind, solar, hydro, or geothermal energy are used for the generation of electricity.

Hydrogen production via biotechnology (Bio-$H_2$), on the other hand, employs the use of either dark fermentative or light dependent hydrogen producing organisms. This method of hydrogen production is an attractive alternative to conventional thermo-chemical and electro-chemical methods since it is a potentially carbon neutral process which is carried out at lower temperatures and pressures, and is therefore less energy-intensive than thermo-chemical and electro-chemical processes. Furthermore, unlike thermo-chemical methods, which involve the conversion of nonrenewable fossil fuels into hydrogen, fermentative hydrogen production can utilize renewable carbohydrate-rich substrates such as waste biomass from municipal, agricultural, and forestry sectors, while light driven biological hydrogen production processes utilize light energy, water, and/or $CO_2$.

## 2 Biohydrogen Production

The term "biohydrogen" refers to hydrogen production by living organisms. Hydrogen production via biotechnology can be classified as follows:

### *2.1 Direct Biophotolysis of Water*

$$2H_2O + \text{light} \rightarrow 2H_2 + O_2 \quad (\text{by cyanobacteria, microalgae})$$

### *2.2 Indirect Biophotolysis of Water*

$$6H_2O + 6CO_2 + \text{light} \rightarrow C_6H_{12}O_6 + 6O_2 \quad (\text{a}) \quad (\text{by microalgae})$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 6CO_2 \quad (\text{b}) \quad (\text{by cyanobacteria})$$

$$2CH_3COOH + 4H_2O + \text{light} \rightarrow 8H_2 + 4CO_2 \quad (c)$$

$$\textbf{Overall reaction}: 12H_2O + \text{light} \rightarrow 12H_2 + 6O_2$$

## 2.3 Photo-Fermentation

$$CH_3COOH + 2H_2O + \text{light} \rightarrow 4H_2 + 2CO_2 \quad (\text{purple bacteria, microalgae})$$

## 2.4 Water-Gas Shift Reaction

$$CO + H_2O \rightarrow H_2 + CO_2 \quad (\text{fermentative bacteria, photosynthetic bacteria})$$

## 2.5 Two-Phase Anaerobic Process

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2 \quad (\text{fermentative bacteria})$$

$$2CH_3COOH \rightarrow 2CH_4 + 2CO_2 \quad (\text{methanogenic bacteria})$$

## 2.6 Hybrid Hydrogen Production System (Dark Fermentation + Photo-Fermentation)

Biohydrogen production methods can also be grouped into light independent processes (dark anaerobic fermentation) and light dependent hydrogen production methods with or without oxygen evolution (bio-photolysis) in terms of the energy sources and electron donors used by microorganisms. Light-dependent hydrogen results from the process of photosynthesis. Among photosynthesizing microorganisms capable of evolving hydrogen most attention is paid to microalgae, heterocyst cyanobacteria, and purple non-sulfur bacteria. Microalgae and cyanobacteria possess two photo-systems and can decompose water to release oxygen. Bacteria with one photosystem (first of all purple and green sulfur and non-sulfur bacteria) are incapable of evolving oxygen, and need more reduced electron donors than water to affect photosynthesis. Biohydrogen processes can convert high carbohydrate content waste streams into useable renewable energy, while reducing waste disposal costs and negative environmental impacts. The main criteria for the selection of

organic wastes are availability, low cost, carbohydrate content, and biodegradability. In this chapter, each potential biohydrogen production method is discussed.

## 3 Light Independent Hydrogen Production—Dark Fermentation

Dark fermentation, which is a naturally occurring process for a variety of microbes, can convert organic material (especially carbohydrate rich ones) into $H_2$, $CO_2$, and organic acids. This is a promising alternative to light dependent processes, particularly when waste biomass is used as a feedstock for the generation of $H_2$. The main advantages of this method over light dependent biohydrogen method is that fermentation does not require a constant light supply, it can be run continuously using inexpensive and commercially available systems and $H_2$ production rates are much higher than photosynthesis-based systems (Levin et al. 2004). On the other hand, there are also some disadvantages as summarized in Table 11.1.

Although fermentative hydrogen production has many advantages as mentioned above, it is necessary to note that there are also some constraints such as thermodynamic limitations, product inhibition, the presence of branched catabolic pathways, media composition, and the nature of substrate, which all have an impact on hydrogen yields. These constraints are discussed below.

### *3.1 Basics of Dark Fermentative $H_2$ Production*

Fermentative $H_2$ production yields only about 10–20 % of the hydrogen potentially available in the substrate (theoretical upper limit: 12 mol $H_2$ $mol^{-1}$ hexose) (Hawkes et al. 2007; Kraemer and Bagley 2007; Hallenbeck and Ghosh 2009). Somewhat higher yields, up to 25 % (3 mol $H_2$ $mol^{-1}$ hexose) can be achieved with thermophilic fermentations using either pure cultures or co-cultures at the expense of volumetric productivities (Panagiotopoulos 2010; Zeidan and van Niel 2009).

Fermentative hydrogen production, where hydrogenase enzymes are involved, is carried out via two metabolic types; facultative anaerobes, such as *Escherichia coli*, and strict anaerobes, like *Clostridia* as shown in Fig. 11.1.

Heterotrophic organisms (bacteria growing on organic substrates) have special problems with respect to the disposition of electrons from energy-yielding oxidation reaction during the anaerobic mode of growth. Various kinds of specific controls are required to regulate electron flow in the metabolism of strict and facultative anaerobes. The ability of "disposing off" excess electrons ($e^-$) in the form of $H_2$ through the activity of hydrogenase is among them.

As shown in Fig. 11.1, substrate is first broken down to pyruvate by Embden-Meyerhof-Parnas pathway (glycolysis) which results in ATP production and reduction of NAD to NADH. Oxidation of NADH is necessary for glycolysis to continue and this is achieved via the production of a variety of reduced products (e.g. ethanol

**Table 11.2** Advantages and disadvantages of dark fermentation processes

| No | The process | Organisms | Key enzyme | Advantages | Disadvantages |
|---|---|---|---|---|---|
| 1 | Dark anaerobic fermentation | Wide range of anaerobic bacteria | Hydrogenase | Substrates: wide range of organics including wastes; highest rates of the process | Low yield of the process (not more than 4 moles $H_2$ per 1 mol of glucose); organic acids, alcohols as by-products; high $H_2$ concentration inhibits the process |
| 2 | Dark aerobic or anaerobic | All chemotrophic diazotrophic bacteria during nitrogen fixation | Nitrogenase | All organic substrates available for aerobic or anaerobic decomposition by diazotrophic bacteria under nitrogen fixing conditions | Low efficiency of the bioconversion, especially under aerobic conditions |
| 3 | Dark anaerobic CO decomposition (water-shift reaction) | Some purple bacteria | Hydrogenase in conjunction with CO-dehydrogenase | The substrate: toxic gas which represents big part of syngas | High sensitivity to inactivation by oxygen; organic substrates and $H_2$ can inhibit the process |

and acetate during facultative anaerobic pathway and ethanol, butyrate, butanol, acetone during strict anaerobic pathway).

Pyruvate, which is the key intermediate, is catabolized in two different ways. Facultative anaerobes degrade pyruvate to formate and acetyl-CoA through the action of pyruvate formate lyase (PFL). Strict anaerobes produce acetyl-CoA, $CO_2$, and reduced ferredoxin with the help of pyruvate ferredoxin oxidoreductase enzyme (PFOR) (Hallenbeck 2009). Thus, facultative anaerobes produce hydrogen from formate by the formate hydrogen lyase (*fhl)* complex which possesses an energy-converting Ni-Fe hydrogenase, a member of the Ech family of hydrogenases (Vignais and Billoud 2007; Vignais 2008) and in the other case, hydrogen production is derived by reduced ferredoxin by a Fe-Fe hydrogenase (Hallenbeck 2009; Hallenbeck and Ghosh 2009). Reducing ferredoxin can lead to additional hydrogen production by re-oxidizing NADH under very low hydrogen partial pressures which is not common in typical hydrogen fermentations. In both facultative and strict anaerobic hydrogen production pathways, some of the acetyl-CoA is used for the

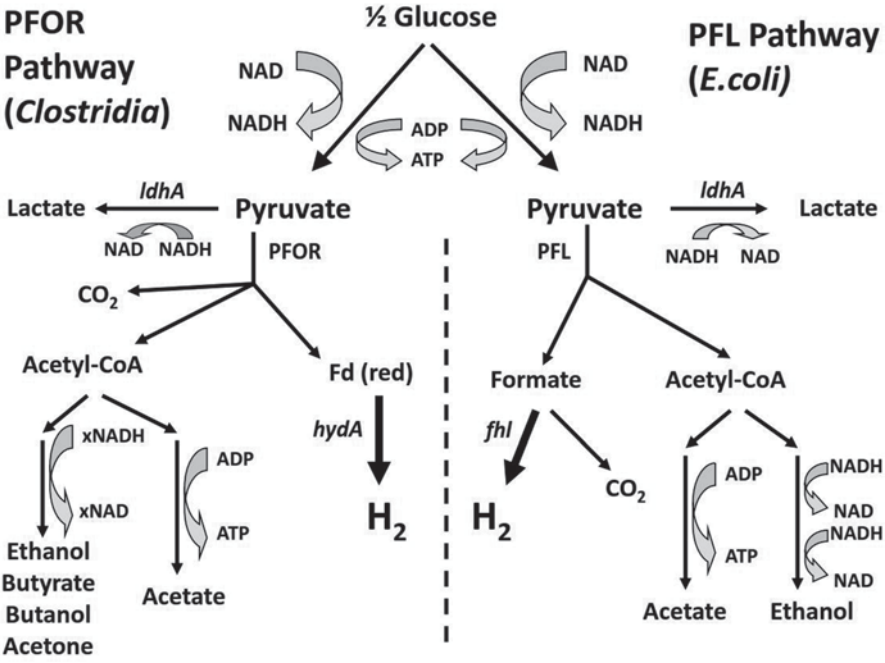


**Fig. 11.1** Metabolic pathways in fermentative $H_2$ production. (Hallenbeck and Ghosh 2012)

production of reduced products and rest is required for ATP synthesis. Even though many organic compounds enable the production of hydrogen during dark fermentation, estimations of potential yields are mostly based on hexose conversions. The theoretical yield per mole of glucose is described as follows:

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3 + 4H^+ + 4H_2 + \Delta G^\circ = -206\,\mathrm{kj\,mol^{-1}}$$

Theoretically, a maximum of 4 moles of $H_2$ per mole of glucose can be produced concurrently with the production of 206 kJ energy per mole of glucose in acetic acid fermentations. The remainder of the hydrogen in the hexose is conserved in the byproduct acetate, and under non ideal circumstances, more reduced products such as ethanol, lactate, or alanine (at high $H_2$ partial pressures). The production of more reduced organic acids and/or alcohols results in lower $H_2$ yield. For example, the conversion of 1 mol of glucose into butyrate is accompanied by the production of only 2 moles of $H_2$. Generally, a mixture of products is produced especially by *Clostridia* and the available $H_2$ from glucose is determined by the butyrate/acetate ratio. The complete oxidation of glucose to hydrogen and carbon dioxide yields 12 mol $H_2$ $mol^{-1}$ of glucose without taking the metabolic energy needed. In practice, hydrogen yield is 2 mol $H_2$/mol glucose (2 mol $H_2$ $mol^{-1}$ glucose only at $PH_2 < 0.1$ kPa) for both PFOR and PFL pathways. The overall yields in these metabolisms are relatively low. This is a natural consequence of the fact that

fermentations have been optimized by evolution to produce cell biomass and not hydrogen. Thus a portion of the substrate (pyruvate) is used in both cases to produce ATP giving a product "acetate" that is excreted. Also, in many organisms the actual yields of hydrogen are reduced by hydrogen recycling due to the presence of one or more uptake hydrogenases, which consume a portion of the hydrogen produced. It is unknown to what extent hydrogen production could be increased through metabolic engineering and manipulation of culture conditions.

### 3.1.1 Microbes Involved

Various kinds of microorganisms take part in the dark fermentative hydrogen generation. *Bacillus, Escherichia, Enterobacter, Ruminococcus, Citrobacter* and *Clostridia* are most common microbial genera capable of producing hydrogen via fermentation (Das 2009; Davila-Vazquez et al. 2008). Many anaerobes are capable of producing hydrogen from hexoses in acetic acid, butyric acid and acetone-butanol ethanol fermentations. *Clostridia* (*C. butyricum, C. welchii, C. pasteurianum, C. beijerincki*) and mixtures have been used in many studies dedicated to hydrogen production. The hyperthermophile *Pyrcoccus furious*, an archaebacterium, is also known to produce $H_2$, organic acids and $CO_2$ from carbohydrates (Fiala and Stetter 1986; Brown and Kelly 1989; Godfroy et al. 2000). There are other cellulotic thermophiles and extreme hyperthermophilic bacteria producing hydrogen such as *Anaerocellum, Caldicellulosiruptor, Clostridium, Dictyoglomus, Fervidobacterium, Spirocheta, Thermotoga and Thermoanaerobacter* (Schröder et al. 1994).

Rumen bacteria are other strict anaerobic bacteria, which are capable of $H_2$ production and other products such as acetate, ethanol, formate and $CO_2$ from carbohydrates. *Ruminococcus albus* is one of the most commonly known one (Innotti et al. 1973). Methanogens have hydrogenase which is usually involved in the oxidation of $H_2$ coupled to CH production and $CO_2$ reduction. On the other hand, it is well known that *Methanosarcina barkeri*, under the conditions of inhibition of $CH_4$ formation, is capable of carrying out so-called water-gas shift reaction (production of $H_2$ and $CO_2$ in stoichiometric amounts from CO and $H_2O$) (Bott et al. 1986). Strict anaerobes have no tolerance over oxygen and do not usually survive low oxygen concentrations, on the other hand, facultative anaerobes are resistant to oxygen. These bacteria are capable of rapidly consuming oxygen and restoring anaerobic conditions. Among these bacteria, *Enterobacter* and other members of *Enterobacteriaceae* are able to produce $H_2$ and are not inhibited by high $H_2$ pressures, but the $H_2$ yield on glucose is lower than that of *Clostridia* (Tanisho and Ishiwata 1994). Hydrogen production by *Escherichia coli* is mediated by the formate hydrogenlyase (*fhl*) complex as shown in Fig. 11.1. *E. coli* can perform a 'mixed-acid fermentation' in which glucose is metabolised to ethanol and various organic acids, including formate. This formate is further disproportionated to carbon dioxide and hydrogen by the formate hydrogenlyase (FHL) complex. Another facultative anaerobe, genus *Citrobacter* is considered under the family *Enterobacteriaceae*. They are gram-negative, non spore forming, facultative anaerobic and motile bacilli employing peritrichous flagella for locomotion and commonly utilizing citrate as their sole carbon source. *Citrobacter*

species, especially *C. freundii* have been shown to produce $H_2$ from CO and $H_2O$ by the water-gas shift reaction under anaerobic conditions (Jung et al. 1999). Some aerobic bacteria like *Alcaligenes eutophus* and *Bacillus licheniformis* are known to produce $H_2$. *B. licheniformis* was reported to produce 0.5 mol $H_2$ per mole of glucose. Use of cell immobilization enhanced $H_2$ yield increasing it up to 1.5 mol $mole^{-1}$ glucose. *Alcaligenes eutophus*, which contains a soluble NAD-reducing hydrogenase, can grow heterotrophically on gluconate and fructose and produces $H_2$ when exposed to anaerobic conditions (Kalia et al. 1994; Kumar et al. 1995).

### 3.1.2 Enzymes Involved

Availability of a hydrogen-producing enzyme is the most crucial aspect of all biohydrogen processes. One should note that the catalytic activity of the various enzymes differs enormously and the quantity or inherent activity of these enzymes could limit the overall process. However, currently there is no evidence proving that the quantity of hydrogen-producing enzyme is the limiting factor in any known system. In contrast, potential catalytic activity far surpasses the amount of hydrogen produced which means that other metabolic factors might be limiting.

Hydrogen-producing enzymes catalyze the simplest chemical reaction:

$$2H^+ + 2e^- \leftrightarrow H_2$$

However, it is known that enzymes capable of hydrogen evolution contain complex metallo-clusters as active sites harboring Ni and Fe atoms. At present, three enzymes carrying out this reaction are known; nitrogenase, Fe-hydrogenase, and Ni-Fe hydrogenase. Like most metalloenyzmes, hydrogenases are quite sensitive to oxygen, high temperature and some other environmental factors. Protein matrix surrounding the metal centers allows hydrogenases to function properly, selectively and effectively (VoIbeda et al. 1995).

### 3.1.3 Feedstock for Dark Fermentative Hydrogen Production

There are two concerns regarding the feedstock that could be utilized i) the range of organic compounds and ii) quality of the feedstock. Carbohydrates are the preferred organic carbon source for $H_2$ production. Glucose or in principle its isomer hexoses or its polymers starch and cellulose, give maximum yield of $4H_2$ per glucose when acetic acid is the by-product

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COO^- + 2CO_2 + 4H_2$$

On the other hand, half of this yield per glucose is obtained with butyrate as the fermentation end product as follows:

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$$

**Table 11.3** Examples for various substrates used for Bio-$H_2$ production and $H_2$ yield

| Substrate | Microorganism | $H_2$ yield mol $H_2$ $mol^{-1}$ glucose equiv |
|---|---|---|
| Glucose | Mixed *Clostridium* sp. | 1.43 |
| Glucose | Acclimatized sludge | 1.66 |
| Glucose | Heat-treated sewage sludge | 0.96 |
| Sucrose | Mixed, undefined community | 1.0–1.9 |
| Pulped sugar beet | Mixed, undefined community | 0.9–1.7 |
| Soluble starch | Mixed, undefined community | 2.14 |
| Wheat starch | Mixed, undefined community | 1.9 |
| Mixed wastes | Heat-treated sludge compost | 0.9–2.4 |
| Mixed wastes | Heat-treated sludge compost wastewater | 2.59 |
| Glucose | *Clostridium butyricum* | 1.4–2.3 |
| Glucose | *Enerobacter aerogens* HU−101 | 1.17 |
| Glucose | *Clostridium acidisoli* | 1.0 |
| Glucose | *Enerobacter cloacae IIT BT 08* | 2.3 |
| Glucose | *Enerobacter cloacae* DM 11 | 3.8 |
| Glucose | *Caldicellulosiruptor saccharolyticus* | 3.3–3.6 |
| Sucrose | *Clostridium butyricum* | 2.78 |
| Molasses | *Enerobacter aerogens* | 1.58 |
| Cellobiose | *Clostridium thermocellum ATCC 27405* | 1.5 |
| Cellobiose | *Clostridium termitidis CT112* | 0.5 |
| Delignified wood fibers | *Clostridium thermocellum ATCC 27405* | 1.6 |
| α-cellulose | *Clostridium thermocellum ATCC 27405* | 1.28–1.9 |
| α-cellulose[a] | *Clostridium thermocellum ATCC 27405* | 0.98–1.29 |
| Dried distillers grains | *Clostridium thermocellum ATCC 27405* | 1.27 |
| Barley hulls | *Clostridium thermocellum ATCC 27405* | 1.18–1.24 |
| α-cellulose | *Clostridium termitidis CT112* | 1.4 |

[a] Continuous fermentation over 3,000 h with a 24 h HRT

Table 11.3 summarizes a wide variety of organic materials employed for the hydrogen generation, using mixed, undefined microbial communities or defined pure cultures. Most common substrates used for biohydrogen production during dark fermentation studies include sugars, such as glucose, fructose, galactose and arabinose, sucrose, xylose, starch, and cellulose and various industrial waste streams that contain heterogeneous mixtures of sugars and starch (Das 2009; Davila et al. 2008).

It is obvious that the cost of raw material or substrate is crucial and plays a significant role for the overall economics of biohydrogen production, in view of this, finding inexpensive feedstock from agro-based wastes and industrial organic wastes is indispensable for the economical sustainability of biohydrogen production.

The majority of research has been directed towards expensive pure substrate or to a much lesser degree solid waste or wastewaters. However, more sustainable feedstock will be needed for a sustainable process. These could be achieved by sugar-containing crop such as sweet sorghum and sugar beet, starch based crops such as corn or wheat, or ligno-cellulosics such as fodder grass and *Miscanthus*. Ligno-cellulosic biomass is a complex of biopolymers that makes up the structural components of plant material. The approximate composition of lingo-cellulose found in most biomass feedstocks is roughly 45–60 % cellulose, 20–40 % hemicellulose, 25 % lignin, and 1–5 % pectin (Demain et al. 2005; Desvaux 2005; Lynd et al. 2005). Cellulose consists of linear, insoluble polymers consisting of up to 25,000 repeating β-1,4 linked β-D-glucopyranose units. Cellulose is a highly ordered molecule consisting of 15–45 crystalline microfibril chains, which in turn associate to form cellulose fibers. In nature, cellulose is found primarily in plant cell walls and is associated with varying degrees of other biopolymers, including: (i) hemi-cellulose, a random, amorphous hetero-polysaccharide composed of typically β-1,3 linked xylans, arabinoxylan, gluco-mannan, and galactomannan; (ii) lignin, a complex hydrophobic network of phenyl-propanoid units; (iii) pectins, composed of α-(1–4)-linked D-galacturonic acid; and (iv) proteins. Ligno-cellulosic biomass is renewable, inexpensive, constitutes a large fraction of waste biomass from municipal, agricultural, and forestry sectors, and thus offers excellent potential as a feedstock for renewable biofuels. Cellulose is, however, difficult to hydrolyze due to its crystalline structure. Current strategies that produce fuel ethanol from lingo-cellulosic biomass (or "second-generation" biofuels) use simultaneous saccharification and fermentation (SSF) or simultaneous saccharification and co-fermentation (SSCF). Both SSF and SSCF require extensive pre-treatment of the cellulosic feedstock by steam-explosion and/or acid treatment, followed by addition of exogenously produced cocktails of cellulolytic enzymes to hydrolyse cellulose chains and release the glucose monomers required for fermentation. These pre-treatments are costly, and some of the by-products generated, for example furfurals, can inhibit downstream processes. Steam-explosion of corn stover, with or without acid treatment, can be a suitable substrate for $H_2$ production. Hydrogen production from fermentable biomass has the advantage over ethanol production that the microflora is able to use a wider range of cellulosic substrates than the yeast.

Fermentative $H_2$ production using cellulose as the sole carbon source is under extensive investigation (Wang et al. 2008; Lo et al. 2008). The mesophilic, cellulolytic bacterium, *C. termitidis* strain CT1112 has displayed a cell generation time of 18.9 h when grown on 2 g $L^{-1}$ α-cellulose (Ramachandran et al. 2008). The major soluble fermentation byproducts were acetate and ethanol. Maximum yields of acetate, ethanol, $H_2$, and formate on α-cellulose are 7.2, 3.1, 7.7 and 2.9 mmol $L^{-1}$ culture, respectively. Although, the generation time was longer when cultured on α-cellulose than on the soluble cellulodextrin cellobiose, acetate and $H_2$ synthesis were favored over ethanol synthesis, indicating that carbon flow to ethanol and formate was restricted. During log phase, $H_2$ was produced at a specific rate of 2.79 mmoles $h^{-1}$ $g^{-1}$ dry weight- of cells on α-cellulose.

The thermophilic, cellulolytic bacterium *Clostridium thermocellum* strain 27405 produced greater amounts of $H_2$ when cultured (in Balch tubes) on cellulosic

substrates compared with the soluble cellulodextran cellobiose, with an average yield of 1.6 mol $H_2$ $mol^{-1}$ glucose equivalent (Islam et al. 2009). The major soluble fermentation byproducts include ethanol, acetate, and formate, with lactate being produced when the pH drops below 6.3. Hydrogen production by *C. thermocellum* 27405 was also investigated using dried distillers grain (DDGS), barley hulls (BH), or *Fusarium* head blight contaminated barley hulls (CBH) as the carbon source in batch fermentation experiments (Magnusson et al. 2008). Overall, DDGS produced the highest concentration of $H_2$ gas at 1.27 mmol $H_2$ $mol^{-1}$ glucose equivalent, while CBH and BH produced 1.18 and 1.24 mmol $H_2$ $mol^{-1}$ glucose equivalent, respectively.

Hydrogen production in a continuous pure culture of *C. thermocellum* 27405 was established in a 5 L working volume fermentor, and growth experiments were maintained for over 3,000 h (Magnusson et al. 2009), substrate concentrations varied from 1 to 4 g $L^{-1}$ and the feed was introduced with continuous $N_2$ gas sparging to prevent clogging of the feed-line; pH and temperature of the reactor were maintained at 7.0 and 60 °C, respectively, throughout the study. At concentrations above 4 g $L^{-1}$, the delivery of α-cellulose was impaired due to feed-line clogging and it became difficult to maintain a homogenous suspension. At a dilution rate of 0.042 $h^{-1}$ and substrate concentration of 4 g $L^{-1}$, the $H_2$ production rate was 5.06 mmol $L^{-1}$ $h^{-1}$. Acetate and ethanol were the major soluble end-products, while lactate and formate were greatly reduced compared to production in batch cultures. Concentrations of all metabolites increased with increasing substrate concentration, with the exception of lactate. Despite a number of short-term electrical and mechanical failures during the testing period, the system recovered quickly, exhibiting substantial robustness. A carbon balance indicated near 100 % carbon recovery. This study shows that long-term, stable $H_2$ production can be achieved during direct fermentation of an insoluble cellulosic substrate under continuous culture conditions.

## 4 Light—Driven Biohydrogen Production

Light energy is essential to hydrogen evolution by photosynthetic cells. Table 11.4 compares various light driven bio-hydrogen processes including enzymes involved. Photoautotrophic green microalgae and cyanobacteria use sunlight and $CO_2$ as the sole sources for energy and carbon. The reducing power for cellular photosynthesis and/or biophotolysis comes from water oxidation under light irradiation.

### *4.1 Bio-Photolysis Based Hydrogen Production*

Bio-photolysis is classified in to two groups; **direct** and **indirect**. Direct photolysis refers to sustained hydrogen evolution under light irradiation. The light energy is absorbed by the pigments at PSII, or PSI or both, which raises the energy level of electrons from water oxidation when they are transferred from PSII via PSI to ferrodoxin. A portion of the light energy is directly stored in hydrogen gas.

**Table 11.4** Various light driven bio-hydrogen production methods

| No | The process | Organisms | Key enzyme | Advantages | Disadvantages |
|---|---|---|---|---|---|
| 1 | Anaerobic photosynthetic under N deficiency | Nitrogen fixing anoxygenic photosynthetic bacteria | Nitrogenase | High rates, especially by purple non-sulfur bacteria, might be activated by near-infrared light; low sensitivity to high $H_2$ pressure | Sensitivity to N sources (due to repression and inactivation of nitrogenase); needs in simple organics as electron donor for photosynthesis; narrow range of organics; inactivation by oxygen; low efficiency (needs in electrons and ATP) |
| 2 | Anaerobic photosynthetic under over reduced conditions | Anoxygenic photosynthetic bacteria and cyanobacteria under anaerobic conditions with reversible hydrogenase (*hox YH*) | Hydrogenase | Relatively high efficiency (does not need ATP) | At present knowledge appears only during short-term experiments under over-reduced conditions; high $H_2$ concentration inhibits the process; under mesophilic conditions a possibility for pathogenic bacteria development |
| 3 | Aerobic photosynthetic | Nitrogen fixing cyanobacteria | Nitrogenase | Converts light energy into $H_2$ fuel in stoichiometry near to $2H_2O<->2H_2+O_2$; low sensitivity to high $H_2$ concentrations; temporal or spatial separation of $O_2$ and $H_2$ production | Low rates of the process; low efficiency (needs in electrons and ATP) |
| 4 | Temporal separation of oxygenic photosynthesis and light-dependent $H_2$ production under sulfur deprivation | Microalgae with Fe-Fe hydrogenase; proved for several species including *Chlamydomonas* and *Chlorella* | Hydrogenase | Converts light energy into $H_2$ fuel; long term process; possibility to repeat the cycle "oxygenic photosynthesis-light-dependent $H_2$ production" | Low rates; high sensitivity to oxygen inactivation; still need experimental studies to determine efficiency of process |
| 5 | Aerobic photosynthetic | Microalgae with Fe-Fe hydrogenase | Hydrogenase | Converts light energy into $H_2$ fuel in stoichiometry near to $2H_2O<->2H_2+O_2$; high efficiency; high rates of the process | Appears only during short-term experiments at the start of anaerobic cultures illumination; under S-deprivation shows decreased rates but for longer period. By-products under S-deprivation are organic acids and ethanol |

**Table 11.4** (continued)

| No | The process | Organisms | Key enzyme | Advantages | Disadvantages |
|---|---|---|---|---|---|
| 6 | Temporal separation of oxygenic photosynthesis with accumulation of polysaccharides and dark fermentative $H_2$ production | Proved for some cyanobacteria with reversible hydrogenase (*hoxYH*) and marine microalgae | Hydrogenase | Converts light energy into $H_2$ fuel; long term process; possibility to repeat the cycle "oxygenic photosynthesis-dark fermentative $H_2$ production" | Low rates; high sensitivity to oxygen inactivation; organic acids (especially acetate) as by-products |

In photosynthesis, the reduced carbon is stored as endogenous carbohydrates, such as starch in microalgae and glycogen in cyanobacteria. Studies on the mechanisms involved in hydrogen evolution have found that the electrons or reducing equivalents of hydrogenase and nitrogenase do not always come from water, but may sometimes originate from the intracellular energy reserve including carbohydrates. The stored energy is released through fermentation of the endogenous carbohydrates in dark conditions, and the excess reducing power can be deposited by hydrogenase on protons ($H^+$) forming molecular hydrogen. Hydrogen evolution from endogenous carbon reserve under dark anaerobic conditions looks very similar to the conventional anaerobic hydrogen fermentation, but the endogenous carbon reserve is made in vivo during photosynthesis. In this sense, the electrons or reducing equivalents in indirect bio-photolysis are derived from water by photoautotrophic cells. This indirect bio-photolysis, therefore, consists of two stages in series: photosynthesis for carbohydrate accumulation, and dark fermentation of the carbon reserve for hydrogen production. This way the oxygen and hydrogen evolutions are temporally and/or spatially separated. This separation not only avoids the incompatibility of oxygen and hydrogen evolution (e.g., enzyme deactivation and the explosive property of the gas mixture), but also makes hydrogen purification relatively easy because $CO_2$ can be conveniently removed from the $H_2/CO_2$ mixture.

In cells of certain green algae (e.g. *Chlamydomonas reinhardtii, Chlorella fusca*) and blue-green algae (cyanobacteria), hydrogen production occurs as a result of light-driven splitting of water during photosynthesis. In direct bio-photolysis, the photosynthetic apparatus captures light and the recovered energy is used to couple water splitting to the generation of a low-potential reductant, which can be used to reduce a hydrogenase enzyme. This is an inherently attractive process since solar energy is used to convert a readily available substrate, water, to oxygen and hydrogen:

$$2H_2O + \text{light energy} \rightarrow 2H_2 + O_2$$

This reaction was first demonstrated with a cell free chloroplast-ferredoxin-hydrogenase system, although the existence of such a reaction in green algae had been suggested earlier (Spruit 1958). Anaerobic conditions are indispensable for this process to occur. A stream of electrons and protons originating from water is generated upon the light energy with wavelength lower than 680 nm and absorbed by photosystem II (PSII). On the other hand, photosystem I (PSI) is induced with light wavelength lower than 700 nm which allows the transportation of electrons from PSII to PSI via chain of reductors called cytochrome bf. Electrons from PSI system are transferred via ferrodoxine to hydrogenase (algae or cyanobacteria) or nitrogenase (cyanobacteria) and these enzymes reduce protons to molecular hydrogen. In direct biophotolysis, neither $CO_2$ nor liquid metabolites are observed. The constant removal of oxygen is required since oxygen inhibits hydrogenase activity irreversibly (Das and Veziroglu 2008).

Photoautotrophic microorganisms, either prokaryotic cyanobacteria or eukaryotic green microalgae, possess chlorophyll *a* and other pigments to capture sunlight energy and use photosynthetic systems (PSII and PSI) to carry out oxygenic

photosynthesis. All oxygenic photosynthetic organisms extract electrons and protons from water and use them to reduce $NADP^+$ and plastoquinone for use as energy sources for metabolism such as the Calvin cycle ($CO_2$ fixation) and other pathways. However, oxygenic phototrophs, such as cyanobacteria and microalgae, can transiently produce $H_2$ under anaerobic conditions *via* proton reduction, catalyzed by a hydrogenase (or nitrogenase) in competition with other intracellular processes. In this case the electrons and protons ultimately produced by water oxidation are redirected at the level of ferredoxin/NADPH into hydrogenase (Kruse et al. 2005).

Hydrogen producing cyanobacteria may be either nitrogen-fixing or non-nitrogen-fixing. The examples of nitrogen-fixing, hydrogen producing cyanobacteria include non-marine *Anabaena* species, marine species of *Anabaena,* such as *Anabaena cylindrica, Anabaena variabilis, Anabaena variabilis* PK84, *Anabaena* AMC41, marine cyanobacteria in the genera *Calothrix, Oscillatori*a, *Gloebacter* PCC7421, and *Synechococcus* PCC602, and the marine species *Aphanocapsa montana.*

Some hydrogen producing species of *Synechococcus*, *Gloebacter,* and *Anabaena* are non-nitrogen-fixing and produce more hydrogen than nitrogen-fixing cyanobacteria. Heterocystous filamentous *Anabaena cylindrica* is a well-known hydrogen producing cyanobacterium, but *Anabaena variabilis* has received more attention in recent years, because of higher hydrogen yield (Liu et al. 2006).

Heterocysts provide an oxygen-free environment for the oxygen-sensitive nitrogenase enzyme that reduces molecular nitrogen into $NH_3$, and protons to $H_2$ (Eq. 11.1). In a $N_2$-containing atmosphere, nitrogen-fixation is the predominant reaction while $H_2$ is a minor byproduct. More $H_2$ can only be formed in the absence of molecular nitrogen according to the Eq. 11.2. The reducing power for $H_2$ evolution is derived from the energy-rich carbohydrate ($CH_2O$) stored in the heterocyst or transferred from neighbor cells. Because of the high energy demand (4 ATP per $H_2$), the energy conversion efficiency from light to $H_2$ by nitrogenase is quite low (< 1 %) (Yoon et al. 2006).

$$N_2 + 8H^+ + 8e + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (11.1)$$

$$8H^+ + 8e + 16ATP \rightarrow 4H_2 + 16ADP + 16Pi \quad (11.2)$$

The maximum specific $H_2$ evolution rate per gram of cell mass or chlorophyll *a* (the pigment content accounts for 2–3 % of cell mass) for the representative nitrogen-fixing cyanobacteria varies between 0.21–3.06 mmol $g^{-1}$ $h^{-1}$. Volumetric productivity of $H_2$, on the other hand, has been reported to be between 0.084–0.93 mmol $H_2$ $L^{-1}$ $h^{-1}$. Surface area is one of the major cost factors for photobioreactors. Hydrogen production is also compared in terms of energy productivity, a general performance parameter for energy generation based on the energy output per volume per time. The energy productivity is calculated by multiplying the volumetric productivity (mmol $H_2$ $L^{-1}$ $h^{-1}$) by the heat of combustion of hydrogen at 25 °C ($\Delta Hc$, $H_2 = -0.24$ kJ $mmol^{-1}$) (Weissmann and Benemann 1977).

Biological $H_2$ production is often conducted in sequential mode under different headspace conditions (argon is preferred during the hydrogen production stage): the

first stage for cell growth, followed by the second stage for $H_2$ evolution. The headspace gas component could be a significant cost factor in large-scale hydrogen production. A $N_2$-free gas phase is needed, and argon plus $CO_2$ is preferred, for higher $H_2$ evolution rate, since nitrogenase uses the reducing power for nitrogen reduction, rather than $H_2$ evolution when nitrogen is present in headspace. *A. variabilis* PK84, for instance, was reported to produce almost 15 times more $H_2$ in $CO_2$-enriched argon than in $CO_2$-enriched air. Therefore, a metabolic stress in the form of nitrogen starvation is often required at the end of the growth period to induce the activity of nitrogenase (Fernando et al. 2002). Light intensity also influences $H_2$ production by cyanobacteria. The most commonly applied light intensity varies between 20 and 100 w $m^{-2}$ (Fernando et al. 2002).

It is clear that there is a need for a significant technological breakthrough for $H_2$ productivity by nitrogen-fixing cyanobacteria. Progress up to a certain extent has been made with the genetic engineering of nitrogen-fixing cyanobacteria. In wild type strains, uptake of hydrogenase enzymes to reoxidize $H_2$ to protons takes place, thus reducing $H_2$ evolution. A genetically modified *A. variabilis* PK84, in which the uptake of hydrogenase was mutated, displayed a four-fold greater (3.1 mmol $g^{-1}$ dry wt $h^{-1}$) $H_2$ production rate compared with the wild type strain (Masukawa et al. 2002). There are reports in literature indicating that energy conversion efficiency of photosynthetically active radiation (PAR) could also be increased from 0.005 % of a wild type to above 1 % for strains with impaired uptake hydrogenases (Masukawa et al. 2002).

It is known that unicellular, non-nitrogen-fixing cyanobacteria, such as *Gloebacter* PCC7421, *Synechococcus* PCC602, and *Aphanocapsa montana* are also able to produce $H_2$. Hydrogen evolution values of these cyanobacteria are lower than the heterocystous nitrogen-fixing strains, and there is a need for a highly reducing gas to protect the hydrogenase from oxygen ($O_2$) inhibition.

Hydrogen production is also possible with green microalgae. Among the green algal species studied (*Scenedesmus obliquus*, *Chlorococcum littorale* and *Platymonas subcordiformis*), *Chlorella vulgaris*, *Chlorococcum humicolum* and *Chlamydomonas reinhardtii* are among the best-known $H_2$ producing algae. The main problem with algal $H_2$ production is $O_2$ inhibition. Various methods to overcome the inhibitive effect of oxygen on hydrogenase have been investigated, with limited success including spatial separation of $O_2$ from $H_2$ production, oxygen scavenging, and gas purging. Two fundamental approaches were developed by Ghirardi et al. (2000) and Melis et al. (2000). One involves the temporal separation of the usually incompatible reactions of $O_2$ and $H_2$ production in green algae, and the second involves the use of classical genetics to increase the $O_2$ tolerance of the reversible hydrogenase enzyme (Ghirardi et al. 2000; Melis et al. 2000).

Algal $H_2$ production could be an ecologically acceptable process, since the raw material, water, comes from a sustainable and renewable source, and $CO_2$ consumption during the process provides advantages with respect to reduction of greenhouse gases, specifically $CO_2$. However, low $H_2$ evolution and strong inhibition due to the $O_2$ on hydrogenase enzyme are major limitations for algal $H_2$ production (Greenbaum 1982). Furthermore, algae are not able to utilize organic waste materials.

Therefore, other biohydrogen methods such as anaerobic dark and photo-fermentations are more advantageous, since simultaneous waste utilization and $H_2$ evolution are possible (Hallenbeck and Benemann 2002).

## 4.2 Water-Gas Shift Reaction

Photo-heterotrophic bacteria are also able to produce hydrogen via water-gas shift reaction. During this reaction $H_2$ is released while carbon monoxide (CO) is oxidized to carbon dioxide ($CO_2$) in the presence of anaerobic bacteria as follows:

$$CO(g) + H_2O(l) \rightarrow CO_2(g) + H_2(g) \Delta G_0 = -20\,\text{kJ mol}^{-1}$$

Both the gram-negative organisms, photo-heterotrophic bacteria (*Rhodospirillum rubrum* and *Rubrivax gelatinosus*) and gram-positive bacteria (*Carboxydothermus hydrogenoformans*) are able to utilize this reaction. Under anaerobic conditions, CO induces the synthesis of several proteins, including CO dehydrogenase, Fe-S protein, and CO-tolerant hydrogenase. Electrons produced from CO oxidation are conveyed via the Fe-S protein to the hydrogenase for hydrogen production.

## 4.3 Photo-Fermentation

Much work has been done on the capacity of photosynthetic bacteria to produce significant amounts of $H_2$. Nitrogen deficient conditions using light energy and reduced compounds are required for the photosynthetic bacteria to evolve molecular $H_2$ catalyzed by nitrogenase (Fig. 11.2). The overall reaction of hydrogen production is given as follows:

$$C_6H_{12}O_6 + 6H_2O + \text{Light energy} \rightarrow 12H_2 + 6CO_2$$

The main advantage of this reaction over biophotolysis is the fact that the lack of PSII in this organism automatically eliminates the $O_2$ inhibition of $H_2$ production. Furthermore, the ability of these organisms to utilize wide variety of organic compounds for hydrogen production is another additional advantage. Some of the photo-heterotrophic bacteria capable of hydrogen production under anaerobic conditions in the presence of light are *Rhodobacter spheroides*, *R. capsulatus*, *Rhodovulum sulfidophilum* W-1S and *Rhodopseudomonas palustris*. The highest conversion efficiency reported in the literature has been obtained when lactic acid was used as the sole carbon source. *Rhodospirillum rubrum* and *Rhodopseudomonas palustris* P4, which express a CO-dependent dehydrogenase (CODH) enzyme, have also been reported to be able to produce $H_2$ from CO or other organic acids (Hallenbeck and Benemann 2002). The optimum growth for the photosynthetic bacteria is reported to be in the range of 30–35 °C and at pH 7.0. Hydrogen production by these bacteria

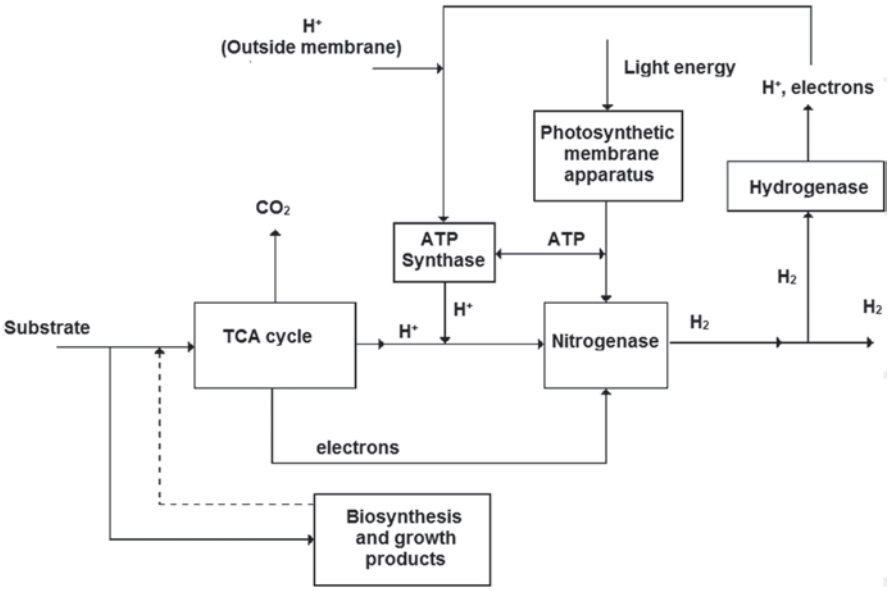


**Fig. 11.2** Hydrogen generation via photofermentation. (Koku et al. 2002)

requires anaerobic conditions under illumination (Bolton 1996). Even though these organisms prefer organic acids as carbon source, other industrial effluents are amenable to $H_2$ production (Koku et al. 2002).

$H_2$ production rates by photo-heterotrophic bacteria are mainly affected by the factors such as light intensity, carbon source, enzymes involved and the type of microbial culture. Nitrogenase, however, is the key enzyme catalyzing $H_2$ production by these bacteria. The presence of oxygen, ammonia and high N/C ratios inhibit the activity of nitrogenase. For example, $H_2$ production by *R. sphaeroides* is completely inhibited at ammonia concentrations above 2 mM (Koku et al. 2002). Under the presence of high nitrogen, metabolic pathway shifts from hydrogen production to the utilization of organic substrates for cell growth which in return prevents the light penetration. For this reason, ammonium concentration in the reactor has to be limited and oxygen should be eliminated. For nitrogen source proteins such as albumin, glutamate, and yeast extract are generally preferred. Uptake of hydrogenase enzymes in photo-fermentative bacteria, oxidizes $H_2$ and are antagonistic to nitrogenase activity, therefore uptake hydrogenase activity should be eliminated for enhanced $H_2$ production. Two to three times more $H_2$ production has been achieved by using hydrogenase deficient mutant cultures of photo-fermentative bacteria (Kars et al. 2008).

Light intensity is another important parameter affecting the performance of photo-fermentations. Increasing light intensity has a stimulatory affect on $H_2$ yields and production rates, but has an adverse effect on the light conversion efficiencies. It was reported that the reduced antenna mutant of *R. sphaeroides* MTP4 produces

$H_2$ more efficiently under high light intensity as compared to the wild type strain. Reduced antenna mutants have been studied for many biotechnological applications, since the benefits deriving from a reduced absorption of light may affect a number of physiological pathways, in different microorganisms. Torzillo et al. (2009) has reported that a great benefit can be derived from the mutants of green algae. Light intensity also affects the consumption rates of organic acids. For example, butyrate consumption requires higher light intensities (4,000 lx) as compared to acetate and propionate. Exposure time to light also affects $H_2$ production. Alternating 14 h light/10 h dark cycles yielded slightly higher $H_2$ production rates and cell concentrations compared to continuous illumination. More frequent exposure to dark/light cycle has a better effect on $H_2$ production (Kapdan and Kargi 2006).

Industrial effluents that do not cause any inhibition on light penetration (colored wastewater) are also amenable for $H_2$ production by photosynthetic organisms. Ammonia content of industrial effluents may also inhibit the nitrogenase enzyme and reduce the $H_2$ productivity. Therefore, pretreatment to remove ammonia and toxic compounds (heavy metals, phenols, etc.) and dilution of high organic matter content (COD) in industrial effluents may be required before using such industrial wastewater during biohydrogen production.

An extensive summary of $H_2$ production studies from some food industry wastewaters has been given by Kapdan and Kargi (2006). Glucose, sucrose, starch, wheat starch, lactose, food waste, potato processing waste, apple, domestic sludge, molasses, rice winery, biosolids, filtrate, sweet potato starch residues, and organic fraction of municipal solid wastes have been used as substrates for $H_2$ production. Tofu wastewater, which is a carbohydrate and protein rich effluent, has also been used for $H_2$ production. Hydrogen yield from tofu wastewater (1.9 L $H_2$ $L^{-1}$ wastewater at 30 °C) has been reported to be comparable to $H_2$ yield from glucose (3.6 L $H_2$ $L^{-1}$ wastewater) using *R. sphaeroides* RV immobilized in agar gel (Zhu et al. 1999). No ammonia inhibition (2 mM) was observed and 41 % of total organic carbon (TOC) was removed. Similarly, the dilution of the wastewater at a ratio of 50 % resulted in an increase in $H_2$ yield of up to 4.32 L $H_2$ $L^{-1}$ wastewater and 66 % TOC removal (Zhu et al. 1999).

Other agro-based waste materials such as potato starch, sugar cane, juice and whey have also been investigated in terms of $H_2$ production using *Rhodopseudomonas* sp. Sugar cane juice yielded the maximum level of $H_2$ production (45 mL [mg DW $h^{-1}$ basis]) as compared to potato waste (30 mL [mg DW $h^{-1}$ basis]) and whey (25 mL [mg DW $h^{-1}$ basis]). There was no $H_2$ production by the photosynthetic bacterium, *Rhodobium marinum* using raw starch as the substrate (Singh et al. 1994).

Use of photo-bioreactors is also critical factor for efficient photobiological $H_2$ production. Most common photo-bioreactors configurations used for $H_2$ production in literature are tubular, flat panel, and bubble column reactors. One of the attempts to increase volumetric hydrogen production in suspended cell bioreactor systems is to keep high biomass inventory in the reactor, but it fails due to the exponential decay of light intensity with increasing density of the cell culture. Therefore, suspension layer thicknesses of 1–5 cm have to be provided in photo-bioreactors. Another attempt to provide higher biomass inventory in the reactor is by cell immobilization

on light transmitting matrices with high surface area/volume ratios. In this case, up to 12 g of cells (dry weight) can be immobilized in 1 L of matrix. Therewith, the rates of $H_2$ evolution per unit volume increase considerably. Thus, *R. sphaeroides* immobilized on a porous glass steadily evolves $H_2$ at a rate of 1.1 L $L^{-1}$ $h^{-1}$ for more than 1,000 h. The maximum volumetric $H_2$ rate attained is 3.8 L $L^{-1}$ $h^{-1}$, with an 80% conversion of the organic acid substrate (Tsygankov et al. 1998). Use of mutant photosynthetic bacteria has also been considered by many researchers to enhance the light conversion efficiency, and hence $H_2$ production rate. Although an improvement has been observed by mutant type, the light conversion efficiency was around 6% (El-Shishtawy et al. 1997), which is still less than theoretical efficiency. The light penetration length is important for the hydrogen productivity. In relation to solar energy driven $H_2$ production, the light conversion efficiency has been reported to be less during mid-day because of high light intensity (1.0 kW $m^{-2}$). In addition, a delay of 2–4 h has been observed in maximum hydrogen production rate (3.4 L $H_2$ ($m^2$ $h^{-1}$) after the highest light intensity at noon with an average light conversion efficiency of 1.4% (El-Shishtawy et al. 1997). A 3.5% light conversion efficiency with an over 0.8 kW $m^{-2}$ light intensity at midday has been obtained using a photo-bioreactor system with light shade bands, whereas photo-inhibition has been observed at 0.4 kW $m^{-2}$ in photo-bioreactors without shade bands Miyake et al. 1999).

Mixing of reactor content is the other important factor affecting $H_2$ production. Some literature reports suggest gas injection using argon gas for mixing, although not cost-effective. On the other hand, it is also known that continuous argon sparging may inhibit the growth of *Rhodopseudomonas* in a pneumatically agitated photo-bioreactor. Re-circulation of reactor content was also a choice for mixing which provides better growth of the culture. A novel flat-panel airlift photo-bioreactor with baffles has provided a significant increase in the biomass productivity and therefore it could also be used for $H_2$ production (Wakayama et al. 2000).

Multi-tubular photo-bioreactors made up of parallel transparent tubes are another preferred reactor configuration, generally used for the cultivation of *Spirulina*. The system is inclined with a 10–30% slope to allow gas bubbles to rise. The hydrogen production rate from lactate using a modified tubular reactor reaches 2 L $m^{-2}$ $h^{-1}$ with light conversion efficiency of 2% in outdoor experiments (Modigell and Holle 1998).

## *4.4 Biohydrogen Production by Hybrid Systems*

Although the theoretical maximum yield of $H_2$ from a single dark fermentation reaction is limited to 4 mol $H_2$ $mol^{-1}$ glucose, yields higher than 4 mol $H_2$ $mol^{-1}$ glucose can be achieved through hybrid systems. Hybrid hydrogen production system is composed of two sequential reactors in which dark fermentation is carried out in the first reactor and then a second photosynthetic reactor is integrated where hydrogen atoms sequestered in low molecular weight VFAs are converted to $H_2$ via photosynthetic organisms. This is required since hydrogen production is possible up to a certain extent in dark fermentation and significant amount of $H_2$ is sequestered

in the form of volatile fatty acids (VFAs) such as acetic acid, butyric acid, propionic acid etc. This type of hydrogen production system strategy includes dark fermentation followed by photo-fermentation or dark fermentation followed by a microbial electrohydrolysis cell (MEC), which is also referred to as 'electrohydrogenesis'. Thermodynamic constraints limit the release of the hydrogen atoms bound up in fermentation end-products by dark fermentation, so integration of dark fermentation with photosynthetic bacteria is needed for the maximization of $H_2$ yield.

The combined use of anaerobic bacteria and purple non-sulfur photosynthetic bacteria for efficient conversion of wastewater into $H_2$ using effluents from three different carbohydrate-fed reactors (CSTR, ASBR, and UASB) has been reported by Lee et al. (2002). The authors report that CSTR effluent is the most suitable for photohydrogen production. Azbar and Dokgoz (2010) have reported the use of a two-stage reactor to maximize the $H_2$ yield from cheese whey wastewater. For this purpose, effluent from a thermophilic anaerobic digester fed with cheese whey has been used in photo-fermentation reactors using *Rhodopseudomonas palustris* strain DSM 127. In this study, overall $H_2$ production yield (for dark + photo fermentation) has been found to vary between 2 and 10 mol $H_2$ $mol^{-1}$ lactose. It is suggested that cheese whey effluent with a co-substrate containing L-malic acid, such as apple juice processing effluents could provide successful hydrogen production.

A hybrid hydrogen production system employing dark-fermentation process followed by a photo-fermentation process has been used by Lo et al. (2008) for hydrogen production from acid-hydrolyzed wheat starch. The effluent from dark fermentation reactor in which hydrolyzed starch was continuously converted to $H_2$ by *Clostridium butyricum* CGS2, was fed into photo $H_2$ production process inoculated with *Rhodopseudomonas palustris* WP3–5 (ToC = 35 °C, pH 7.0, light 100 W $m^{-2}$ irradiation). Combining enzymatic hydrolysis, dark fermentation and photo fermentation has led to a marked improvement of overall $H_2$ yield, up to 16.1 mmol $H_2$ $g^{-1}$ COD or 3.09 mol $H_2$ $mol^{-1}$ glucose, and COD removal efficiency (ca. 54.3 %), suggesting the potential of using the proposed integrated process for efficient and high-yield bio-$H_2$ production from starch feedstock. Similar experiments have been conducted using *Enterobacter cloacae* DM11 in the first stage, followed by photo-fermentation by *Rhodobacter sphaeroides* strain OU001 (Nath et al. 2008). The yield of $H_2$ in the first stage has been approx. 3.3 mol $H_2$ $mol^{-1}$ glucose (approx. 82 % of theoretical), while the yield of $H_2$ in the second stage is between 1.5–1.7 mol $H_2$ $mol^{-1}$ acetic acid (37–43 % of theoretical). The combined yield of $H_2$ in the two-stage process is 4.8–5.0 mol $H_2$ $mol^{-1}$ substrate, significantly higher than the 3.3 mol $H_2$ $mol^{-1}$ glucose obtained in the dark fermentation alone.

## 5 Conclusions

$H_2$ fuel is clearly a promising solution for energy security as a sustainable alternative energy carrier and also a reliable choice against climate change. Biotechnology seems to provide much more environmentally friendly alternative $H_2$ production in

comparison to the conventional hydrogen production methods (thermo-chemical and electro-chemical), which are dependent on fossil fuels or highly energy intensive. Numerous reports show that each biohydrogen process has its advantages and disadvantages in terms of technology and productivity. In order to compete with aforementioned conventional hydrogen production methods, there is a need for intensive work on both enhancement of biohydrogen yield and energy efficiencies of the respective processes. It is particularly imperative to address several techno-economic challenges for cost-effective production as well as commercial application of biohydrogen.

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# Chapter 12
# Evaluation of *Senecio glaucus* L. and its Root-Associated Fungi for Bioremediation of Crude Oil Polluted Soils

**Fariba Mohsenzadeh and Abdolkarim Chehregani Rad**

**Abstract** Environmental pollution with crude oil is a common disaster in many countries. Bioremediation of petroleum contamination in soils is based on the stimulation of petroleum hydrocarbon-degrading fungal and microbial communities. Earlier researches have shown that there are some petroleum-resistant plants and their root associated fungal strains were grown in oil-polluted soils. *Senecio glaucus* (Asteraceae) is one of the plants that was collected from the crude oil-polluted sites of Abadan refinery in Iran. The root-associated fungi of this species were determined and results showed the presence of six species associated with the roots of the plants growing in the polluted areas, but only three of them were found in non-polluted soils. The fungi were cultured in oil-contaminated media and results showed that all the studied fungi were resistant to low oil pollution (1 % w/w) and a few species, especially *Fusarium* species, showed higher resistance to oil pollution (10 % w/w) and it seems that they may be suitable for bioremediation in highly polluted areas. Bioremediation tests with *Senecioa glaucus*, with and without fungal strains, showed that application of both plant and its root-associated fungal strains was more effective than application of plant and fungi individually. Results indicated that fungal strains had the main role in bioremediation of crude oil-polluted soils but plant root exudates lead to an enhancement of the process.

**Keywords** Bioremediation · Petroleum pollution · Root-associated fungi · *Senecioa glaucus*

**Abbreviations**

| | |
|---|---|
| PDA | Potato dextrose agar |
| TOG | Total oil and grease |

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## 1 Introduction

Soil pollution with crude oil is a global disaster. It is a common phenomenon in oil bearing and industrial countries (Merkel et al. 2004a, b). There are several soil cleaning methods including burning, washing, chemical and physical treatments and also bioremediation (Garcia et al. 2000). Bioremediation is use of plants, microorganisms or both to remove or detoxify environmental contaminants. This phenomenon has been intensively studied during the past two decades, because of the need for a low-cost, *in-situ* alternative to more expensive technologies (Merkel et al. 2004a, b; Chehregani and Malayeri 2007; Chehregani et al. 2008). In petroleum-polluted conditions, applying plants or microorganisms or their combinations can help to convert hydrocarbons to non-toxic forms (Cunningham et al. 1996). Bioremediation has been applied for soil cleaning from crude oil (Wiltse et al. 1998; Radwan et al. 1998; Merkel et al. 2005), motor oil (Dominguez-Rosado and Pichtel 2004), and diesel fuel pollution (Chaineau et al. 2000), but the removal efficiency is highly variable and is related to applied organism potency and environmental conditions (Angehrn et al. 1998). Since bioremediation of crude oil-contaminated soils is mainly due to biodegradation by the microbial populations that are associated with the rhizosphere of plants, or are associated and attached with roots, the root system of the plants play an important role (Frick et al. 1999; Mohsenzadeh et al. 2009). Plants can influence degradation of oil indirectly by changing the physical and chemical conditions of the soil (Cunningham et al. 1996). Roots exude organic and inorganic substances to its surroundings during normal metabolism, which act as nutrients for soil microorganisms, thus increasing the degradation of toxic organic chemicals (Anderson et al. 1993).

It has been shown that some tropical grasses and legumes are also resistant to crude oil pollution and root surface increases in some graminoid plants including *Brachiaria brizantha*, *Cyperus aggregatus* and *Eleusine indica* under polluted soil condition (Merkel et al. 2005).

The microorganisms are economically and environmentally important because they help in the remediation and rehabilitation of oil-polluted soils (Yateem et al. 1997; Eggen and Majcherczykb 1998; Nicolotti and Egli 1998, Obuekwe et al. 2005; Dritsa et al. 2007; Friedrich et al. 2007). Some researches have shown that some fungal species are resistant to petroleum pollutants and they are capable of removing soil pollutants. The keratinolytic fungi, especially *Trichophyton ajelloi* have been shown as a potential tool for assessment of soil petroleum hydrocarbon pollution and associated bioremediation progress (Ulfig et al. 2003). Some fungal strains namely *Alternaria alternate*, *Aspergillus flavus*, *Curvularia lunata*, *Fusarium solani*, *Mucor racemosum*, *Penicillium notatum* and *Ulocladium atrum* were isolated from the soils in the oil- polluted areas in Saudi Arabia (Hashem 2007). Eggen and Majcherczykb (1998) showed that white rot fungus, *Pleurotus ostreatus,* could remove polycyclic aromatic hydrocarbons (PAH) in polluted soil (Eggen and Majcherczykb 1998). Little attention has been paid to the role of plant root associated fungal species in the environmental biotechnology and bioremediation of petroleum pollution, especially in Middle East region (Yateem et al. 1999; Hashem 2007).

Numerous sites are contaminated worldwide with crude or refined oil in different countries and also in Iran. Since in different areas, various ecological conditions with different climates are present, we need to apply native plants or microorganisms for bioremediation of newly subjected areas. The aim of this paper is to evaluate the ability of *Senecioa glaucus* and its root-associated fungal species for the remediation of crude oil-polluted soils.

## 2 Materials and Methods

*Senecio glaucus* L. was collected from the Abadan oil refinery, located near the coast of the Persian Gulf. It was completed in 1912 and was one of world's largest oil refineries before it was largely destroyed in 1980. The refinery had a capacity of 635,000 b $day^{-1}$ in 1980 and formed a refinery complex with important petrochemical plants. Its capacity has increased steadily from 1988 onwards and is now listed as 429,000 barrels per day (68,200 $m^3$ $day^{-1}$) crude oil.

Soil characters of the refinery were evaluated as sandy loam containing 85% sand, 10% loam, 4% sludge and 1% organic material, with pH 6.1. The crude oil used in the refinery contains 14.6% saturated hydrocarbons, 39% aromatic hydrocarbons, 44.4% polar compounds (Refinery office data). The refining activities in this region lead to a high degree of oil pollution in some areas. Identification of soil pollution was done visually as well as through experimental assays. *S. glaucus* was collected from the oil polluted areas in the refinery. The taxonomical identity was carried out in Bu-Ali Sina University.

Plant root samples with 1 cm length were harvested and dried at room temperature after washing with distilled water. Samples were incubated in 1% sodium hypochloride followed by ethanol 70% (3 s), for removing the peripherally attached microorganisms. These were washed with distilled water (three times) and dried using sterile paper (Mohsenzade et al. 2009). The samples were kept in petri dishes containing sterile PDA media, incubated in an incubator at 25 ± 2 °C for 3 days. Different fungal colonies were isolated and transferred to PDA media. Fungal colonies and their mycelia were examined under light microscope after preparations and were identified using morphological characters and taxonomical keys (Nelson et al. 1983; Gilman 1998; Watanabe 2002). The root-associated fungi for the plants were collected from the oil-polluted area and compared with the non-polluted ones to find out oil resistant fungal species.

The growth assay was done to find the fungal species resistant to oil contamination of the soil by comparing the growth rates of fungal strains, as colony diameter, on the oil contaminated and control Petri dishes. Test dishes were prepared by adding different concentrations of crude oil (1%, 4%, 10%) to warm sterile PDA and then left on a shaker. In order to have a uniform concentration of oil in all plates, the solution was thoroughly mixed with a magnetic stirrer, right before it was added to the plates. Three concentrations of Oil/PDA mixture (1%, 4%, and 10% w/w) were prepared. Pure PDA was used in the control plates. All dishes were inoculated

with 4 mm diameter plugs of the isolated fungal mycelia taken from agar inoculums plate. The dishes were incubated at 25 ± 2 °C in an incubator. Fungal mycelia on the plates (colony diameter) were measured using a measuring tape after 4 days and compared with the control plates (Mohsenzade et al. 2009).

*S. glaucus* belonging to the family Asteraceae is a common plant on the oil polluted sites in Abadan area, with a higher frequency than others, especially in the central region of the oil-polluted areas. It is petroleum resistant, therefore its root-associated fungi were chosen for this investigation. 80 pots were prepared and filled with 2 kg sterile garden soil. These were divided in to 16 groups (depending on the fungi isolated). Each group included five pots. The groups were containing following agents separately as follows:

(1) Sterile soil, (2) Sterile soil + Plant, (3) Sterile soil + *Altenaria,* (4) Sterile soil + Plant + *Altenaria,* (5) Sterile soil + *Fusarium acuminatum,* (6) Sterile soil + plant + *F. acuminatum,* (7) Sterile soil + *F. equiseti,* (8) Sterile soil + plant + *F. equiseti*, (9) Sterile soil + *F. reticulatum,* (10) Sterile soil + Plant + *F. reticulatum,* (11) Sterile soil + *penicilinium,* (12) Sterile soil + Plant + *Penicilinium,* (13) Sterile soil + *Rhizoctonia,* (14) Sterile soil + Plant + *Rhizoctonia,* (15) Sterile soil + all above mentioned fungi, (16) Sterile soil + Plant + all above mentioned fungi.

In the group with plants each pot had two seedlings. Crude oil was applied to all pots, at a pollution level 5 % w/w. The pots were kept under same conditions in greenhouse at Bu-Ali Sina University. *S. glaucus* plants were removed after 3 months at the end of the growing period. The soil of experimental and control pots was homogenized separately and stored at 4 °C in the refrigerator until further analysis. At the end of experiment concentrations of oil were determined in the soil of experimental and control pots (after 3 months) and decrease in the oil was compared in the control and experimental pots.

The soil samples in the pots after 3 months were collected separately. Each soil sample, without plant segments, was homogenized and stored at 4 °C until further processing. TOG was analyzed according to the EPA method 9071A and EPA Method 3540B (USEPA 1994). Fifteen grams of each soil sample were acidified with hydrochloric acid to pH 2 in two replicates and dehydrated with magnesium sulphate monohydrate. After 15 min, samples were transferred to paper extraction thimbles and placed in a Soxhlet type apparatus. TOG was extracted with dichloromethane for 8 h and filtered through filter paper (Whatman No. 4) with 1 g sodium sulphate. The solvent was evaporated with a rotary evaporator and the weight of dry extract was determined. Percentage of TOG was calculated based on dry weight of soil and its decrease was compared in the control and experimental pots.

The results were subjected to statistical analysis of variance (ANOVA) followed by the least significant difference test (LSD) that was performed between control and experimental groups (Chehregani et al. 2005). Each data was represented as the means ± SD of five samples for experimental and control groups.

**Table 12.1** Comparison of root-associated fungal species in *Senecioa glaucus* plants growing in oil polluted and non-polluted areas

| Fungi in petroleum polluted area | Fungi in non-polluted area |
|---|---|
| *Alternaria, Fusarium acuminatum, F. equisetti, F. reticulatum, Penicillinium, Rhizoctonia* | *Alternaria, Penicillinium, Rhizoctonia* |

**Table 12.2** Growth behavior of isolated fungi in the PDA media containing different concentrations of crude oil (Data expressed as diameter of colony—mm)

| Experimetal groups microorganism | Non-contaminated (control) | 1% oil | 4% oil | 10% oil |
|---|---|---|---|---|
| Alternaria | 49±6 | *28±8 | *18±6 | *14±4 |
| Fusarium acuminatum | 34±5 | 42±10.3 | 50±10 | 48±12 |
| F. equiseti | 12±2 | *46±4 | *63±4 | *85±8 |
| F. reticulatum | 33±9 | 45±6 | 61±7 | 55±5 |
| Penicillinium | 40±5 | 30±4 | 24±4 | 11±2 |
| Rhizoctonia | 88±1.2 | *67±9 | *42±3 | *18±5 |

Each value represents the mean±SE of 3–5 samples
* Data significantly different from the control ($p \leq 0.05$)

## 3 Results

The root-associated fungi were collected from *S. glaucus*, isolated and identified by morphological characters and taxonomical keys (Table 12.1). The results of identification of plants root associated fungi showed the presence of six fungal species in the roots of the plant in oil-polluted soils namely; *Alternaria sp., Fusarium acuminatum, F. equisetti, F. reticulatum, Penicillinium sp., Rhizoctonia sp.,* but three of them *Alternaria* sp*., Penicillinium* sp*., Rhizoctonia* sp. were found to be associated with the roots of the plant in non-polluted soils as well. The plant species used here had different fungal species as its root-associated fungi in the polluted and non-polluted areas (Table 12.1).

The growth activity of the isolated fungal strains was carried out under different concentrations of crude oil and was expressed as the diameter of the colony (Table 12.2). The results showed that all studied fungi were resistant to petroleum pollution and they made sufficient colonies in 1% crude oil concentration; meanwhile, only some of them continue their growth activity at 10% petroleum pollution. Among the fungi determined here, *Fusarium equiseti, F. reticulatum* and *F.cuminatum* had the highest resistance to crude oil pollution (with 48 and 55 mm diameter of colonies) and *Penicillinium sp.* was the most sensitive one (with 11 mm diameter of colony) in the 10% crude oil polluted PDA.

*S. glaucus* was chosen as a resistant plant to crude oil pollution for this study because it is a common plant in the polluted area and grows successfully on polluted soils. It is a perennial herb which can propagate by means of seeds and underground gemma. After 3 months bioremediation using seed growing plants and their root-associated fungal strains, decrease of concentrations in crude oil were determined

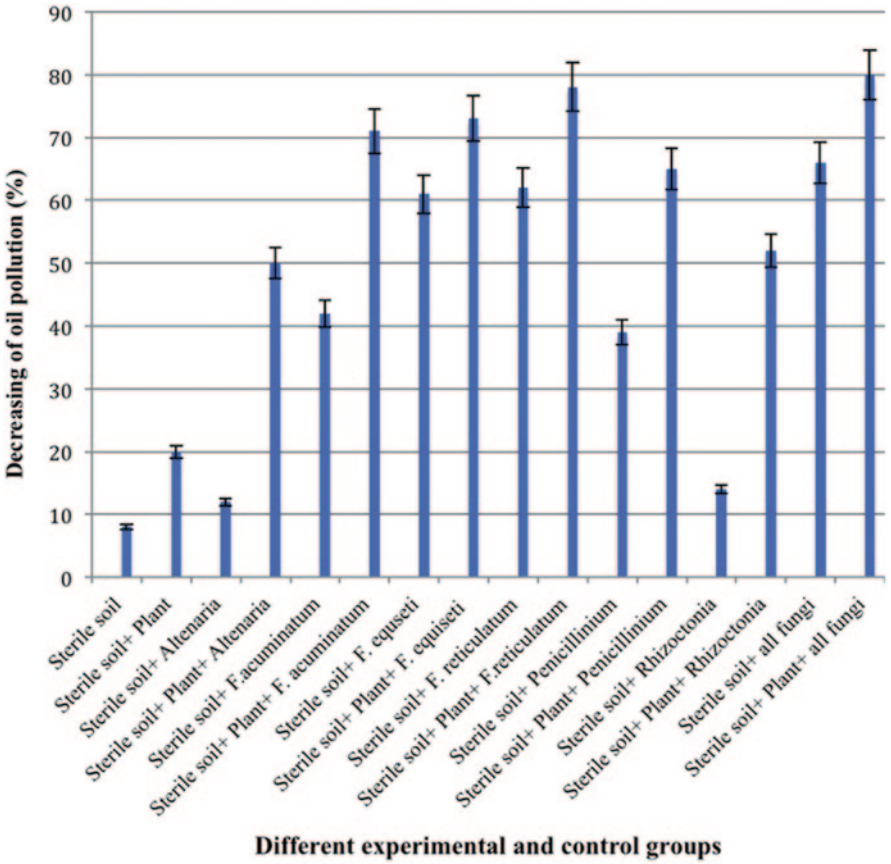


**Fig. 12.1** Decrease of crude oil concentration (%) in the polluted soils after bioremediation by *Senecio glaucus* and its root-associated fungal strains. All pots were subjected to 5% w/w petroleum pollution at the beginning of experiment. Data indicated that amounts of petroleum pollution decreased due to bioremediation. Decrease in petroleum in control pots is the result of evaporation. Decrease of pollution in the experimental pots is significantly different from the control ($P \leq 0.01$). Each data represent the means ± SE of five samples

in the soil of experimental pots and compared with control ones. The data showed that the concentration of petroleum pollution decreased considerably in the all experimental pots except in the control one (Fig. 12.1). The data showed that decrease in the experimental pots containing plant together with all fungal strains was more than other groups (up to 80%). Meanwhile, the decrease of crude oil pollution was also considerable in the pots containing plant with *Fusarium equiseti* and *F. reticulatum* added (up to 74% and 78%). The data showed all fungal species were capable of decreasing petroleum pollution (Fig. 12.1), but they were more effective when applied as associates with the plant. *Altenaria sp.* cause decrease up to 14% but when applied with plant, pollution was decreased up to 50% and also *Rhizoctonia* reduced soil pollution up to 14% solely and when applied with plant, decrease went up to 52%.

## 4 Discussion

The soil pollution with crude oil and its derivers is a major environmental problem in many countries (Klokk 1984). Serious risks can occur to the public health and environment when soil is polluted by crude oil (Nicolotti and Egli 1998). Results of this investigation showed that crude oil, in the concentrations up to 10 % cannot kill this plant species. These results are in accordance with those of an earlier study. Merkel et al. (2005) showed that several legumes and graminoids were found on the oil-polluted soils with about 5 % pollution. He reported that an indirect effect of crude oil in the soil is confined to a more or less marked reduction in plant growth and biomass Merkel et al. (2004a, b). Some other reports too showed the same results about other plant species like *Festuca rubra, Puccinellia maritime* (Baker 1999), *Trifolium rubra* (Klokk 1984), and different legumes and grasses (Nicolotti and Egli 1998).

Study on fungal species has revealed that *Alternaria, Penicillium* and *Rhizoctonia* were the common fungi that have been observed in the roots of *S. glaucus* plants, both in the polluted and non-polluted soils; but in the oil-polluted soils three additional fungal strains (*Fusarium acuminatum, F. equisetti, F. reticulatum*) were observed. Our investigations enlightened the fact that fungal variation in petroleum-polluted area was more than the non-polluted (Table 12.1). This means that roots of the plant species had more fungi yielded in the polluted areas than non-polluted ones. This goes in line with the finding of some prior researches (Anderson et al. 1993; Hashem 2007). It seems that the fungal species use oil compounds as nutrients and crude oil pollution causes an increase in the fungal variation in the oil-polluted soils. Similar findings have been reported by other researchers (Yateem et al. 1997; Eggen and Majcherczykb 1998; Nicolotti and Egli 1998; Obuekwe et al. 2005; Dritsa et al. 2007).

*In vitro* growth test of the fungi in the PDA media containing different concentrations of crude oil showed a species-specific response. Most of the studied fungal strains were able to grow in 1 % w/v oil pollution and therefore should be considered for a possible use for the remediation of light soil pollution. Some fungal species were inhibited by high oil concentrations (10 % w/w). These species included: *Alternaria*, *Penicillinium* and *Rhizoctonia* while others actually grew very well in oil-contaminated media, even at very high concentrations. These fungal species are; *Fusarium acuminatum, F. equiseti,* and *F. reticulatum*. It seems that crude oil could supply essential nutrients for these fungi and they are more effective in oil degradation. Our results are in accordance with the finding of other researchers about other different fungal species (Yateem et al. 1997; Eggen and Majcherczykb 1998; Nicolotti and Egli 1998; Obuekwe et al. 2005; Dritsa et al. 2007).

Bioremediation of oil-contaminated soil is mainly based on biodegradation in the rhizosphere and the root-associated fungi are one of the most important factors in this process (Frick et al. 1999). The results of our study propose the above-mentioned fungi for the future remediation tests and this is the first report about their remediation capacity. It means that the data of this study indicated that isolated strains of *Fusarium acuminatum, F. equseti* and *F. reticulatum* may have the potential for bioremediation of crude oil in highly polluted conditions (up to 10 % pollution).

*S. glaucus* as one of the abundant plants distributed on the polluted areas. It was therefore chosen for bioremediation test together with its root associated fungi, their ability to remove crude oil from polluted soils has been evaluated. Highest decrease of crude oil concentration has been observed in the pots containing plant added *Fusarium equiseti, F. acuminatum* and *F. reticulatum* (Fig. 12.1). Although all subjected fungal strains decreased petroleum concentration in soils but application of plant together with its associated fungal strains was more effective (Fig. 12.1). It means that plant root exudates increase the petroleum biodegradation driven by fungal strains that is in confirmity with earlier reports (Garcia et al. 2000; Mohsenzadeh et al. 2005; Mohsenzade et al. 2009). Phytoremediation of petroleum pollution is a cost-effective green technology; there are more advantages, when it comes to the use of native plants and fungi (Schroder et al. 2002). This is the first report on the ability of *S. glaucus* and its root-associated fungi for remediation of petroleum polluted soils.

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# Chapter 13
# Root and Shoot Peroxidase Activity in *Festuca arundinacea* in Light Oil-Contaminated Soil

**Zahra Ghaffari, Sahar Shademan, Zahra Sobhani-Damavandifar and Dariush Minai-Tehrani**

**Abstract** Peroxidases are a group of enzymes that occur especially in plant cells. They are classified as oxido-reductases and are given the official EC number 1.11.1. For many of these enzymes the optimal substrate is hydrogen peroxide, but others are more active with organic hydroperoxides such as lipid peroxides. Peroxidases can contain a heme cofactor in their active sites, or redox-active cysteine or selenocysteine residues. Toxic molecules such as superoxide and hydroxide radicals can be found in cells due to the presence of oxygen. These are byproducts of aerobic respiration. They are eliminated by a number of enzymes present inside the cell such as peroxidases. Some oil-producing countries may encounter the risk of soil pollution by oil, during transportation, extraction and refining of crude oil. Oil-contaminated soil can be hazardous to plants and soil microorganisms. Among the plants, grasses such as *Festuca arundinacea* (Tall fescue) and legumes have high potential on removal of oil from contaminated soil. In the process of phytoremediation of crude oil, some morphological, enzymatic and physiological changes were observed in plants. In this study, the effect of light crude oil (5 % v/w) in soil on the activity of peroxidase was studied and compared with control. Our results showed that in both roots and shoots, the $K_m$ and $V_{max}$ of the enzyme were changed. The contaminated soil caused delay of germination and chlorosis in plants. In the contaminated soil, the $K_m$ of root peroxidase was determined to be about 55.5 μM while it was 91 μM in control. The $V_{max}$ of root's enzyme was 2 and 6 nmol/mg protein/min in contaminated soil and control, respectively. The $K_m$ of enzyme in shoots was determined to be about 36 and 42 μM in contaminated soil and control, respectively, while the $V_{max}$ in control was about 1.4 nmol/mg protein/min and it decreased to

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1 nmol/mg protein/min in contaminated soil. The specific activity of enzyme in root control was $5.3 \times 10^{-3}$ U/mg protein while it was $3.5 \times 10^{-3}$ U/mg protein in contaminated roots. The specific activity of enzyme in shoots was $1.8 \times 10^{-3}$ and $1.7 \times 10^{-3}$ U/mg protein in control and contaminated soil respectively. Our results propose that in the root grown in contaminated soil, the plant uses peroxidase isoform in comparison with control roots, while in the shoots the same peroxidase was used in the plant in both contaminated and control.

**Keywords** *Festuca arundinacea* · Light crude oil · Peroxidase · Enzyme · Pollution

## 1 Introduction

Peroxidases are a group of enzymes that catalyze oxidation-reduction reactions. As such, they are classified as oxido-reductases. They are a group of enzymes that occur especially in plant cells and catalyze the oxidation of a substance by peroxide. Peroxidase can be used for treatment of industrial waste waters. For example, phenols, which are important pollutants, can be removed by enzyme-catalyzed polymerization using horseradish peroxidase. Thus, phenols are oxidized to phenoxy radicals, which participate in reactions producing polymers and oligomers that are less toxic than phenols. A number of reviews have addressed the structural and catalytic properties of the various peroxidase isoforms in general (Hofmann et al. 2002; Wood et al. 2003) and specifically in plants (Dietz et al. 2002). Experimental evidence exists for a triple peroxidase function in plant cell biology as (1) antioxidant, (2) modulator of cell signaling pathways, and (3) redox sensor. In 1996, cDNA sequences encoding a barley 1-Cys peroxidase (Stacy et al. 1996) and a 2-Cys peroxidase were published and identified as peroxidase. Contamination of soil by crude oil and its byproducts cause changes in physico-chemical and biological properties of soil. Depending on chemical composition and concentration, the oil influences on soil enzymatic and microbiological activity. Crude oil and its by-products are widely used by human for various reasons, including home heating and fueling of vehicles. During the past century, the use of petroleum products increased; this has resulted in the contamination of soil and water by petroleum and its by-products. The spilling of crude oil into the soil causes damages to the environment and changes the biological and physico-chemical properties of the soil. Some petroleum components are toxic for living organisms, however, some plants and microorganisms are able to biodegrade the crude oil hydrocarbons into products less toxic than the parent compounds (Cerniglia 1992). In this study, the effect of oil-contaminated soil on the activity of peroxidase was studied and compared with control (non-contaminated soil).

## 2 Materials and Methods

Light crude oil (API gravity = 40) was obtained from an oil processing factory of Sarkan in the west of Iran and added to the dry soil with concentrations of 1–5 % (w/w). The soil and the oil were mixed to make homogenized contaminated soil and transferred to 1 l pots. Each sample consisted of 500 g of dry soil. Animal manure was added to the soil as fertilizer before seeding. Fifty seeds of Tall Fescue were planted in the soil. The control sample was also prepared as above but did not receive oil. After 2 months, the plants were removed from the soil and the roots washed with water to dislodge excess soil adhering to the roots. Roots and shoots were separated and kept frozen at −10 °C for further experiments. One gram of roots or shoots was homogenized in 10 ml of NaCl (0.15 M) by a homogenizer to destroy the cells. The homogenized solution was centrifuged (3000 × *g* for 10 min) to remove unbroken cells. The supernatant was used as cell free extract. The enzyme assay was performed in 0.1 M phosphate buffer (pH 7.0). The reaction was started by adding 100 µl of guaiacol solution to the test tubes containing 100 µl of enzyme solution (cell free extract) and 100 µl of different concentrations of $H_2O_2$ as substrate. The final volume was always 2 ml. The colorimetric change of guaiacol to tetraguaicol was detected during 10 min using Perkin-Elmer visible spectrophotometer. Results were the average of at least two separate experiments and expressed as mean ± Standard deviation (± SD).

## 3 Results

Figure 13.1 shows the number of seeds germinated in the vegetated samples 30 after planting. In 0 % sample, the number of germinated seeds was higher than in the other samples while it was lower in 5 % sample. However the difference number of germinated seeds between the control and 5 % sample was not significant. The dry biomass of roots and shoots was measured at the end of the experiment (Fig. 13.1). The separation of roots from the soil showed that the distribution of roots in the soil has decreased by increasing the crude oil concentration. The higher root biomass was observed in 0 % sample, in which the roots were well distributed in the soil. The lower root biomass was observed in 5 % sample. The total dry biomass (roots + shoots) was also high in 0 %, while it was low in 3 and 5 % samples. A sudden decrease in total dry biomass was observed in 1 % sample in comparison with that in the control (0 %) sample and the difference was significant between control and all of contaminated samples (Fig. 13.1). The length of leaves decreased by increasing crude oil concentration; the shorter leaves were observed in 5 % sample, while the tallest in 0 % sample (Fig. 13.2). The number of green plants at the end of experiment was lower in 5 %, while the higher was observed in 0 % followed by 1 % samples (Fig. 13.2).

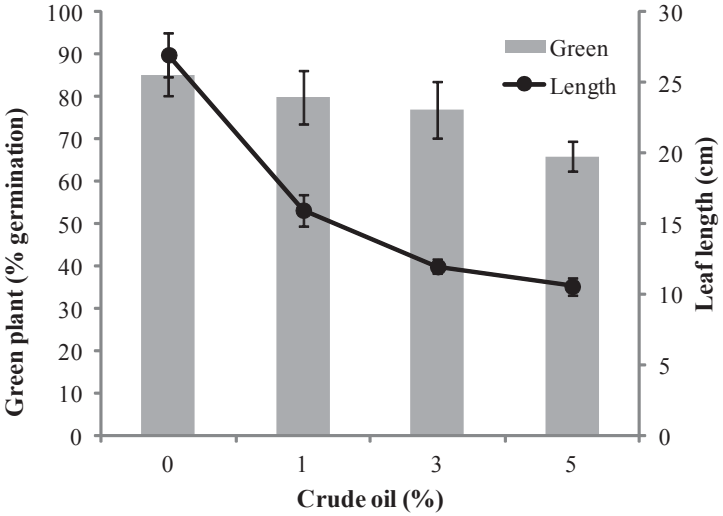


**Fig. 13.1** Number of germinated seeds in different concentrations of crude oil after 30 days of seeding and total dry biomass (shoots + roots) and dry biomass of roots after 120 days of planting. Average values ± standard deviation (±SD), $p < 0.05$

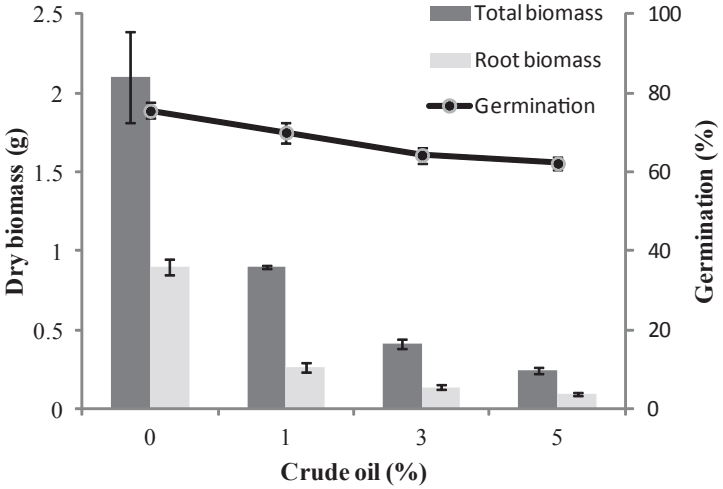


**Fig. 13.2** Length of leaves measured 60 days after planting; Green plants were counted at the end of experiment (120 days)

**Peroxidase Activity** The peroxidase activity was determined in both roots and shoots of *Festuca arundinacea* which was in contact of crude oil-contaminated soil (the concentration of oil in soil was 5% w/w) and control (non-polluted soil). Figures 13.3 and 13.4 show the reciprocal of velocity (1/V) against the reciprocal of the substrate ($H_2O_2$) concentration (1/(S)) in shoots and roots. In shoots, the $K_m$ of enzyme was determined to be 36 and 42 μM in contaminated soil and control,

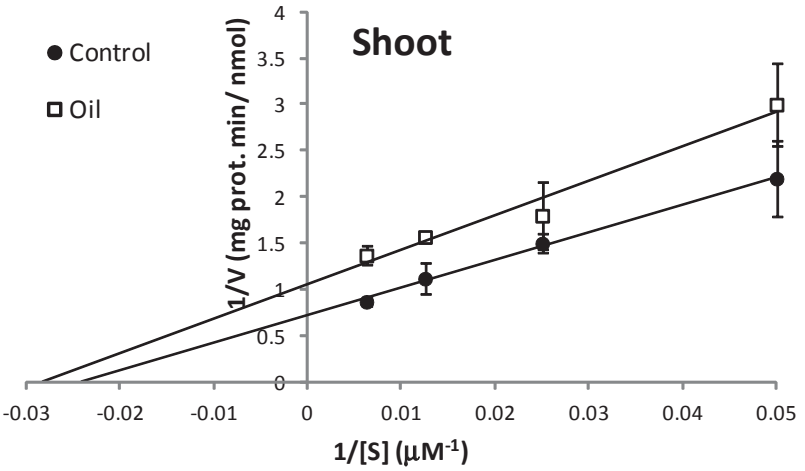


**Fig. 13.3** Determination of $V_{max}$ and $K_m$ of peroxidase in the shoots of plants grown in oil-contaminated and control soil samples

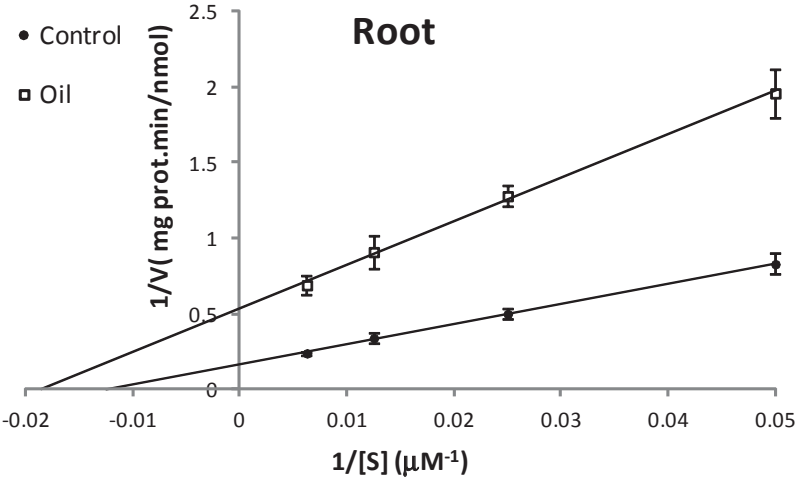


**Fig. 13.4** The line-weaver plot of the enzyme in roots of contaminated and control samples

respectively (Fig. 13.3), while in the control sample, the $V_{max}$ of the enzyme was about 1.4 nmol/mg protein/min and it decreased to 1 nmol/mg protein/min in contaminated soil. In the contaminated soil, the $K_m$ of root peroxidase was determined to be about 55.5 μM while it was 91 μM in control. The $V_{max}$ of root's enzyme was 2 and 6 nmol/mg protein/min in contaminated soil and control, respectively (Fig. 13.4). The specific activity of the enzyme in root control was $5.3 \times 10^{-3}$ U/mg protein, while it was $3.5 \times 10^{-3}$ U/mg protein in contaminated roots. The specific activity of the enzyme in shoots was $1.8 \times 10^{-3}$ and $1.7 \times 10^{-3}$ U/mg protein in control and contaminated soil, respectively.

## 4 Discussion

Our results showed that crude oil either in low (1%) or high (5%) concentration could reduce the number of germination, the length of leaves and the plant dry biomass in both roots and shoots. Increasing the crude oil concentration increased the changes in the plant. Maximum effect of crude oil on the plant was observed at 5% concentration. The poor distribution of roots in 5% contaminated soil proposed that the pollution could affect the root growth which was in direct contact with toxic compound of oil. The presence of crude oil in the soil delayed the germination and caused chlorosis in plants (Gong et al. 2001; Adam and Duncan 2002; Ogboghodo et al. 2004). The germination was observed in all the oil-contaminated soils suggesting that the plant could tolerate 5% contamination in the soil. Tolerance is defined here as the ability of the plant to grow in oil-contaminated soil. This does not necessarily means that the plant is healthy. Some reports showed that diesel fuel could highly decrease *Poa trivialis* germination in the 5% concentration (Adam and Duncan 1999, 2002). The other report indicated that *Poa trivialis* can tolerate up to 15% crude oil in the soil (Minai-Tehrani 2008).

In this study, peroxidase enzyme was chosen for studying the effect of oil-contaminated soil on enzyme activity in plant. Determination of peroxidase activity may be a good index of study of environmental stresses on plants (Fan and Krishnamurthy 1995; Riser-Roberts 2010). The activity of this enzyme changes in the oxidative stress conditions (Heinonsalo et al. 2000; Bučková et al. 2010; Kathi and Khan 2011). Our results showed that the kinetics parameters ($K_m$ and $V_{max}$) of enzyme in roots of treated samples were different from control sample. This propose that in the root grown in contaminated soil, the plant uses peroxidase isoform in comparison with control roots, while in the shoots the same peroxidase was used in the plant in both contaminated and control. Previous reports also showed that crude oil can affect peroxidase activity in some plants such as lentil (Naemi et al. 2011; Masakorala et al. 2013).

## 5 Conclusion

In conclusion, crude oil-contaminated soil could delay germination, early chlorosis and reduce the length of roots and shoots of *Festuca arundinacea*. Crude oil can induce not only macroscopic changes but also biochemical changes in the root and shoots of plant. The activity of peroxidase in both roots and shoots of treated sample was changed. The enzyme activity change was more significant in the root than the shoots.

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