# INDEX

<table>
<thead>
<tr>
<th>CHAPTER NUMBER</th>
<th>CHAPTER NAME</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter-1</td>
<td>Microsporidian Parasite Impact on Humans Health</td>
<td>1-4</td>
</tr>
<tr>
<td>Chapter-2</td>
<td>Microbial Production of Nanoparticles and their Applications</td>
<td>5-26</td>
</tr>
<tr>
<td>Chapter-3</td>
<td>Diverse Groups of Isolated Bacteria for Biodesulfurization of Petroleum Products in Petroleum Refinery Industry</td>
<td>27-51</td>
</tr>
<tr>
<td>Chapter-4</td>
<td>Solid State Fermentation: A Source of Bioactive Molecule Production</td>
<td>52-55</td>
</tr>
<tr>
<td>Chapter-5</td>
<td>Enzymatic Degradation of Lignocellulosic Biomass: A Brief Update</td>
<td>56-69</td>
</tr>
<tr>
<td>Chapter-6</td>
<td>The Emerging Prospects of Global Anti Microbial Resistance: Pros and Cons</td>
<td>70-82</td>
</tr>
</tbody>
</table>

**Published in:** June 2018  
**Online Edition available at:** http://openaccessebooks.com/  
**Reprints request:** info@openaccessebooks.com  
**Copyright:** @ Corresponding Author
Microsporidian Parasite Impact on Humans Health

Dipti Kashyap*; Abrar Ahmad

1Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow, India

2Environmental Biotechnology Division, CSIR-IITR, Lucknow, India

*Corresponding to: Dipti Kashyap, Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow, India

Email: hdgen31@gmail.com

Abstract

Microsporidiosis is an emerging and opportunistic infection coupled with a broad range of clinical syndromes in humans. Microsporidian parasite infectivity has been identified in a wider range of human populations that includes persons with HIV infection, travellers, children, organ transplant recipients, and the elderly. Human immunodeficiency virus-positive patient with chronic diarrhoea, anorexia, and lethargy revealed the presence of numerous refractile bodies resembling microsporidian spores. Questions still exist about whether Microsporidia infections remain unrelenting in immune-competent individuals, re-activate during conditions of immune compromise, or may be transmitted to others at risk, such as during pregnancy or through organ donation. Therefore, this book chapter highlights the research on microsporidiosis in humans.

Key Words: Microsporidiasis; Emerging; Opportunistic; Immunodeficiency; Immune-competent

1. Introduction

The term microsporidia refers to a group of obligate intracellular protozoan parasites belonging to the phylum Microspora. Their host range is extensive, including most invertebrates and all classes of vertebrates [1]. More than 100 microsporidial genera and almost 1,000 species have now been identified [2]. The first human case of sufficiently substantiated microsporidial infection was reported in 1959 [3]. Yet, as part of the budding deadly disease of HIV infection, microsporidia have gained attention as opportunistic pathogens. To date, five genera (Enterocytozoon spp., Encephalitozoon spp., Pleistophora spp., and Nosema spp.), as
well as unclassified microsporidial organisms (referred to by the collective term Microsporidium), have been associated with human disease, which appears to predominantly affect immunocompromised persons [4]. *Enterocytozoon bieneusi* has been well documented as a cause of chronic diarrhoea in human immunodeficiency virus-infected patients [5]. The potential sources and means of transmission of human microsporidial infections are uncertain. Preliminary connections have indicated that microsporidial species first identified in patients with AIDS will not be restricted to this patient group.

2. Taxonomy and Biological Characteristics of Microsporidian parasite

The Microsporidia are ancient eukaryotes lacking mitochondria, current data suggests that they are linked to the Fungi [6,7]. With the completion of the *E. cuniculi* genome, the phylogenetic relationship between microsporidia and fungi has been further solidified by the presence of numerous genes that on phylogenetic analysis cluster the Microsporidia with the Fungi [8]. The developmental stages preceding the spore are structurally simple cells, a thick wall consisting of an electron-dense proteinaceous exospore, an electron-lucent chitinous endospore layer, and a plasma membrane renders the spores environmentally resistant. The spore wall encloses the uni-or binucleate infective spore content (sporoplasm), an exceptional extrusion apparatus (the polar tubule) for injecting the sporoplasm into new host cells, a complex membrane system (termed lamellar polaroplast) surrounding the straight section of the polar tubule, some rough endoplasmic reticulum, and free ribosomes. The extrusion apparatus consists of a polar tubule that lies coiled inside the spore and is attached to an anchoring disk. The tubule is averted when triggered by appropriate environmental stimuli, e.g., small-intestinal fluid, and is capable of penetrating a host cell to inoculate the sporoplasm into the host cell cytoplasm [9,10]. The life cycle of microsporidia includes three distinct phases: first, the infective phase, i.e., the spore stage, stimulation of the spore necessary to trigger the extrusion of the polar tubule, and inoculation of infective spore content (termed sporoplasm) into a host cell; second, the proliferative vegetative phase, termed merogony (schizogony), during which the parasites multiply intracellularly; and third, the intracellular sporogony, during which infective spores are formed [11,12].

3. Epidemiology

The epidemiology of human microsporidiosis may differ according to host immune status and the infecting species of microsporidia [13,14]. With antigens obtained from cultures of murine-derived strains of *Encephalitozoon cuniculi*, serologic surveys for human antibodies to *Encephalitozoon cuniculi* performed in the 1980s suggested that travellers and residents in tropical countries may have increased exposure to this organism, but clinical correlation and definitive epidemiologic data were lacking [15,16]. In humans, different host-parasite interactions may be observed depending on the microsporidial species and the competence of
the immune response. In immunocompetent and otherwise healthy persons, acute intestinal, self-limiting microsporidiosis may occur [17], but systemic microsporidiosis has not been satisfactorily documented in a previously healthy person (Table 1).

**Table 1: Reports on Microsporidiosis infection in Humans**

<table>
<thead>
<tr>
<th>Microsporidial species</th>
<th>Detection of parasite</th>
<th>Clinical Manifestation</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nosema connori</em></td>
<td>Autopsy</td>
<td>Disseminated infection</td>
<td>Margileth et al., (1973) [18]</td>
</tr>
<tr>
<td><em>Pleistophora</em> spp.</td>
<td>Histological examination</td>
<td>Myositis</td>
<td>Ledford et al., (1985) [19]</td>
</tr>
<tr>
<td><em>Enterocytozoon</em> <em>bienueusi</em></td>
<td>Stool specimen</td>
<td>Diarrhea</td>
<td>Sandfort et al., (In press) [20]</td>
</tr>
<tr>
<td><em>Nosema</em> <em>corneum</em></td>
<td>Histological examination</td>
<td>Keratitis</td>
<td>Arison et al., (1966) [21]</td>
</tr>
<tr>
<td><em>Encephalitozoon</em> <em>cuniculi</em></td>
<td>Cerebrospinal fluid</td>
<td>Seizures</td>
<td>Matsubayashi et al,(1959) [3]</td>
</tr>
</tbody>
</table>

4. Conclusion

Microsporidia have also successfully adapted to the mammalian host. The long-term evolutionary host-parasite interactions have resulted in a "well-balanced" relationship and generally low pathogenicity of the parasite, manifesting in dormant or mildly symptomatic infection in mammals. Current data suggest that microsporidia are important pathogens capable of causing opportunistic infections in strictly immunodeficient HIV-infected patients. Seroepidemiologic surveys have provided confirmation for the occurrence of latent microsporidial infection in healthy persons. Human microsporidiosis appears to be adventitious and chiefly related with an increasing centre of population of immune-deficient individuals. Altogether, a strong confirmation exists for an increasing commonness of microsporidiosis in animals and humans. Proper care has to be taken to further check its spread to HIV-infected persons. Awareness programmes should be launched to check its severe and large scale spread. More research has to be carrying out to further establish immunopathological Biochemistry, Microbiology and Molecular Biology.

5. References


Abstract

Nanoparticles are particles that have size of 100 nm or less with one or more dimensions has gained larger attention due to their characteristic and unique properties apart from wide range of applications over their other counterparts. The physical, chemical, biological and hybrid ways of synthesis of nanoparticles is dependent on the requirement and type of nanoparticles however for clinical and biological application the chemical methods have proven to be toxic to the living system therefore better and safer alternatives are chosen like biological methods of production of nanoparticles. In biological methods the use of microorganism for production of nanoparticles is gaining lots of attention for being economical, rapid and safer alternative to physical and chemical methods. The wide range of microorganism and their potential to adapt in different environment gives them an edge over other ways. The microbial production of nanoparticles is the part of microbial growth that involves two processes: reduction process and precipitation process. The latter is further achieved by either nucleation or crystal growth. The entire production is controlled by controlling the growth parameter of the microbes. Thus the process is simpler and economical but is slow and time consuming as compared to chemical ways, however the quality and quantity of the nanoparticle is far better in biological methods than in chemical methods.
1. Introduction

Nanoparticles are those entities of matter that have one or more dimensions ranging from 100 nm or even less. These particles have higher surface area but smaller size making them a better alternative for application than their bulk counterparts [1,2]. The physical, chemical, biological and hybrid ways of synthesis of nanoparticles is dependent on the requirement and type of nanoparticles [3-6].

Since physical and chemical methods are more fastidious and give high yield of nanoparticles, they are most popular ways for nanoparticle synthesis, however the toxicity in the living system due to use of chemicals greatly limits their biomedical applications, particularly in clinical use. Secondly it was found that biogenic nanoparticles had greater potentials to include wider varieties and different shapes, compositions, coatings and structures of nanoparticles with special properties as compared to their chemical counterparts [7]. Thirdly it was reported that even if synthetic nanoparticles are not used directly to the living system yet their accumulation was found because of use of certain daily products like consumer products which contains trace amount of nanoparticles that can lead to their accumulation into the living system which is harmful for both prokaryotic and eukaryotic system [8-9]. By using microorganisms for synthesis of nanoparticles, a reliable, nontoxic and eco-friendly methods is designed that is of utmost importance to expand the biomedical applications of nanoparticles and also keeping in mind the environmental hazard the accumulation of synthetic nanoparticles can lead to.

Biological entities of matter have tremendous property to produce variety of potential nanoparticles. If fully understood and deciphered, these entities can be used for large scale production of almost all types of nanoparticles at industrial level manufacturing. The biologically aided synthesis not only decreases the consumption of energy and toxic chemicals but also opens the path for environmentally friendly green manufacturing [10].

The use of bacteria among all biological systems for production of metal and metal oxide nanoparticles of various sizes, compositions and properties are well documented. For example the use of Bacillus sp. for reduction of Tellurium to Rosette- aggregated rod shape nanoparticles of size approximately 30x200 nm and Selenium to 200 nm spherical nanoparticles [11,12]. Another example is of Shewanella oneidensis, a specialized bacterium with a property of reducing metals like Tellerium to spherical nanoparticles of size 50-80 nm [13] and Magnetospirillum magneticum that produces magnetic nanoparticles of 30-120 nm [14].

Despite of the fact that there are sufficient examples of different types of biological entities that can produce variety of nanoparticles of varying properties, yet there is a huge knowledge gap in understanding the mechanism behind the formation of those nanoparticles and the mechanism to control the final product is still unclear. There is still not sufficient information
that can lead to standardization of the process of formation of nanoparticles with specific desired properties, concentration and size. Similarly there is no possible information that can provide the ways to standardize the final product when the nanoparticles are used in a process. This knowledge gap refrains the use of biological agents for manufacturing of nanoparticles at industrial level. The bacterial based nano-manufacturing for mass production is precluded because of insufficient knowledge.

This chapter is an attempt to explore the available information about microbial production of nanoparticles as a salient need to develop a mechanistic understanding of the processes that lead to the formation of solid state nanoparticles by bacteria. The fundamentals that are derived from the natural microbial process are used in production of these nanoparticles, will be explored and studied to strengthen the further knowledge. Similarly the content will also cover the examples with their mechanism of production of nanoparticles so that the fundamentals can be well explained and can be used as a document for further research studies (Figure 1)

2. Mechanism of Production of Nanoparticles

The nanoparticles from microbes are produced by microbial enzymatic reactions that are far superior to the chemical reactions and far more rapid in production of reactant free specie for nanoparticle production. The green approach is far more suitable as the production of nanoparticles of different size and type can be calibrated by changes in pH, temperature and pressure, the conditions that defines the growth of majority of bacteria that are used to produce nanoparticles. The biochemistry that lies behind the phenomena is simple enzymatic actions that are involved in production of nanoparticles. By calibrating the ambient conditions the catalytic sites of interactions are changed. This produces highly reactive species that leads to higher catalytic action, strong binding between the enzyme and metal precursor and increased binding potential because of increase in specific surface area. The nanoparticles are hence formed when the microbial enzymes feed on the metal precursor as substrate from the environment and reduce it to reactive metal specie that acts as precursor for formation of nanoparticles. The synthesis of nanoparticles can be intercellular or extracellular depending on the location of formation of nanoparticles [17,18]. In the intercellular synthesis the metal
substrate from the environment is transported into the cell where microbial enzymes acts on it and reduce it to reactive specie leading to formation of nanoparticle inside the cell whereas in extracellular the metal substrate is trapped on the cell surface and the enzyme is transported or excreted out of the cell where it reacts with the metal ion on the cell surface to reduce it to reactive species thereby the nanoparticle is formed on the cell surface [19]. In general the use of microorganisms leads to the nanoparticle formation by two distinct approaches. First is: Bottom up approach in which the supersaturated solution is made to saturate more till it settles down in some phase and nanoparticles of particular size is produced. Second is: Top down approach in which the organic polymer produced by microorganism leads to the nucleation of first reactive specie also called as nanoparticle seed. These organic polymers modify the nucleation process of the nanoparticle seed by either favouring it or by inhibiting it, either way it can stabilize the molecule to produce the nanoparticle of particular size.

The bacterial production of metallic nanoparticles is by two processes:

a. Reduction Process

b. Precipitation Process

The precipitation process is further achieved by two ways

a. Nucleation

b. Crystal growth

From the above two processes, the reduction process is most studied and documented than precipitation process by nucleation or by crystal growth.

2.1. Reduction process of nanoparticle synthesis by microbes

The microbes use their reducing agents in formation of nanoparticles from its precursor molecule. These reducing equivalents can be taken by inorganic compounds as in lithotrophs or by organic compound as in organotrophs thus these precursors of nanoparticles act as substrates for reducing agents. The reduction of metals to their corresponding sulphides by metal reducing bacteria is an example of such reduction mechanism. The variety of metal nanoparticles produced by microorganisms are deposited in the cytoplasm, periplasm, and extracellular area or on the cell surface. These nanoparticles are produced either by energy conserving metal reduction dissimilation process or by cell building assimilation or by both as in co-metabolism. Such nanoparticles generally helped in remediation process as most of them lowered the concentration of toxic compounds. Usually such reduction processes occur in growth phase of microbial culture but some of them were reported in stationary phase too or can be produced extracellular by isolated microbial enzyme from the growing culture [15]. With the biological
chemical reduction method the M$^+$ stage is converted to M0 active stage or formation of free radicle is there that initiates the further production of large amount of nanoparticles in low cost and less time. Secondly it is easier to tune the formation of nanoparticles of varying size and tune by just changing the reducing agent, the dispersing agent, temperature and time. However in case of microbial production of nanoparticles no reducing agent is added from outside as the biological entity itself has reducing agent in large amount in growing microbial culture, that are highly reactive and capable of producing the nanoparticles in less time. However the dispersing agent can be added to give the desired size to the nanoparticles. Since no chemical agent like reducing agent is added from outside the amount of impurity is lesser than in chemical production of nanoparticles. So this process is used in biological manufacturing of nanoparticles. Once the nanoparticles are formed they are then precipitated by crystal growth or by nucleation (Figure 2).

![Figure 2: Example of Microbial Synthesis of Nanoparticles](image)

2.2. Precipitation of nanoparticles

There are many ways of separation of Nanoparticles like poly condensation of sol to gel or gel to sol state sedimentation as seen in metallic nanoparticles. The aggregation of crystals can be by simple drying after the liquid phase sedimentation. In order to form mono disperse nanoparticle of particular size it is important that crystal grows at very slow and steady rate from the rapidly generating seed particles. Once the desired nanoparticles is formed then dispersing agent is added to avoid further aggregation like addition of citrate as dispersing agent in formation of gold nanoparticles. Once the desired size nanoparticles is achieved then it is separated by various methods like the sedimentation method of nanoparticles by co-precipitation or alkaline precipitation, by gel filtration, by gel electrophoresis and by centrifugal separation.

On the basis of fact that precipitation of nanoparticles is most fruitful method for sedimentation of desired nanoparticles from colloid with varying sizes of nanoparticles, new tech
niques are being used in this process. When the precipitation is done under high gravity conditions, it produces larger amount of nanoparticles and that too in low cost. This technique is finding its application for industrial production of nanoparticles because of its low cost and environment friendly green approach. This technique is called HIGH GRAVITY REACTIVE PRECIPITATION (HGRP) [16].

3. Production of Nanoparticles by Microbes

The microbes are those biological entities that are well studied for their constant environment interactions. Therefore they have wide range of energy producing processes ranging from organotrophy to lithotrophy in presence of oxygen or in absence of it, thus using different bio-mechanism as per the environmental need. This basic fact led to the use of microbes for production of nanoparticles by lithotrophy in presence of air or in absence of it. The commonest nanoparticles produced by microbes are discussed here in details to give an insight of the biosynthesis of nanoparticles by the microbes (Table 1). The nanoparticles are divided under four categories: Metallic nanoparticles (Au, Ag, Alloy, and metal nanoparticles), Oxide nanoparticles (metallic and non-metallic oxide nanoparticles), Sulphide nanoparticles and other miscellaneous nanoparticles. Each type when studied in details helped in understanding the mechanism behind the production of nanoparticles by microbes.

3.1. Metallic nanoparticles

The metallic nanoparticles show different optical property that is of the fundamental attraction and characteristics of nanoparticle. In general these properties are size dependent ranging from 1-100 nm. The metallic nanoparticles have different physical and chemical properties than bulk metals and so they exhibit optical characteristics for example 20 nm Au-Np exhibit wine red colour, Ag-Np exhibit yellowish grey and Pd-Np; Pt-Np exhibit black colour.

The metallic nanoparticles are produced by microbes as a result of reduction of metal to free radicle specie that aggregates to form nanoparticles. Thus M⁺ is converted to M⁰ in presence of reducing agent of microbial origin or system.

The environmental toxicity is also largely due to accumulation of heavy metals that are toxic to microbes as well, however there are certain microbes that are resistant to these heavy metals and can use them as substrate in one or the other biochemical reaction for generation of energy as ion efflux from the cell by membrane proteins that function either as ATPase or as chemiosmotic cation or proton anti transporters that causes chemical detoxification. This led to the research behind production of nanoparticles from heavy metals as well for example production of Palladium, Mercury, Platinum etc. nanoparticles apart from gold and silver or their alloy nanoparticles.
<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Nanoparticle</th>
<th>Culture Temperature (oC)</th>
<th>Size (nm)</th>
<th>Shape and Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shewanella oneidensis</td>
<td>Fe$_3$O$_4$</td>
<td>28</td>
<td>40–50</td>
<td>Rectangular and Extracellular</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>Sb$_2$O$_3$</td>
<td>25–60</td>
<td>2–10</td>
<td>Spherical and Intracellular</td>
</tr>
<tr>
<td>Lactobacillus sp.</td>
<td>TiO$_2$</td>
<td>25</td>
<td>8–35</td>
<td>Spherical and Extracellular</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>BaTiO$_3$</td>
<td>25</td>
<td>4-5</td>
<td>Spherical and Extracellular</td>
</tr>
<tr>
<td>Brevibacterium casei</td>
<td>PHB (Polyhydroxybutyrate)</td>
<td>37</td>
<td>100–125</td>
<td>Intracellular</td>
</tr>
<tr>
<td>E. coli</td>
<td>CdS</td>
<td>25</td>
<td>2–5</td>
<td>Wurtzite crystal and Intracellular</td>
</tr>
<tr>
<td>Rhodobacter sphaeroides</td>
<td>ZnS</td>
<td>Unknown</td>
<td>10.5+/-0.15</td>
<td>Spherical and Extracellular</td>
</tr>
<tr>
<td>Desulfobacteraceae</td>
<td>FeS</td>
<td>Unknown</td>
<td>2</td>
<td>Spherical and Extracellular</td>
</tr>
<tr>
<td>Brevibacterium casei</td>
<td>Au, Ag</td>
<td>37</td>
<td>10–50</td>
<td>Spherical and Intracellular</td>
</tr>
</tbody>
</table>

Table 1: Common examples of Nanoparticles produced from microbes

The extracellular synthesis of gold nanoparticles by fungus *Fusarium oxysporum* and actinomycete *Thermomonospora* sp. and the intracellular synthesis of gold nanoparticles by fungus *Verticillium* sp. has been reported by Mukherjee, Sastry and co-workers [20,21,22]. Similarly it has been demonstrated that gold nanoparticles can be produced intercellularly inside the bacterial cell when it is incubated in media with Au$^{3+}$ ions [23]. The study was also done in microbial synthesis of monodisperse Au nanoparticles from alkali tolerant *Rhodococcus* sp. in alkaline environment under bit high temperature [24]. The synthesis of Au nanoparticles in different structure was reported by Lengke et al. They claimed to produce Au nanoparticles of different structures like spherical, cubical and octahedral from filamentous cyanobacteria by using Au(I)- thiosulfate and Au(III) chloride complexes [25,26]. Similarly Lactate degrading bacteria, Lactobacillus was reported to produce nanocrystals and nanoalloys at the time of microbial log phase of growth by Nair and Pradeep [27].

The other metallic nanoparticles that are being produced rapidly by microbes are silver nanoparticles that has more importance in biomedical sector as they have antimicrobial activities that led to the development of biomimetic approach for their production. Since Vedic ages, silver is known for its antimicrobial activities and so the use of silver utensils for eating was very common and application of silver vark on sweets to prevent bacterial and fungal growth on them were common practices. It has been proved that they not only show antimicrobial activity against Gram negative and Gram positive bacteria but also against highly tolerant and multi resistant strains like methicillin resistant *Staphylococcus aureus* [28]. Various mi-
Bacteria are known to reduce the Ag$^+$ ions to form silver nanoparticles mostly spherical in shape [29–31]. The research group has also reported to produce nanoparticles from a specific strain isolated from a silver mine of Pseudomonas asututzeri which they tagged as AG259. This strain produced silver nanoparticles within periplasmic space when placed in concentrated aqueous solution of Silver Nitrate. The bacterium produced free reactive Ag$^0$ species from silver nitrate solution by reduction of Ag$^+$. It was found that the Ag nanoparticles so formed were deposited in periplasmic space in bacterial cell [32]. On other hand when fungi, Verticillium, Fusarium oxysporum, or Aspergillus flavus, were employed, the synthesis of Silver nanoparticles were in the form of a film or they were released in solution or they aggregated on the cell surface [33].

After the production of silver and gold nanoparticles, nanoparticles in form of alloy hold numerous application in the field of electronics, alloy coatings, as catalyst in reactions and as optical material for communication etc. [34].

Moving ahead with alloy nanoparticles, Senapati et al. reported that fungi *F. oxysporum* can synthesise hybrid alloy of Ag-Au in presence of Co-factor NADH secreted indigenously by the microbe that even decides the composition of the alloy [35]. Similar hybrid alloy of Ag-Au is also reported to be synthesised by yeast cells by Zheng et al. that after the synthesis of alloy, did the characterisation by fluorescence microscope and transmission electron microscope that indicated that the alloy is produced extracellularly in form of polygons. Similarly by the same group the electrochemical study stated that the vanillin sensor was a modified glass carbon electrode with Ag-Au alloy coatings that enhanced the electrochemical activity of vanillin by five folds [36]. After the report of synthesis of polygonal hybrid alloy of Ag-Au, there were reports of synthesis of core shell alloy nanoparticles of Ag-Au that were synthesised by fungus Fusarium semitectum and these nanoparticles were found to be highly stable in suspension for many weeks. This study was done by Sawle et al. [37].

In the genre of metallic nanoparticles is the new edition of heavy metal nanoparticles synthesised by metal resistant microbes. The use of metal ion-reducing bacterium Shewanella algae for production of Platinum nanoparticles in periplasm of 5nm size is a microbial biochemistry of reduction of Platinum chloride to Platinum free radicles at room temperature and pH within an hour in presence of Lactate as electron donor [38]. Similarly Mercury nanoparticles of size 2-5 nm were prepared by Enterobacter sp. at slightly alkaline pH of 8 and lower concentration of mercury lead to increase in chemical detoxification [39]. The palladium nanoparticles could be synthesised by the sulphate reducing bacterium, Desulfovibrio desulfuricans, and metal ion-reducing bacterium, *S. Oneidensis* mentioned earlier [40]. Similarly the metal resistant bacteria that use Hydrogen as electron donor are capable of reducing large amount of heavy metals like Chromium, Uranium, and Cobalt etc. [41].
3.2. Magnetic and Non-magnetic Oxide Nanoparticles

Magnetic oxide nanoparticles and nonmagnetic oxide nanoparticles are important types of compound nanoparticles that are synthesized by microbes. The magnetic nanoparticles have gained so much importance because of their unique micro configuration and super paramagnetic properties. Biocompatible magnetic nanoparticles like Fe₃O₄ (magnetite) and Fe₂O₃ (Maghemite) are found to be clinically safe. Since they are biocompatible, they can be used for clinical application as in targeted cancer treatment, sorting and manipulation of stem cell, site directed drug delivery, targeted gene therapy, targeted DNA analysis, and identification by magnetic resonance imaging (MRI).

The microbes used for production of such nanoparticles are Magnetotactic bacteria. These bacteria are capable of synthesizing intracellular magnetic particles that comprises of iron oxide, iron sulfides, or even both [42,43]. These magnetic particles being of microbial origin are enveloped by phospholipids and proteins organic membranes that can easily disperse them in aqueous solutions. Furthermore, an individual nanoparticle or magnetite is a mini magnet that contains a single magnetic domain that yields higher magnetic properties [44]. The members of the family Magnetospirillaceae are the bacteria that are found to produce the maximum number of magnetic nanoparticles or to say it this way that to date the maximum number of magnetotactic bacteria belong to this family. These bacteria are found in fresh water sediments and they were segregated from other fresh water bacteria by differential growth medium and magnetic isolation techniques. The bacteria can be chemoorganotroph or chemolithotroph. The first isolated bacteria of this family was *Magnetospirillum magnetotacticum*, identified as strain MS-1 [45]. Mostly cultured magnetotactic bacteria are mesophilic and tend not to grow much above 30°C however uncultured magnetotactic bacteria were mostly at or below 30°C with only few reports describing thermophilic magnetotactic bacteria. These bacteria tend to form magnet aggregates lined in form of chain along the geometric north of the earth and often cluster in periplasm or intercellular spaces that help the bacteria to move in oxygen gradient under the influence of Earth’s magnetic field. It was reported that magnetic Fe₃O₄ nanomaterials with mesoporous structure were synthesized by co-precipitation method using yeast cells as a template [46,47] that led to precipitate out the magnetic oxide nanoparticle from the growing bacteria without its lysis.

Besides magnetic nanoparticles, large number of nanoparticles were produced from nonmagnetic elements too. The mechanism of production remains the same. It was reported by Jha and co-workers that biosynthesis of Sb₂O₃ nanoparticles can be mediated by Saccharomyces cerevisiae and this green process is economical and reproducible [48]. Similarly Bansal et al. used *F. oxysporum* (Fungus) to produce SiO₂ and TiO₂ nanoparticles from aqueous anionic complexes SiF₆²⁻ and TiF₆²⁻, respectively [49].
3.3. Sulphide and other Nanoparticles

The next in the generation of nanoparticles used extensively in biomedical fields as cell labelling agents, for protein targeting and for developing quantum dots as they exhibit novel electronic and optical properties [50]. These are Sulphide nanoparticles of CdS nanoparticle is the commonest example that act as quantum dots in technical applications apart from labelling agent. These quantum dots were formed by the reaction of Cd\(^{2+}\) ions with sulphide ions which were produced by the enzymatic reduction of sulphate ions to sulphide ions (SO\(_4^{2-}\) to S\(^{2-}\), Cd\(^{2+}\) S\(^{2-}\) → CdS). The sulphate reducing bacteria use sulphur as electron donor that act as reducing agents to reduce metal sulphates to their corresponding metal sulphides.

It was found that *Clostridium thermoaceticum* could precipitate CdS from CdCl\(_2\) on the cell surface as well as in the medium in the presence of Cysteine Hydrochloride in the growth medium as Sulphide source [51]. Similarly *Klebsiella pneumonia* and *E. Coli* were reported to form CdS on cell surface when grown in media with Cd\(^{2+}\) ions [52]. The production of CdS and other commonly produced nanoparticles of ZnS and PbS were synthesised from *Rhodobacter sphaeroides* and *Desulfobacteraceae* and the diameter of the nanoparticles were controlled by the culture time [53-55]. Production of magnetic nanoparticles like Fe\(_3\)S\(_4\) or FeS nanoparticle from uncultured magneto tactic sulphate reducing bacteria was also reported [56,57]. The sulphide nanoparticles can also be generated extracellular by the fungus Fusarium oxysporum when exposed to aqueous solution of metal sulphate [58].

**Other Nanoparticles:** In nature the compounds are never in free form they are always bound to one another for stability and better interaction, such compounds are called biopolymers that can be synthesised by other biopolymers like proteins or by using microbes for example Pb-CO\(_3\), CdCO\(_3\), SrCO\(_3\), PHB, Zn\(_3\)(PO\(_4\))\(_2\), and CdSe nanoparticles were reported to be synthesized by microbes like *Fusarium oxysporum* [59,60] and Yeast [61] that can form nanoparticles in form of crystals or in form of powder.

4. Microbial Biochemistry of Production of Important Nanoparticles

The microorganism are the biological entities that have more than one mechanism for living and they can use many different ways to produce nanoparticles. The metal ions are first trapped metal ions are first trapped on the surface or inside of the microbial cells that are then reduced to nanoparticles in the presence of enzymes. The exact mechanism of intracellular formation of nanoparticles is not well understood, however the presence of silver and gold nanoparticles on the surface of the algal mycelia supports the theory. The precursor ions of these nanoparticles are found to be trapped in surface of microbial cell via electrostatic interaction between positive charge on ions and negative charge on microbial cell surface where the enzymes reduces the metal ions to form gold and silver radicles that further forms nuclei and grow through further reduction and accumulation. Some workers speculated that the syn-
thesis of silver nanoparticles in *B. licheniformis* is mediated by nitrate reductase enzyme. The possible mechanism involving this enzyme could be reduction of silver ions to reactive silver specie because of electron activity due to reduction of nitrate ions. This generates Co factor NADH, a powerful reducing agent that further reduce silver ions [62]. It has to be noted that the synthesis of metal nanoparticles in presence of enzyme reductase is directly dependent on NADH and if not in presence of enzyme then only NADH in system acts as an important factor.

The formation of heavy metallic nanoparticles from heavy metal ions like Hg$^{2+}$, Cd$^{2+}$, Ag$^+$, Co$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, Pb$^{2+}$, and Zn$^{2+}$ as discussed earlier is due to the metallophilic microbes that have potential to synthesise heavy metal nanoparticles in presence of toxic heavy metals. These bacteria develop heavy metal resistance in order to survive in the heavy metal toxicity. The microbes were thus well adapted in heavy metal environment and developed metal homeostasis gradually. This generated a unique genetic and proteomic responses in this bacteria to toxic environment like mines, waste rock piles, metal processing plants drains or natural mineralized zone of earth. Such responses were uncommon in bacteria inhabiting the normal surrounding [63,64,65].

The formation or bio mineralization of bacterial Magnetic Nanoparticles and its molecular mechanism is hypothesized to be a multistep process as following:

1. The mechanism of vesicle formation: It is proposed that the process vesicle formation resembles the process of formation of mesosomes in eukaryotes that is an energy dependent process and utilises GTPase enzymes at time of invagination. Similarly the invagination of cytoplasmic membrane forms vesicle in presence of GTPase enzyme. These vesicles are seeds to bacterial magnetic nanoparticles that are surrounded by phospholipids and protein organic membranes because of invaginations of cytoplasmic membranes.

2. The linear arrangement of vesicle: the arrangement of formed vesicles is in the linear form along with cytoskeletal filaments. This form linear chains of small magnets surrounded by organic membranes called as magnetosomes.

3. Accumulation of iron ions: The accumulation of ferrous ions occurs into the vesicles with the help of iron transporters that are transmembrane proteins or by siderophores, maintaining the external ion concentration by the process called biomineralization, however the internal ion concentration is maintained by simple cellular oxidation-reduction system.

4. Nucleation: This process is the final stage. The magnetosomes bounded by organic membranes form magnetite crystals because of the process of nucleation. There are various proteins associated with the bacterial magnetic particle membrane that play functional roles involved in magnetite generation. The last step involves accumulation of supersaturating iron
concentrations inside the cell. The high concentration causes partial reduction and dehydration in case ferrohydrite is used for production of magnetite crystals or else it is the maintenance of reductive conditions and the oxidation of iron to induce mineralization to magnetite.

The formation of magnetic nanoparticles from bacteria like *Shewanella oneidensis* has already been discussed earlier, however the mechanism that involves the production of magnetites consists of both passive and active mechanisms. It involves following two steps:

1. Production of Fe\(^{2+}\): The utilization of ferrohydrite by bacteria as a terminal electron acceptor for active production of Fe\(^{2+}\) and the pH value surrounding the cells rises probably due to the bacterial metabolism of amino acids.

2. Localization of Iron ions: The localized concentration of Fe\(^{2+}\) and Fe\(^{3+}\) at the net negatively charged cell wall, cell structures, and/or cell debris is through a passive mechanism that induces a local rise of supersaturation of the system with respect to magnetite, causing the magnetite phase to precipitate. Thus the precipitation of nanoparticle crystals by simple low cost and efficient method is only feasible because of microbial interaction.

Next in the line are quantum dots or CdS nanoparticles produced from sulphur reducing bacteria that follows the following steps:

1. Breakage of Cysteine bridges on Cell surface of bacteria: The proposed mechanism can be because of disulphide (cysteine) bridges in cell structure of the bacteria and may be because of cleavage of S–H bond and formation of a new bond, that is, –S–Cd bond where Cd is from Cd-thiolate (Cd–S–CH\(_2\)COOH) causing the nanoparticle production on the surface.

2. Interaction of Cd-thiolate group: It has to be noted that the –COOH groups from the cadmium-thiolate complexes do not react with the –NH\(_2\) groups of protein on the cell surface of the bacteria because of electronegative potentials but interact with hydrogen bond.

3. Capping of CdS Nanoparticles: The capping the CdS nanoparticles is because they are bonded to –NH\(_2\) groups by hydrogen bond [66] and one of the oxygen atoms of the carboxylic group (–COOH) forms the coordinate bond between the oxygen atom and Cd\(^{2+}\) ions [67], thus on grounds of electric potentials it competes with the thiol group of cysteine bridges to assemble onto the surfaces of the CdS nanoparticles causing its capping. This leads to accumulation of CdS nanoparticles on the surface of bacteria.

4. Application of Nanoparticles Produced from Microbes and Future Prospects

There are many applications of nanoparticles but when it comes to biomedical application the nanoparticles risk analysis needs to be done. To overcome this problem the green production of nanoparticles was done. As a result the use of nanoparticles from such biologi-
cal producers makes them a safer option in nanomedicines and nanotherapeutics involving safe delivery of drugs, proteins or targeting of oncogenes or immune systems etc. The field of nanomedicines in diagnosis and treatment, primarily of human diseases is an upcoming avenue in the field of research thriving for continuous improvement and standardization. The biosynthesis of nanoparticles by microbes makes it a safer option in this field of nanomedicines as the green chemistry procedure is found to be clean, biocompatible, nontoxic and environmentally safe. The production of desired nanoparticle can be manipulated as to its intracellular and extracellular synthesis that employs the use of microbes, right from bacteria to actinomycetes depending upon the location where the nanoparticles to be formed.

The second property of these green nanoparticles is that by simple manipulations of pH, temperature and other growth conditions like substrate concentration and exposure time, the rate of intercellular production of nanoparticles and their sizes can be calibrated. Some changes occur in exponential phase and some occur in lag and few in stationary phase of microbial growth (Figure 4).

Thus these green particles have some chief applications as mentioned below;

1. **In Cancer targeted treatment:** The use of Iron nanoparticles like Magnetite and Maghemite for targeted cancer treatment as already been reported as they are biocompatible and has role gene therapy and DNA analysis. They also hold application in MRI imaging and stem cell sorting that further helps in tracking the oncogenesity. It was also found that when these magnetosomes were used on mammalian immune system then they showed neutral behaviour without altering the immunology of the host [68]. In another experiment directly the drug duboroxin, an anti-tumour drug was loaded on magnetosomes and it was found to effectively target and kill the tumour cells without effecting the normal cells [69]. Thus proving that these magnetosomes can be effective carriers of drug, gene or any other therapeutic for cancer treatment. Similarly Silver nanoparticles were found to be anti angiogenic and exhibited caspase dependent apoptosis of the tumour cell line. Thus it can be seen that these green nanoparticles are capable of acting in more than one way.
2. **The targeted drug delivery**: The sulphide nanoparticles, the iron nanoparticles were reported to be the best drug delivery carriers as they bear all the properties of a good carrier, being smallest in size, bear large surface area, are biocompatible and inert and most importantly can cross the blood brain barrier and surface epithelial junctions without being rejected. These carriers had potential to distribute the drug at the targeted site without causing its accumulation elsewhere and the probability of drug toxicity is reduced. They have improved pharmacokinetics and biodistribution of therapeutic agents. The use of magnetosomes and the bacterial cell as whole with magnetosomes as carriers for drug delivery has been used extensively. The bacterial cells with magnetosomes can be derived to the targeted area under the influence of magnetic field, however for this MRI imaging is very important that can show the movement of bacteria to the targeted site. Once the bacteria reaches the targeted site then the magnetosomes on its surface deliver the drug to the target and the treatment of the disease is there [70]. Similarly the use of gold as therapeutic agent has been since a very long time and their nanoparticles are far more effective than the compound because of smaller size, high surface to volume ratio, unique optical and electronic properties and are tuneable. These gold nanoparticles can be easily modified by binding ligands that exhibit gold affinity like thiols, amines and phosphines that increase the reactivity of these particles. This has made them more promising for drug and gene delivery. Thus the nanoparticle-mediated targeted delivery of drugs modifies chemotherapy by reducing the dosage of less specific and highly toxic anticancer drugs and by using chemo drugs with better specificity that enhances the efficacy of therapy and causes low toxicity in the system. The process will be less expensive and fastidious. Secondly it will be biocompatible so chances of rejection is also ruled out. Thus the upcoming trends is of
nanomedicines to solve the problems in cancer therapy that arises due to heterogeneity, non-targeted therapy and development of drug resistance in cancer patients.

3. Antimicrobial agent: The use of silver nanoparticle as antimicrobial agent is already know. However the only concern of use of these silver nanoparticles were toxicity that can result in their accumulation so the biomedical application of these chemically synthesised silver nanoparticles was restricted. But when the green chemistry approach of synthesis of silver nanoparticles from fungi like *F. oxysporum* was studied, it was found that these nanoparticles are highly reactive but biocompatible so the risk of toxicity was reduced. The silver nanoparticles also acted as carriers for major antibiotics like ampicillin, kanamycin, chloramphenicol and erythromycin highly reactive to Gram positive and Gram negative bacteria. When these antibiotics were loaded with Silver nanoparticles the antimicrobial activity enhanced without bringing any change in media. Recently a new type of work was done by researchers where they incorporated these green nanoparticles in a textile to prevent it from *Staphylococcus aureus* [71] infection. Similarly the beauty products also have these biocompatible silver nanoparticles.

4. Biosensors: The optical and electronic properties of nanoparticles make them an efficient biosensors. The single ion reactivity can be detected making these biosensors highly sensitive. It was reported that when conventional glucose biosensor was compared with gold nanoparticle based biosensor then the activity of glucose oxidase for smaller amount of sample was increased by folds, making the sensor highly sensitive to even a drop of the sample. Thus the use of such glucose sensor is now common in biomedical applications [72]. Similarly the use of Gold Silver alloy nanoparticles in modified glassy carbon electrode whose commercial application is as Vanillin biosensor in testing purity and amount of vanilla extract or vanillin from vanilla beans or vanilla tea [73]. The modifications were also done in conventional first enzyme based biosensor with enzyme Horseradish Peroxidase, making it more sensitive and highly specific. The modified Horseradish Peroxidase biosensor contains Selenium nanoparticles produced from Bacillus species. These H$_2$O$_2$ biosensors had high sensitivity and affinity for H$_2$O$_2$. The highly reactive Se- NP, with large surface to volume ratio, is stable at room temperature and has good adhesive ability, and biocompatibility that led to enhancement of the HRP- biosensor. These sensors exhibited good electrocatalytic activity towards the reduction of H$_2$O$_2$ due to the good adhesive ability, and biocompatibility of Se-NP [74]. Another effective biosensor is that of Gold nanoparticles being largely used in cancer targeting [75] because of its surface plasmon resonance properties of light scattering.

5. As reducing and catalytic agents: The nanoparticles being a highly reactive species act as effective reductants and catalyst in many chemical reactions. Their high surface to volume ratio and electronic properties facilitate the chemical process. It has been reported earlier the use of silver nanoparticles with antibiotics to enhance the antimicrobial activity. Similarly the magnetosomes capping on bacteria or their formation enhance the microbial activity like
enhancement of desulphurisation of complex polymer by *Pseudomonas* sp. when coated with magnetite [76] or enhancement in detoxification of heavy metals by the magnetotactic bacteria with magnetosomes. The magnetic nanoparticles bearing high surface energy caused their strong adsorption on the cells where they behave as catalyst and just like enzymes can be procured back similarly in presence of an external magnetic field these particles were always in suspended form in the solution and can be collected back thus the cells with nanoparticles can be used several times making the use of nanoparticles as reductants or catalyst for any chemical reactions more economical affair.

6. **As a tracer and imaging particle**: The optical and electronic properties of nanoparticles make them and efficient tracer molecule in detection of complex biochemical pathways. These particles exhibit different light scattering patterns at different sizes like gold nanoparticles exhibit optical activity at different sizes and it is this property that was exploited for biomolecular recognition with help of single gold nanoparticle functionalised with biotin to which when streptavidin binds. The reactivity of gold nanoparticles produces high light scattering wherever the binding of biotin with streptavidin will take place and the biomolecule can be recognised [77]. Similarly as discussed earlier that iron nanoparticles since are magnetic in properties, in presence of magnetic field when tagged with a biomolecule helps in knowing the bioassay of that biomolecule and so they act as effective biological label. Competitive chemiluminescence, enzyme immunoassays using antibodies immobilized onto bacterial magnetic particles, modified biosensors, and were developed for the rapid and sensitive detection of small molecules, such as environmental pollutants, hormone, and toxic detergents [78]. Apart from magnetic particles acting in presence of magnetic field there are certain specialised nanoparticles like that of gold quantum dots of Au67 that can trace DNA directly in one step process under influence of magnetic field [79].

The MRI imaging in presence of magnetic particles has proven to be more effective than conventional imaging of cancer targeted treatment. Similarly Cadmium Sulphide nanoparticle tags are extensively used in DNA hybridisation experiments in electrochemical stripping method [80]. The nanoparticle tracers are also being used for environmental concerns. The tracers are the most direct ways of diagnosing environmental problems of groundwater contaminations or for knowledge of natural gas and oil productions by tracing the subsurface fluid flow pathway. The nanoparticle tracers are more effective as they are path sensitive and highly specific so they never diffuse out of the specified flow channel and the time taken to cover the distance between the two points is very less. The green nanoparticle tracers are tuneable and so the chances of their aggregation or sticking to the narrow porous channels is greatly reduced and far more avoidable [81].

The above mentioned applications are milestones in field of nanomedicines and biomedical treatments and lay the foundations for better prospects in the field of therapeutics by
modifying the existing processes or by producing more varieties of nanoparticles employing green technologies. The recent advances focus around manipulations at microbial molecular level involving alterations at genomic and proteomic levels to produce highly efficient nanoparticle that can be used extensively for biomedical application. Secondly the manipulations at molecular level can help in standardizing the process so that the large and commercial scale production of nanomedicines can be facilitated as boon in health care sector.

Apart from the prospective applications of the nanoparticles from microbes still there are certain consequences that need to be overcome so that the microbial production of nanoparticles becomes the best commercial process that can be used in large scale. The microbial production of nanoparticles is still less rapid and slow process as compared to physical or chemical ways of production of nanoparticles. Secondly lot of effort is required to improve the synthesis efficiency and effort to control the particle size and morphology. The reduction of synthesis time and making the process tuneable will make this biosynthesis route much more attractive. The desired particle size and the nature of nanoparticles are two important issues in the evaluation of monodisperse nanoparticle synthesis. This requires an effective dispersing agent along with microbial reductants. To identify more and more dispersing agents that are eco-friendly is the area of study. Thirdly it was seen that the shelf life of the nanoparticles produced by microbes was very intangible as such the decomposition rate was nearly rapid and after certain time, they decomposed. Thus, the tangibility of nanoparticles production by biological means needs extensive study and standardization. It has already been seen that the control of particle size is in physical and chemical ways is easily feasible however with biological ways the control of particle size can be by varying parameters like the type of microbes, their stage of microbial growth, pH, substrate concentration, temperature, the concentration of source of target nanoparticles, the reaction time and the capping or coatings with different nanoparticles or by adding an untargeted ion that can act as dispersing agents, can lead to control of particle size and monodispersity. Sometimes the coating with lipids and proteins also confer the physiological stability of the nanoparticles making them more biocompatible and with longer shelf life, that is important for biomedical applications

The research is currently revolving around manipulating cells at the genomic and proteomic levels, because that will help in creating microbes that can produce stable and biocompatible nanoparticles with longer shelf life. With a better understanding of the mechanism at the cellular and molecular level, the isolation and identification of compounds, better reductants and production conditions could be explored. This further helps in reducing the reaction time and increasing the efficacy of the process and the product that is nanoparticle, important for biomedical applications. The microbial approach to production of biocompatible nanoparticles that are economical, nontoxic and safer to the environment further strengthen the nanomedicines mediated therapeutics.
As it is said what you give to the nature comes back to you so if we give a healthier green approach to the environment then environment will also keep us healthy. The latest technologies and research should mow focus more on green approaches like use of flora and fauna for innovations rather than using the consumables for research that will exhaust one day and lead to the accumulation of toxic substance. Therefore to conclude it can be said that the tiny factories (microbes) are harbours of most skilled technicians (nanoparticles) for Dynamos (energy efficient process/products) that is by employing microbes the nanoparticles can be produced and these nanoparticles can be used for different applications that are necessary for the environment wellbeing.

5. References


7. Rai M, Gade A, Yadav A. Biogenic nanoparticles: an introduction to what they are, how they are synthesized and their applications. In Metal nanoparticles in microbiology 2011 (pp. 1-14). Springer Berlin Heidelberg.


40. Kashefi K, Lovley DR. Reduction of Fe (III), Mn (IV), and toxic metals at 100 C by Pyrobaculum islandicum. Applied and Environmental Microbiology. 2000; 66(3): 1050-1056.


43. Bazylnski DA, Frankel RB, Heywood BR, Mann S, King JW, Donaghay PL, Hanson AK. Controlled Biomineralization of Magnetite (Fe (inf3) O (inf4)) and Greigite (Fe (inf3) S (inf4)) in a Magnetotactic Bacterium. Applied and Environmental Microbiology 1995; 61(9): 3232-3239.


57. Bazylinski DA, Frankel RB, Heywood BR, Mann S, King JW, Donaghay PL, Hanson AK. Controlled Biomineralization of Magnetite (Fe (inf3) O (inf4)) and Greigite (Fe (inf3) S (inf4)) in a Magnetotactic Bacterium. Applied and Environmental Microbiology. 1995; 61(9): 3232-3239.


Diverse Groups of Isolated Bacteria for Biodesulfurization of Petroleum Products in Petroleum Refinery Industry

Abrar Ahmad¹; Mohd Jahir Khan²; Satya Prakash Gupta³; Mohd Mabood Khan⁴; Mohammad Kashif⁵; Mahmood Ahmad Khan⁶ and Anjum Bee⁷

¹Environmental Biotechnology Division, CSIR-Indian Institute of Toxicology Research, Mahatma Gandhi Marg, Lucknow 226001, India.
²School of Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India.
³Department of Biotechnology, DDU, Gorakhpur University, Gorakhpur, India.
⁴Department of Pathology, King George Medical University, Lucknow, India.
⁵Plant Molecular Biology and Genetic Engineering Division, CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, India.
⁶Department of Biochemistry, UCMS & GTB Hospital, Dilshad Garden, Delhi 110095, India.
⁷Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Rae Bareli Road, Lucknow 226025, India.

*Correspondence to: Abrar Ahmad, Environmental Biotechnology Division, CSIR-Indian Institute of Toxicology Research, Mahatma Gandhi Marg, Lucknow 226001, India.
Email: abrar.ahmadg@yahoo.com

Abstract

Various energy sources are required for different needs of daily life including use of electrical appliances and moving the automobiles and other transport machinery etc. A major source (~82%) for energy production is fossil fuels, and nearly half of it comes from petroleum. Incidentally, the naturally occurring crude oils contain numerous sulfur compounds, which upon combustion convert into sulfur-dioxide that lead to various environmental and health problems. For this reason, several countries have prescribed the guidelines to reduce the sulfur content in transportation fuels 0 to 10 ppm by the year 2020. In India also, Euro IV guidelines (maximum sulfur level at 50 ppm) has been implemented in 13 major metropolitan cities. While in other parts of the India, Euro III (50-350 ppm) stage is being followed.
Conventionally, organic sulfur from crude oil-derived fuels is removed by a ‘hydrodesulfurization’ (HDS) process, wherein the crude oil fractions are reacted with hydrogen at high pressure (150-3000 psi) and temperature (290-455°C) in the presence of metal catalysts i.e. CoMo/Al₂O₃ or NiMo/Al₂O₃. In this process, the organic sulfur gets converted into hydrogen sulfide and total sulfur concentration in oil-fractions can be reduced from 1-5% to 0.1%. Many sulfur compounds e.g. 4, 6-dimethyl dibenzothiophene (4, 6-DMDBT) are refractory to desulfurization and are often removed by ‘deep HDS’, where higher temperatures and pressures are used that makes the process very expensive.

Bio-desulfurization (BDS) has been suggested as an alternative method for achieving low sulfur levels in the crude oil fractions. It is remarkable for its mild operating conditions, greater reaction specificity afforded by the nature of biocatalysts and for not requiring molecular hydrogen. For this reason, biodesulfurization of thiophenic compounds e.g. dibenzothiophene and benzothiophene has been studied extensively. Several diverse groups of microorganisms have been isolated and reported for these groups of organosulfur compounds (Thiophenic and non-thiophenic) found in crude oil. They have shown Biodesulfurization mostly by reductive 4S pathway and some by related to degradative pathway like Kodama pathway. Studies on non-thiophenic compounds, however, are rare. Microbes for broad substrate range organo sulfur compounds desulfurization are being isolated, but little progress has been made. Isolated strain like Rhodococcus sp., Mycobacterium sp., Bacillus sp., Pseudomonas sp. have shown limited substrate range for organosulfur compounds found in crude oil/Diesel/Petrol. Recently isolated our lab isolate Gordonia sp. IITR100 has shown broad substrate range and biodesulfurized model compounds Dibenzyl sulfide, thianthrene, Dibenzothiophene, Benzonaphthothiophene.

Hence we could say that we can try from even more new sources for microbe’s isolation which could full fill the demand of industrial activity of 3.2-100 µmol/dry cell weight. Diverse group of microbes even from different sources may be explored to obtain the even enhanced features bacterium from industrial points of view. In this book chapter, I have shown various microbes isolated for Biodesulfurization along with nature of problem of desulfurization/Biodesulfurization.

**Keywords:** desulfurization; hydrodesulfurization; organosulfur compounds; biodesulfurization; diverse group of microbes

1. **Sulfur Compounds in Crude Oil**

Sulfur is nearly ubiquitous in fossil fuels, where it arises both as inorganic (e.g., elemental sulfur, hydrogen sulfide and pyrites) and as organic sulfur (e.g., sulfur atom or moiety present in a wide variety of hydrocarbons for example, disulfides, mercaptans, sulfones, thiols, thiophenes, thioethers and other more complex forms) that can account for approximately to 100% of the total sulfur content of petroleum liquids, crude oil and many petroleum distillate fractions. Organic sulfur in crude oils can range from 5 wt % down to about 0.1 wt % [1]. Al-
though the organic forms are predominant with over 200 sulfur containing organic compounds [2]. These compounds can be divided in three groups: (I) aliphatic and aromatic thiols and its oxidation products (bisulfides); (II) aliphatic and aromatic thioethers; (III) heterocycles based in thiophenic ring: thiophene, benzothiophene (BT), dibenzothiophene (DBT) and its alkyl derivatives [3].

Petroleum recovered from different reservoirs varies widely in compositional and physical properties [4] having different concentration of sulfur (0.4 to 5.0 wt %). In general, a crude oil from a region of a higher density contains a higher percent of sulfur [5].

The organosulfur compounds in petroleum include thiols, thioethers, and thiophenes, but the sulfur compounds that predominate in the so-called heavy fractions, where sulfur content is the highest, are primarily the condensed thiophenes [2]. In some cases the sulfur content is very high, e.g., the crude oil in Utah and California at the USA and in Germany contain 13.9, 5.5, and 9.6% of sulfur, respectively [3]. Sulfur in gasoline is mainly found in thiophenic and non-thiophenic compounds, and in diesel oil is found in benzo and dibenzothiophenes [6]. Total sulfur content varies on average, organic and inorganic sulfur comprise equal amounts [7], although there are some exceptions [8]. Organic sulfur is present in several forms; the principal moieties are sulfides, disulfides, thiols and thiophenes, while inorganic moieties are found in the form of pyrites [7].

While refiners gain some advantage in using higher quality crude, the price premium more than cancels the cost advantage for low-sulfur fuel refining [9]. Since there are many different sulfur-containing compounds in petroleum-derived fuels, the sulfur content is usually expressed as the weight percent (wt %) of sulfur in the fuel as demonstrated on 78 different crude oil types [10]. As mentioned before, the most important constituents are organic sulfur compounds, but inorganic sulfur can also be present. An overview of the range of organic sulfur contents in crude oils found in different countries over the world is given here in Figure 1.1 below.

Sulfur compounds in crude oil include thiols, sulfides, polysulfides, thiophenic and alkyl substituted isomers of thiophenic compounds containing a variety of aromatic rings (i.e., polycyclic aromatic sulfur heterocycles such as thiophene, benzothiophene, dibenzothiophene and benzonaphthothiophene) which are carcinogenic. The molecular structures of some of these sulfur components are presented in Scheme 1.1.

Distribution and amount of organic sulfur compounds reflect the reservoir and maturity of the oil. Chemically immature oils are rich in sulfur and often have a high content of non-thiophenic sulfur compounds [12]. Mercaptans in crude oils are generally of low molecular weight (less than eight carbon atoms). They are readily removed from crude oil during refinery processing and are negligible in petroleum products.
Figure 1.1: Average organic sulfur contents in crude oils [11].

Scheme 1.1: Different types of sulfur-containing organic compound identified in crude oils

Aliphatic sulfides (cyclic or acyclic) are major components of the sulfur-containing fraction of petroleum products, e.g. diesel fuels and heating oils. Aromatic sulfides are of lower concentration in the heavier cuts. Thiophenic sulfur is normally the most plentiful form but as mentioned before, depending on the reservoir history of the oil, other sulfur compounds are often present in appreciable quantities. The unsaturated five member heterocyclic ring, thiophenes, is an important constituent of high-sulfur oils and its derivatives are the most abundant sulfur compound in distillates and residues, including heavy fuel oils and bitumens.

2. Problems due to Sulfur Compounds in Crude Oil

Most of the hydrocarbons mined from the Earth are burned for energy and since most
liquid and solid (i.e., oil and coal) reserves are contaminated with sulfur, direct combustion of this fuel will lead to the release of vast amounts of sulfur oxides into the atmosphere [13,14]. These oxides (together with acidic nitrogen oxides) are responsible for poor air-quality, acid rain [15] and for ozone layer depletion [16].

SO₂ is also responsible for various health hazards, such as respiratory tract cancer and cardio-respiratory diseases [16]. SO₂ concentrations >100ppm in the atmosphere are harmful to the respiratory system of humans and a short-period exposure to 400-500 ppm is lethal [17]. The mixing of SO₂ with dust in the atmosphere or with fog increases the noxious effect. The plant kingdom is also very sensitive to the SO₂ concentration; exposure to 1-2 ppm SO₂ provokes damages in few hours [17].

The presence of sulfur in oil has been related to the corrosion of pipeline, pumping, refining equipment, and with prior breakdown of combustion engines. Sulfur also contaminates many catalysts which are used in the refining and combustion of fossil fuels. Burning of gasoline and diesel emits sulfur dioxide (SO₂) or sulfate particulate matter leading to acid rain, which has harmful effects on aquatic and forest ecosystems, as well as on agricultural areas [18].

In oil spill accidents, some sulfur heterocyclic compounds are introduced in the environment. Some of these compounds (e.g. benzothiophene and its derivatives) present carcinogenic, mutagenic activities and acute toxicity to the organisms living in that ecosystem and environment. Condensed thiophenes are bioaccumulated in organism tissues, which associated with their mutagenic, carcinogenic and toxic potential; considerably contribute to the negative effect [7].

3. Biodesulfurization

Since 1935, when an early account of microbial desulfurization of crude oil was published [19,20] there have been expended lots of interest in applying biodesulfurization (BDS) processes in the oil industry to demonstrate this ability of microorganisms. In the early 1950s, a series of U.S. patents were issued covering the use of bacteria to reduce the sulfur content of petroleum. However, early attempts were not found to work because of inability to control the bacteria [18]. From 1970s to 1980s, the U.S. Department of Energy (DOE) and other organizations have sponsored work to explore this technology. The isolated bacteria were not appropriate for commercial BDS technologies at that time, because they attack the hydrocarbon portion [21]. In the late 1980s, bacteria that could liberate sulfur from DBT (as the model sulfur compound) without attacking the hydrocarbon were identified [22,23]. During these decades significant contributions have been made to construct and operate a large scale oil biodesulfurization process that involve many challenges and in order to develop biological desulfurization processes numerous attempts have been made up until the present time.
Biological processes require relatively mild conditions (low pressures and low temperatures), which could be a major advantage of BDS. It can be noticed that biocatalytic desulfurization offers the petroleum industry several benefits over hydrodesulfurization (HDS) processes: capital cost savings, operating cost saving, flexibility to handle a wide range of petroleum streams, more rapid engineering and construction time, safer and milder conditions. Testimonies of Naser [24] have indicated that by BDS technology, the small refinery at Woodlands (Texas) achieved capital cost savings of approximately 50 percent and operating cost saving of 10-20 percent. In addition to cost savings, BDS will result in up to 80 percent less greenhouse gas emissions over HDS, because bioprocess operates at essentially room temperature and pressure. The scheme of the biocatalytic desulfurization process is presented in Figure 1.3. The biocatalyst is mixed with water/media and transferred to the bioreactor. The biocatalyst slurry and petroleum containing sulfur compounds are mixed with oxygen and stirred continuously in a tank reactor. The petroleum is desulfurized and separated from the aqueous/biocatalyst output stream. The biocatalyst and water are separated and the sulfur by-product is removed from the process in the aqueous phase as sulfate, which can be disposed of sodium sulfate or ammonium sulfate. After the spent biocatalyst is removed the biocatalyst/water mixture is recycled to the bioreactor.

Basic microbiological researches have been done on the BDS mechanisms which have made a lot of progress in the recent years. Whereas significant removal of organic sulfur compounds from oil has not been demonstrated to date. To enable technological applications, to improve in knowledge on this field, the flux through this pathway must be enlarged considerably on activity, selectivity and stability of microorganisms applied in the process. For commercial applications, mixtures of microorganisms may be needed [10]. Therefore, currently genetic and metabolic engineering efforts are applied by researchers. They tried to identify and clone the genes involved in the sulfur removal pathway, therefore efficiency of biological process could be enhanced by increasing the number of copies of the genes, altering the gene to produce a more active or efficient product and increasing the amount of expression from each gene [25,26,27,28,29]. For example recombinants of Rhodococcus erythropolis containing multiple copies of the dsz-genes were used to study the conversion of sulfur compound [30,31].

4. Biodesulfurization Application

An industrial-scale process for petroleum biodesulfurization using aerobic microorganisms has not yet been demonstrated. However, through an improved understanding of the biochemistry and genetics of the desulfurization pathway, it is anticipated that improved biocatalysts with activities suitable for an industrial process will be developed [1]. Until the present date, studies on sulfur oxidative pathways have mainly been focused in model compounds, which limit the ability to demonstrate the commercial potential of BDS [32]. However, some
reported works involved several fractions of crude oil refining, including gasoline and diesel [33-38]. Efforts to increase the rate of sulfur removal from aromatic sulfur heterocycles have been possible due to the use of genetic engineering techniques or the use of immobilization matrices [39].

The selection of the petroleum feedstock in biodesulfurization will play a large role in the overall economic viability of the process. BDS may be utilized as a pre-treatment to crude oil before entering pipelines. It may also be applied as an alternative to hydro treating the crude at the refinery or it may be applied in the polishing of refinery products such as diesel or gasoline. As pre-treatment, the BDS unit may be used to treat marginally sour crudes (0.6-0.7% S), converting them to sweet crudes (<0.5% S). For this application, the extent of desired desulfurization is quite low, and this may serve as an attractive initial niche for BDS [40]. Inherent to all of the current bio processing of fossil feedstock’s schemes is the need to contact a biocatalyst-containing aqueous phase with an immiscible or partially miscible organic substrate [41]. Factors such as liquid-liquid and gas-liquid mass transport, amenability for continuous operation and high throughput, capital and operating costs, as well as ability for biocatalyst recovery and emulsion breaking, are significant issues in the selection of a reactor for aqueous-organic contacting [40]. Biodesulfurization studies of fossil fuels usually involved intact cells as biocatalyst, which avoids the Dsz enzymes purification and facilitate the BDS industrialization. The immobilization of cells can be used to desulfurize DBT efficiently [42] being the life-time of immobilized cell biocatalysts more than 600 h [43].

Traditionally, impeller-based stirred reactors are used for such mixing, because of their ease of operation and wide acceptance in the chemical and biological processing industries. This kind of reactors promotes the contact between the aqueous and organic phases by imparting energy to the entire bulk solution, achieving water or oil droplet sizes of 100-300 µm in diameter when surfactants are not present. To obtain droplets of about 5 µm, the energy consumed by the reactor will be 5-fold higher [44]. In BDS processes, oil is mixed with an aqueous medium that contains biocatalytically active bacterial cells. Recovery of oil from the oil-water-bacteria mixture follows the biodesulfurization step as a separated batch process [45].

To date, there are some microbial desulfurization studies at laboratory scale involving petroleum fractions and coal. Energy BioSystems Corporation (ECB) was the only commercial venture dedicated to the development of biodesulfurization technology [1]. EBC’s concept for a biodesulfurization process was not only to treat diesel, but also to produce a value-added surfactant byproduct to achieve a more economical process [13]. There was a plan to construct a demonstration-scale biodesulfurization process at the Petro Star refinery in Valdez, Alaska. The date for the construction of a demonstration plant was progressively postponed [1]. For the industrial application, DBT desulfurization was optimized by using response surface meth-


5. Oil Biodesulfurization

Early biodesulfurization research used model compounds like DBT, sometimes in aqueous systems bearing little resemblance to the conditions the biocatalyst would encounter in commercial applications [47]. In fact, the desulfurization rates of diesel oil were much smaller than those obtained for pure DBT [33].

Biodesulfurization of petroleum results in total sulfur removals between 30 and 70% for mid-distillates [48] 24 to 78% for hydro treated diesel [33,49,50], 20-60% for light gas oils [51,52] and 25-60% for crude oils [53]. Taking into account that BDS can be a complementary method to HDS, the study of fractions of pre-desulfurized oil is important. Grossman et al. (2001) reported a treatment by Rhodococcus sp. strain ECRD-1 of middle distillate oil whose sulfur content was virtually all substituted DBTs containing 669 ppm of total sulfur. Analysis of the sulfur content of the treated oil revealed that 92% of the sulfur had been removed, reducing the sulfur content from 669 ppm to 56 ppm.

In addition, studies of desulfurization with Rhodococcus erythropolis I-19, involving hydrodesulfurized middle distillate oil, showed that after 0, 1, 3 and 6 h, the sulfur concentrations were 1850, 1620, 1314 and 949 ppm, respectively. The first 230-ppm drop in total sulfur, observed after 1 h, corresponded primarily to a biotransformation of DBT and midboiling-range sulfur compounds. Between 1 and 3 h, another 300-ppm sulfur reduction occurred, with some evidence for more highly alkylated DBTs being affected. At 3 h, most of the DBT and much of the C1-DBTs were consumed. Between 3 and 6 h, desulfurization shifted to the higher-boiling-range sulfur compounds, resulting in an additional 365-ppm drop in total sulfur. Analysis of this middle distillate oil biodesulfurized from 1850 to 615 ppm sulfur showed the majority of the remaining sulfur to be thiophenes (75%), with 11% sulfides, 2% sulfoxides and 12% sulfones [31]. More recently, Zhang et al. (2007) reported a total reduction of 97% (to 6 µg ml-1) of the sulfur content of previous hydrodesulfurized diesel oil.

There are also some studies on desulfurization of oil fractions involving thermophilic bacteria such as Paenibacillus sp. [54]. When Paenibacillus sp. strains A11-1 and A11-2 were cultured in the presence of light gas oil containing 800 ppm of total sulfur at a high temperature, the bacteria grew well. Light gas oil is known to contain small amounts of sulfur and limited species of heterocyclic organosulfur compounds composed mainly of alkylated DBT derivatives. In conformity with the stimulated bacterial growth, the content of sulfur in the oil phase was significantly decreased, indicating that both Paenibacillus strains can desulfurize at high temperatures from the processed light gas oil.

However, these strains presented a very low desulfurization rate [55] lower than the
desulfurization rate obtained with *R. rhodochrous* [54]. The use of thermophilic bacteria has some advantages since it is not necessary to cool-down the oil fractions after the HDS, which makes this process less expensive [56].

Another advantage is the fact of reducing the possibility of contamination by undesirable bacteria that can negatively affect the BDS process [57]. Although the obtained removals are significant, this level of desulfurization is insufficient to meet the required sulfur levels for all oil derivatives [35].

8. The need for Enhancement of Biodesulfurization

Clean fuels research including desulfurization has become an important subject of environmental catalysis studies worldwide. Sulfur content in diesel fuel is an environmental concern because sulfur is converted to (sulfur oxides) \( \text{SO}_x \) during combustion, which not only contributes to acid rain, but also poisons the catalytic converter for exhaust emission treatment. The problem of sulfur removal has become more apparent due to the increasing high sulfur contents in remaining reservoirs of crude oils and the low limit of sulfur content required in finished fuel products by regulations specified by the authorities. It is impossible to have clean atmospheric air, or in particular to reduce air pollution from the transport sector, without removing sulfur from fuels. No significant air pollution reduction strategy can work successfully without reducing sulfur to near-zero level. The Environmental Protection Agency of the United States (EPA) has set a target to reduce the sulfur content of diesel from 500 ppm (2010 regulation) to 15 ppm for the year 2012 [58].

Hydrodesulfurization (HDS) involves the catalytic reaction of hydrogen and the organic matter in the feed, at pressures ranging from 5 up to 10 MPa and temperatures between 300 and 350°C, depending on the oil fraction and the required level of desulfurization [59,60]. Due to the high costs and inherent chemical limitations associated with HDS, alternatives for this technology are of great interest to the petroleum industry. The benefits of sulfur reduction would be more important than the costs, even though required refinery investments continue to be significant. The U.S. EPA found human health benefits and environmental profits due to sulfur reduction ten times higher than the costs [61]. Furthermore, a European study showed that ultralow sulfur fuels significantly reduce total fuel costs by increasing fuel economy from which the considerable potential for greenhouse gas emission reductions adds further payback to the health, environmental, and social benefits of sulfur reduction.

The global refining industry has spent about $37 billion on new desulfurization equipment and an additional $10 billion on annual operating expenditures for sulfur removal through the last 10 years to meet the new sulfur regulations [62]. More than 70 million barrels of crude are worldwide produced each day, of which half is considered to be "high sulfur" (>1%) Energy [63]. This concern will become more serious owing to the decreasing availability of low-
sulfur fuels. So in many areas of the world industry needs new technologies to reduce sulfur to the ultralow levels now acceptable in use. Current costs might be reasonable; the refining industry continues to develop more active catalysts and novel processes for the removal of sulfur in order to reduce costs even more.

9. Attempts Made for Improved Biodesulfurization

9.1. Search for new strains

In order to develop biologically enhanced desulfurization technology, a range of bacteria have been previously isolated which have been shown to be able to remove sulfur from organic compounds that commonly exist in petroleum [65]. However, this activity is unlikely to be sufficient for commercial applications where the requirement is for microorganisms with high activity and selectivity for different sulfur compounds [66]. To achieve this requirement, there needs to be about a 500-fold increase in the rate of biodesulfurization of currently used bacteria [1]. Therefore due to a range of problems (one of which is the low desulfurization capability of the bacteria employed, which makes the process uneconomical) this technology area needs to be enhanced by new developments. Bacteria, which exist in the oil fields, have a great potential to degrade sulfur compounds in fuel [67]. Research over the last few years has shown that oil fields contain microbial communities that influence the petroleum quantity and yield of petroleum produced and its quality. Professionals in the petroleum industry today clearly understand the critical role that regulating microbial activity has in enhancing the beneficial effects. Accordingly newly isolated strains of bacteria continue to attract attention for their potential application to desulfurization. Since DBT is a typical recalcitrant organic sulfur compound in petroleum, it has been a model reactant in the treatment of oil [68, 69]. In 1985 a strain of *Pseudomonas* that could desulfurize DBT was described by Isbister and Koblynski, but unfortunately before the metabolic pathway could be fully characterized, this strain was lost [70]. After 40 years of research effort, Kilbane isolated a suitable bacterium named *Rhodococcus erythropolis* IGTS8 (Kilbane, 1990). Since then many researchers have isolated bacteria capable of degrading DBT via the 4S pathway and a variety of DBT-desulfurizing bacteria have been reported. A list of which has been given by Mohebali and Ball, table 1.1.
**Table 1.1:** List of isolated bacteria those are capable of selectively degrading DBT and its derivatives via the 4S pathway [71].

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agrobacterium</em> sp. strain MC501</td>
<td>Constanti et al. (1994)</td>
</tr>
<tr>
<td><em>Arthrobacter</em> sp. strain ECRD-1</td>
<td>Lee et al. (1995)</td>
</tr>
<tr>
<td><em>Arthrobacter sulfureus</em></td>
<td>Labana et al. (2005)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> strain WU-S2B</td>
<td>Kirimura et al. (2001)</td>
</tr>
<tr>
<td><em>Bacillus brevis</em> strain R-6</td>
<td>Jiang et al. (2002)</td>
</tr>
<tr>
<td><em>Bacillus sphaericus</em> strain R-16</td>
<td>Jiang et al. (2002)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> strain Fds-1</td>
<td>Ma et al. (2006)</td>
</tr>
<tr>
<td><em>Corynebacterium</em> sp. strain SY1</td>
<td>Omori et al. (1992)</td>
</tr>
<tr>
<td><em>Corynebacterium</em> sp. strain P32C1</td>
<td>Maghsoudi et al. (2000)</td>
</tr>
<tr>
<td><em>Corynebacterium</em> sp. strain ZD-1</td>
<td>Wang et al. (2006)</td>
</tr>
<tr>
<td><em>Desulfovibrio desulfuricans</em></td>
<td>Yamada et al. (1968)</td>
</tr>
<tr>
<td><em>Gordona</em> sp. strain CYKS1</td>
<td>Rhee et al. (1998)</td>
</tr>
<tr>
<td><em>Gordona</em> sp. strain WQ-01</td>
<td>Jia et al. (2006)</td>
</tr>
<tr>
<td><em>Gordonia</em> alkanivorans strain 1B</td>
<td>Alves et al. (2005)</td>
</tr>
<tr>
<td><em>Gordonia</em> sp. strain F.5.25.8</td>
<td>Duarte et al. (2001)</td>
</tr>
<tr>
<td><em>Gordonia</em> sp. strain ZD-7</td>
<td>Li et al. (2006)</td>
</tr>
<tr>
<td><em>Gordonia</em> alkanivorans RIPI90A</td>
<td>Mohebali et al. (2007)</td>
</tr>
<tr>
<td><em>Klebsiella</em> sp.</td>
<td>Dudley &amp; Frost (1994)</td>
</tr>
<tr>
<td><em>Mycobacterium</em> sp. strain G3</td>
<td>Nekodzuka et al. (1997)</td>
</tr>
<tr>
<td><em>Mycobacterium</em> sp. strain X7B</td>
<td>Nekodzuka et al. (1997)</td>
</tr>
<tr>
<td><em>Mycobacterium</em> sp. strain ZD-19</td>
<td>Li et al. (2003)</td>
</tr>
<tr>
<td><em>Mycobacterium</em> goodii strain X7B</td>
<td>Chen et al. (2008)</td>
</tr>
<tr>
<td><em>Mycobacterium phlei</em> strain SM120-1</td>
<td>Srinivasaraghavan et al. (2006)</td>
</tr>
<tr>
<td><em>Mycobacterium phlei</em> strain GTIS10</td>
<td>Kayser et al. (2002)</td>
</tr>
<tr>
<td><em>Nocardia globelula</em></td>
<td>Wang &amp; Krawiec (1994)</td>
</tr>
<tr>
<td><em>Nocardia globerula</em> strain R-9</td>
<td>Jiang et al. (2002)</td>
</tr>
<tr>
<td><em>Nocardia asteroids</em></td>
<td>Olson (2000)</td>
</tr>
<tr>
<td><em>Paenibacillus</em> sp. strain A11-2</td>
<td>Konishi et al. (1997)</td>
</tr>
<tr>
<td><em>Pseudomonas abikonensis</em> strain DDA109</td>
<td>Yamada et al. (1968)</td>
</tr>
<tr>
<td><em>Pseudomonas jianii</em> strain DDC279</td>
<td>Yamada et al. (1968)</td>
</tr>
<tr>
<td><em>Pseudomonas jianii</em> strain DDE 27</td>
<td>Yamada et al. (1968)</td>
</tr>
<tr>
<td><em>Pseudomonas delafeldii</em> strain R-8</td>
<td>Jiang et al. (2002)</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp. strain ARK</td>
<td>Honda et al. (1998)</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> strain CECT5279</td>
<td>Alcon et al. (2005)</td>
</tr>
<tr>
<td><em>Rhodococcus erythropolis</em> strain IGTS8</td>
<td>Kilbane (1992)</td>
</tr>
<tr>
<td><em>Rhodococcus</em> sp. strain UM3</td>
<td>Purdy et al. (1993)</td>
</tr>
<tr>
<td>Strain</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Rhodococcus sp. strain UM9</td>
<td>Purdy et al. (1993)</td>
</tr>
<tr>
<td>Rhodococcus erythropolis strain D-1</td>
<td>Izumi et al. (1994)</td>
</tr>
<tr>
<td>Rhodococcus sp. strain ECRD-1</td>
<td>Lee et al. (1995)</td>
</tr>
<tr>
<td>Rhodococcus erythropolis strain H-2</td>
<td>Ohshiro et al. (1995)</td>
</tr>
<tr>
<td>Rhodococcus sp. strain X309</td>
<td>Omori et al. (1995)</td>
</tr>
<tr>
<td>Rhodococcus sp. strain B1</td>
<td>Denis-Larose et al. (1997)</td>
</tr>
<tr>
<td>Rhodococcus erythropolis strain I-19</td>
<td>Denis-Larose et al. (1997)</td>
</tr>
<tr>
<td>Rhodococcus erythropolis strain KA2-5-1</td>
<td>Folsom et al. (1999)</td>
</tr>
<tr>
<td>Rhodococcus sp. strain P32C1</td>
<td>Kobayashi et al. (2000)</td>
</tr>
<tr>
<td>Rhodococcus sp. strain T09</td>
<td>Maghsoudi et al. (2000)</td>
</tr>
<tr>
<td>Rhodococcus sp. strain IMP-S02</td>
<td>Matsui et al. (2001)</td>
</tr>
<tr>
<td>Rhodococcus sp. strain DS-3</td>
<td>Akbarzadeh et al. (2003)</td>
</tr>
<tr>
<td>Rhodococcus sp. strain XP</td>
<td>Labana et al. (2005)</td>
</tr>
<tr>
<td>Rhodococcus sp. strain 1awq</td>
<td>Yu et al. (2006)</td>
</tr>
<tr>
<td>Rhodococcus erythropolis strain XP</td>
<td>Ma et al. (2006a)</td>
</tr>
<tr>
<td>Rhodococcus erythropolis strain DS-3</td>
<td>Yu et al. (2006)</td>
</tr>
<tr>
<td>Rhodococcus erythropolis strain DR-1</td>
<td>Ma et al. (2006b)</td>
</tr>
<tr>
<td>Rhodococcus erythropolis strain NCC-1</td>
<td>Li et al. (2007a)</td>
</tr>
<tr>
<td>Rhodococcus erythropolis strain LSSE8-1</td>
<td>Li et al. (2007b)</td>
</tr>
<tr>
<td>Sphingomonas sp. strain AD109</td>
<td>Zhang et al. (2007)</td>
</tr>
<tr>
<td>Sphingomonas subarctica strain T7b</td>
<td>Darzins &amp; Mrachko (1998)</td>
</tr>
<tr>
<td>Xanthomonas sp.</td>
<td>Gunam et al. (2006)</td>
</tr>
<tr>
<td>Gordonia sp. IITR100</td>
<td>Ahmad et al. (2014)</td>
</tr>
</tbody>
</table>

9.2. Bacteria, characterized for the desulfurization of model thiophenic compound i.e. dibenzo thiophene and benzothiophene

Heterocyclic compounds such as dibenzo thiophene, benzothiophene and their alkylated derivatives have been found to be very recalcitrant against the attacks by the chemical catalysts. Henceforth, development of the catalysts capable of desulfurizing these HDS-resistant thiophenes is now needed for deeper desulfurization of petroleum fractions. Biodesulfurization (BDS) in which microbial catalysts are used to remove sulfur from the petroleum fractions has come to public notice as an environmentally benign process (Monticello et al., 1995) as mentioned earlier. It may be the best choice to employ a microbial catalyst capable of desulfurizing dibenzo thiophene, benzothiophene, benzonaphthothiophene and their alkyl derivatives in the BDS process.

Many microorganisms such as Rhodococcus sp. IGTS8 [72], Rhodococcus erythropolis
D-1 [73] and N1-36 [74] have been reported to mediate desulfurization of dibenzo thiophene. The reaction is accompanied by the production of sulfite ions and 2-hydroxybiphenyl. In nature, many bacterial strains capable of desulfurizing dibenzo thiophene have also been reported to desulfurize benzothiophene. On the other side, it has been shown that benzothiophene is also susceptible to microbial transformation. Fedorak et al. have reported that a *Pseudomonas* strain BT1 converted benzothiophene to benzothiophene-2, 2-dione and 3-methyl benzothiophene to its sulfoxide and sulfone [75]. Boyd *et al.* (1993) have demonstrated that a toluene-degrading mutant strain *Pseudomonas putida* UV4 transforms benzothiophene to three dihydrodiols; cis-4, 5-dihydro benzothiophene, cis- and trans-2, 3-dihydro benzothiophene. However, none of these bacteria produced desulfurized metabolites from benzothiophene and its derivatives. *Gordonia* sp. strain 213E (NCIMB 40816) desulfurizing benzothiophene had been isolated and it had been shown that this strain cannot grow in a mineral salt medium containing dibenzothiophene as the sole sulfur source [76]. Thermophilic *Paenibacillus* sp. strain A11-2 that desulfurize dibenzo thiophene [77], was found to degrade benzothiophene. *Klebsiella* sp. 13T was found to remove 22-53 % of sulfur from different petroleum oils [78].

*G. alkanivorans* strain 1B can remove selectively the sulfur from DBT producing 2-hydroxybiphenyl the only detected metabolic product. In equimolar mixture of DBT/ BT as a source of sulfur in the growth medium, *G. alkanivorans* strain 1B utilized both compounds in a sequential way; BT was the preferred source of sulfur. When BT concentration was decreased to a very low level, then only DBT was utilized as the source of sulfur for bacterial growth [79]. *Rhodococcus* sp. KT462, which can grow on either benzothiophene (BT) or dibenzothiophene (DBT) as the sole source of sulfur, was newly isolated and revealed that strain KT462 has the same BT desulfurization pathway as that reported for *Paenibacillus* sp. A11-2 and *Sinorhizobium* sp. KT55. The desulfurized product of DBT produced by this strain, as well as other DBT-desulfurizing bacteria such as *R. erythropolis* KA2-5-1 and *R. Erythropolis* IGTS8 was 2-hydroxybiphenyl. A resting cells of this strain can degrade various alkyl derivatives of BT and DBT [80]. Sinorhizobium sp. KT55 was the first Gram-negative isolate to be capable of utilizing benzothiophene as the sole source of sulfur. Benzothiophene desulfurization pathway of this strain is 3-benzothiophene sulfoxide, 3-benzothiophene sulfone, 3-benzo[e][1, 2] oxathiin S-oxide, 3-o-hydroxystyrene [81,82].

A benzothiophene-desulfurizing bacterium with novel desulfurization pathway was isolated and identified as *Gordonia rubropertinctus* strain T08. Metabolites formed were detected to be benzothiophene sulfoxide, benzothiophene sulfone, benzo[e]-[1,2]oxathiin S-oxide (BT-sultine), benzo[1,2]oxathiin S,S-dioxide (BT-sultone), o-hydroxystyrene, & 2-coumaranone, but not 2-(2’-hydroxyphenyl)ethan-1-al, that has been reported to be a desulfurized product of mesophilic Nocardio forms [83]. Naphtho [2, 1-b] thiophene (NTH) is an asymmetric structural isomer of dibenzo thiophene (DBT), NTH derivatives can also be detected in diesel oil
following hydrodesulfurization treatment. *Rhodococcus* sp. strain WU-K2R could grow in a medium with NTH as the sole source of sulfur. WU-K2R could also grow on NTH sulfone, benzothiophene (BTH), 3-methyl-BTH, or 5-methyl-BTH as the sole source of sulfur but could not utilize DBT, DBT sulfone, or 4,6-dimethyl-DBT. On the other hand, WU-K2R did not utilize NTH or BTH as the sole source of carbon. Desulfurized NTH metabolites were identified as NTH sulfone, 2′-hydroxynaphthylethene, and naphtha [2, 1-b] furan. Moreover, since desulfurized BTH metabolites were identified as BTH sulfone, benzo[c] [1,2] oxathiin S-oxide, benzo[c] [1,2] oxathiin S, S-dioxide, o-hydroxystyrene, 2-(2′-hydroxyphenyl) ethan-1-al, and benzofuran, thus WU-K2R desulfurized NTH and BTH through the sulfur-specific degradation pathways with the sulfur selective cleavage of carbon-sulfur bond [84]. The biodesulfurization of DBT by resting cells of *Pseudomonas putida* CECT5279 was enhanced by 140 % in a batch process and 122 % in a fed-batch process, in the presence of a co-substrate 1.5 % acetic acid [85].

9.3. *Bacteria desulfurizing sulfidic and thiophenic organosulfur compounds*

Large reserves of different heavy crude oils which have high viscosity, correlated to the average molecular weight of the material and to the asphaltene content have been reported [86, 87]. Transportation of such heavy and viscous oils by pipeline requires the addition of low molecular weight hydrocarbon diluents, which are increasingly expensive and hard to manage. Decreasing average molecular weight of the crude oil on-site before it is sent by pipeline to the refinery would decrease reliance on diluents and make transportation more economic and easier. In particular, reducing the molecular weight of the asphaltene fraction could contribute to a decrease in the viscosity of the oil. According to the molecular model proposed by [88], the asphaltenes found in different heavy crude oils are polycyclic aromatic structures linked by aliphatic chains of various lengths. Ethers sulfides and esters have been identified as linking structures in these aliphatic bridges [89]. Selective chemical cleavage of aliphatic sulfide bonds results in as much as a fourfold reduction in the molecular weight of heavy asphaltene fractions [89]. Biocatalytic cleavage of these carbon-sulfur bonds should achieve the same effect.

The use of a biocatalyst avoids the problems of available technologies for viscosity reduction such as avoiding liberation of hydrogen sulfide, thermal visbreaking and the production of unstable oil. The focus of petroleum biodesulfurization research has been on the aromatic compound dibenzothiophene and on alkyl-substituted dibenzothiophenes. Several bacteria can remove the sulfur from dibenzothiophene by the 4S pathway, leaving the carbon structure intact as 2-hydroxybiphenyl [90,91,92]. Infact, this kind of attack would not reduce the molecular size of asphaltenes and therefore is inappropriate for the viscosity reduction approach. Instead, the ideal biocatalyst would affect selective carbon-sulfur bond cleavage in a broad range of aliphatic sulfides, but would not attack aromatic sulfur compounds such as
dibenzothiophene. Thus, the goal is not to achieve general biodesulfurization of the crude oil, but rather to target cleavage of key carbon-sulfur bonds that would depolymerise asphaltenic molecules and decrease the crude oil viscosity.

There are comparatively few reports of the degradation of aliphatic sulfides, particularly of carbon-sulfur bond cleavage in high molecular weight representatives of this type of compounds. Smaller compounds, including methyl, ethyl, propyl and butyl sulfides [93,94], 2-chloroethyl ethyl sulfide [95,96], are cleaved at the sulfur atom. Jenisch-Anton et al. (2000) showed bacterial degradation of the n-alkyl chain of phytanyl octadecyl sulfide and Van Hamme et al. (2003) showed fungal oxidation of the sulfur atom in dibenzyl sulfide, but carbon-sulfur bond cleavage was not observed in these larger compounds. A mutant of the dibenzothiophene-desulfurizing bacterium *R. erythropolis* strain IGTS8 is able to use di-n-octyl sulfide as a sulfur source, but this compound is not a substrate for the wild-type organism [97]. Recently, Van Hamme et al. (2004) used the fluorinated compound bis-(3-pentafluorophenylpropyl) sulfide to isolate *Rhodococcus* sp. strain JVH1, which uses this compound as a sulfur source by a mechanism analogous to the 4S pathway, although JVH1 does not use dibenzothiophene as a sulfur source.

Compared to sulfur-containing ring structures such as thiophenes and DBTs, relatively little information is available on the microbial metabolism of compounds with sulfur moieties present within alkyl chains. These structures are important as bridges in the high-molecular-weight asphaltene components of petroleum [98]. Therefore, biological attack on sulfides is of considerable interest for biological heavy-oil viscosity reduction. Bioremediation efforts directed towards Yperite [bis (2-chloroethyl) sulfide; mustard gas] contamination have prompted some work in this area. For example, IGTS8 is able to use 1-chloroethyl sulfide as the sole sulfur source [99]. Rhee et al. (1998) reported that the DBT-desulfurizing *Gordona* strain CYKS1 can also use dibenzyl sulfide (DBS) as the sole sulfur source, but the intermediate metabolites were not identified. In a study on fungi, Itoh et al. (1997) reported that *T. versicolor* IFO 30340 and *Tyromyces palustris* IFO 30339 metabolize DBS to benzyl alcohol and benzyl mercaptans. Similarly, *Rhodococcus* sp. strain SY1 reportedly converts dibenzyl sulfoxide to benzyl alcohol and toluene [100], scheme 1.6.
Scheme 1.6: Pathways reported for the desulfurization of DBS by [101,102].

9.4. Bacteria desulfurizing non-thiophenic organosulfur compounds

Studies on the desulfurization of non-thiophenic sulfur compounds like 1, 4-dithiane or thianthrene, however, are scarce [103,104]. Thus, characterization of three bacterial strains *Rhodococcus erythropolis* EPWF, *Pseudomonas* sp. K1oA and *Rhodococcus* sp. K1bD has been reported, which were able to grow on 1, 4-Dithiane under sulfur limiting conditions [103]. No metabolites, however, were detected in either of the culture extracts. Further, while the desulfurization of 1, 4-dithiane by *Rhodococcus* sp. K1bD was inhibited by >90% in presence of DBT [103], results with other bacteria are not available. Similarly, growth of an isolated strain *Rhodococcus* sp IGTS8 by using TA as sulfur source [105], and its oxidation by a thermophilic Sulfolobus acidocaldarius strain, has been shown [106]. But, again no information is available about the formed metabolites. In another study, oxidation of TA to TA-monosulfoxide by the ligninase from *Phanerochaete chrysosporium*, in the presence of hydrogen peroxide, has also been reported [107]. No reports, however, are available where in influence of other sulfur compounds on the desulfurization of TA has been evaluated.

9.5. Genetic engineering approaches to improve biodesulfurization

Even with this number of isolated bacteria, the desulfurization activity of naturally occurring bacterial cultures is not high enough for the requirements of industry and a successful commercial process is still awaited. To achieve this, therefore we need to isolate new species and identify the genes responsible for desulfurization and manipulate the system involved by genetic engineering techniques and it is highly likely that future biodesulfurization research will focus on development of this promising research area along these lines. One of the popular strategies in metabolic engineering of the bacteria is to change host strains in order to take
advantage of another strain’s properties to get a higher metabolic rate. For example, several research studies have focused on over expression of the enzymes involved in microbial desulfurization.

The DNA encoding the Dsz enzymes can be transferred into a host cell [108] or the gene amplified with designed primers [109]. Matsui et al. (2001) used a recombinant strain capable of desulfurizing both DBT and BT as the sole source of sulfur [110, 111]. To improve the uptake of sulfur compounds in oil fractions, Watanabe et al. (2003) transferred the dsz genes from Rhodococcus erythropolis KA2-5-1 into the Rhodococcus erythropolis MC1109. The desulfurization activity of the new strain was about twice that of the previous strain. In order to enhance the expression of the genes involved, the dsz genes from Rhodococcus erythropolis DS-3 were integrated into the Bacillus subtilis and yielded recombinant strains with higher desulfurization efficiency [112]. Li et al. (2007) enhanced the desulfurization ability of Rhodococcus erythropolis DR-1 by removing the gene overlap in the operon.

Other efforts were made include (1) supplying the oxidoreductase gene from Vibrio harveyi, which supplements the DszD activity in Trans, and enhanced the activity by around 7 fold [113]. (2) By supplementing the existing dsz genes in R.erythropolis KA2-5-1by a plasmid that contained two additional copies of dszABC genes and one dszD gene which led to an increase by 4 fold [114]. (3) By making a transposon mutant in the membrane leading to enhancement in the uptake of organosulfur compounds and increase in the desulfurization activity by 2 fold [115]. (4) By supplying the dszB gene in a plasmid where 16 nucleotides in the 5'-untranslated region were changed led to an increase in the activity by 9 fold [116]. (5) By expressing the desulfurization genes from Mycobacterium sp.G3 under phsp60 promoter leading to an increase in activity by 1.2 fold [117,6] changing the order of desulfurization genes from dszABC to dszBCA which led to an increase in activity by 12 fold [118]. Separately, in a chemostat approach a gain of function phenotype i.e. capability to additionally desulfurize 5-methyl benzothiophene and octyl sulfide appeared due to a mutation V261F in dszC gene [119].

10. Bottlenecks for Biodesulfurization Application

A significant stumbling block to the commercialization of BDS is the rate at which whole bacterial cells can remove sulfur [120]. Biocatalyst activity, the oil/water volume ratio and biocatalyst stability constitute the most important technical bottlenecks in the development of biodesulfurization processes. The highest bioconversion values were obtained by unspecific aerobic microorganisms such as Rhizobium meliloti [1200 mg DBT removed (g⁻¹(DCW) h⁻¹)]. However, these destroy the hydrocarbon structure of the sulfur compound [121]. The involvement of three enzymes and two coenzymes in biocatalytic desulfurization makes the use of isolated free or immobilized enzymes difficult. Consequently, schemes using whole cells ap-
pear more feasible because they will allow cofactor regeneration in situ. Another problem to the implementation of a BDS process is the fact that the sulfur requirement of bacteria is low when compared to the level of sulfur found in fuels. In *Rhodococcus* sp., the cells were found to require 0.1 mM of sulfur for normal growth [122]. Only 1% of bacterial dry weight is sulfur [123], which implies a very low need in relation to this element. The sulfur content in fossil fuels is about 100 mM and thus, the bacteria cease desulfurization before its total removal.

The utilization of organic solvents and emulsifiers supports protein solubilisation and enzymatic reactions in hydrophobic environments [124]. These compounds allow the organo-sulfur compounds, which have very low water solubility, to be more available to enzymes and microbial cells [125].

As mentioned above, changing the genes of the host cell for those involved in desulfurization is a popular strategy in metabolic engineering to take advantages of desirable properties another strain such as its physical properties, growth properties or higher intrinsic metabolic rate. Since these are not yet understood, a better understanding of the factors that contribute to the biodesulfurization pathway is needed so as to achieve high level expression of the gene [1] and future research to isolate new strains and identify the biocatalyst would be helpful to develop this promising research area.

Despite significance progress in biodesulfurization, the desired rates and broad substrate range are yet to be realized and more efforts are needed in this direction. Recently, we described characterization of a bacterium *Gordonia* sp. IITR100, isolated from a petroleum-contaminated soil which affects the desulfurization of several organosulfur compounds thiophenic (DBT), aliphatic (DBS) and non-thiophenic (thianthrene) [126,127,128,129]. Pathway and genes of desulfurization of DBS, Thianthrene, Benzonaphathothiophene have been characterized by *Gordonia* sp. IITR100 which is in accordance of 4S pathway [126,12,128,129,130]. Final metabolites of Dibenzyl sulfide was well characterized by the using the analytical technique of GC/GC-MS, and LC/LC-MS, was found to be Benzoic acid. Optimization of this strain has also been reported for desulfurization of crude oil, petrol and diesel. 76% sulfur reductions in crude oil, 98% sulfur reduction in diesel were reported by *Gordonia* sp. IITR100 [131]. Finding such a industrially applicable bacterium, this IITR100’s whole genome sequence have been elucidated recently, [132].

Here it can be concluded that if efforts were being made, microbes may be employed for industrial Biodesulfurization which would be cheap in comparison to current process of costly Hydrodesulfurization and deep desulfurization. So more emphasis should be given to microbes which have shown potential for wide substrate range, high temperature tolerance and better activity for sulfur removal from crude oil, diesel and petrol.
11. References


Fermentations have been playing a very important role in society since the knowledge helped in production of valuable products. Since inception, fermentation has supported in production of variety of value added products like beverages, solvents, enzymes, vitamins, growth factors etc.

Recently, there has been a drastic diversification of usage of variety of substrate replacing traditional substrates for fermentation. Now a days Solid State Fermentation has gained momentum for production of bioactive molecules due to its benefits over traditional fermentation substrates.

1. Introduction

Bioconversions popularly labelled as fermentations can be defined as breakdown of complex organic substances into simple substances either by micro-organisms directly or by their enzymes to produce economically important products.

Pathway of Production: - The bioconversion of complex substrate to variety of secondary metabolites requires multistep enzymatic reactions. Basically the complete bioconversion can be categorized in two stages:-

(a) Production of Primary Metabolites

(b) Production of Secondary Metabolites

(a) Production of Primary Metabolites: - This is a primary or preliminary stage where all catabolic reaction takes place. Complex molecules are broken down into their constituents. The immediate products of primary breakdown are called primary metabolites. Primary metabolites are products which are essential for the microbes for self growth and sustainment as
the microorganisms multiply and increase the total biomass of the fermentative stuff.

**(b) Production of Secondary Metabolites** :- These are the additional compounds produced after the organisms complete log phase and initiates stationary phase. The secondary metabolites are also called as Bioactive compounds due to their beneficial applications in pharmaceuticals, food and health market [1,2,3].

Secondary metabolites have a wide range of applications. The economically important products are antibiotics, peptides, enzyme and growth factors [4,5,6].

**Figure1:** Underlying principle of fermentation

2. **Fermentation Types:- Submerge Fermentation and Solid State Fermentation**

The Submerge Fermentations are well known traditional style of fermentation exemplified by wine production, enzyme production etc. where the substrate is in liquid medium. The most common substrate are molasses, corn steep liquor, broths etc. Since utilization of substrate is fast hence the nutrients are required to be constantly replenished. Generally, bacterial growth is highly favoured in nutrients dissolved in liquid medium.

SSF:- Agriculture and animal productions have been flourishing since decades [7] resulting in generation of wastes which remain underexploited. The valorization of such agro industrial wastes is the upcoming alternative as fermentation substrates in SSF. The commonly used substrates for SSF are categorized in the final **Table1.**
### 3. References


11. Maragkoudakis PA, Mountzouris KC, Psyrras, D., Cremonese, S., Fischer, J., Cantor, M.D. and Tsakalidou, E.


Enzymatic Degradation of Lignocellulosic Biomass: A Brief Update

Mohd Jahir Khan1*; Abrar Ahmad2; Mahmood Ahmad Khan3; Mohammd Kashif 4; Anjum Bee5; Mohamad Yusof Maskat6

1 School of Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India
2 Environmental Biotechnology Division, CSIR-Indian Institute of Toxicology Research, Mahatma Gandhi Marg, Lucknow 226001, India
3 Department of Biochemistry, University College of Medical Sciences and GTB Hospital, University of Delhi, Dilshad Garden, Delhi 110095, India
4 Plant Molecular Biology and Genetic Engineering Division, CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, India.
5 Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar University, Vidya Vihar, Rae Bareli Road, Lucknow 226025, India.
6 School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Selangor DE, Malaysia.

*Correspondence to: Mohd Jahir Khan, School of Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India
Tel: +91-7210007791; Email: khanmohdjahir@gmail.com

Abstract

Lignocellulose comprises mainly cellulose, hemicellulose and lignin is the Earth’s most abundant renewable source. It is a promising feedstock from which to produce biofuels, chemicals, sugars, and materials. Lignocellulose is complex biopolymer therefore a cost effective consolidating bioprocessing microbes that directly convert lignocellulose into valuable end products are exploited. Microbes degrade lignocellulose by producing a battery of enzymes that work synergically. In the near future, processes that uses lignocellulolytic enzymes could lead to new, environmentally friendly technologies. Diverse mechanisms are used by organisms particularly glycoside hydrolases to deconstruct lignocellulose. Lignin depolymerisation is achieved by white-rot fungi and certain bacteria, using peroxidases and
laccases. This study reviews an overview of enzymatic degradation of cellulose, hemicellulose and lignin. In addition, production of lignocellulolytic enzymes by different microorganisms are also outlined.

**Keywords**: Biomass; Lignocellulose; Biodegradation; Enzymatic degradation

1. **Introduction**

Production of ethanol from cellulosic biomass utilizes enzyme was first carried out in 1980s by US Department of Energy. Although enzymatic hydrolysis of cellulose displayed several advantages such as high yield, low energy cost and operating conditions, it was thought that the technology was too risky for industry to pursue at that time. Later, advancement in biotechnology reduced processing cost made cellulosic ethanol competitive [1]. Lignocellulose is about half of the matter produced by photosynthesis and considered as an alternative source of energy. It is composed of cellulose, hemicellulose and lignin, strongly intermeshed and chemically bonded by covalent and non-covalent forces [2-4]. Besides these, some other materials such as proteins, pectin and ash are present in very less quantity. The proportion of cellulose, hemicellulose and lignin varies depends on the source of origin (Table 1). Forestry, agriculture, pulp/paper industry and municipal solids are the main source of lignocellulose biomass. Although a huge amount of lignocellulose produced, the only small portion is used in value-added products like ethanol, food additives, organic acids and pharmaceutical building blocks, rest being considered waste [5-8]. These valuable materials are treated as waste since a long time in some developing counties, which raises numerous environmental concerns [9-11].

Hydrolysis of lignocellulose into simple sugars can be achieved either by enzymes or chemically with sulfuric or other acids. While enzymatic hydrolysis displayed several advantages over acid hydrolysis as it entails lesser energy and mild environmental conditions with a generation of fewer fermentation inhibitors but it seems to be a bottleneck due to the high costs of enzyme production. Therefore, continuous efforts have been made for cost-effective production and search for new sources of enzymes. The complex structure of lignocellulose makes it recalcitrant for enzymatic degradation. Additionally, some enzyme absorbed with condensed lignin by non-specific linkages which decreases hydrolysis yield [12-15].

2. **Lignocellulytic Enzymes Produced by Microorganisms**

Different range of microorganisms, mainly fungi and bacteria have been identified over several years which are producing lignocellulolytic enzymes. These microorganisms depolymerize lignocellulose via a series of hydrolytic and or oxidative enzymes comprising lignin peroxidases, magnese peroxidases, versatile peroxidases, laccases, endoglucanases, cellulbiohydrolases and β-glucosidases. These enzymes broadly studied in a laboratory as submerged and solid culture processes ranging from flask shake to large scale [29-31]. Since bacterial systems lacking lignin peroxidases, therefore, biodegradation of lignocellulose in bacteria is es-
sentially a slow process. Grasses are more susceptible than wood for actinomycete and play a substantial role in humification processes together with bacteria in soils and composts [32-34]. Bacterial enzymes can cleave alkyl-aryl ether bonds in oligomeric and monomeric aromatic compounds, released by fungi during lignin decomposition [35-37]. Therefore, degradation of lignocellulose by prokaryotes is of ecological important while in fungi it is of commercial significance.

Table 1: Percent dry weight composition of lignocellulose materials in some common feedstocks.

<table>
<thead>
<tr>
<th>Lignocellulosic material</th>
<th>Cellulose (wt%)</th>
<th>Hemicellulose (wt%)</th>
<th>Lignin (wt%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty palm fruit bunch</td>
<td>59.7</td>
<td>22.1</td>
<td>18.1</td>
<td>[16]</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>43.8</td>
<td>27.0</td>
<td>22.6</td>
<td>[17]</td>
</tr>
<tr>
<td>Paper</td>
<td>85-99</td>
<td>0</td>
<td>0-15</td>
<td>[18]</td>
</tr>
<tr>
<td>Hardwood</td>
<td>40-55</td>
<td>24-40</td>
<td>18-25</td>
<td>[18, 19]</td>
</tr>
<tr>
<td>Softwood stems</td>
<td>45-50</td>
<td>25-35</td>
<td>25-35</td>
<td>[18,19]</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>41.3</td>
<td>30.8</td>
<td>7.7</td>
<td>[20]</td>
</tr>
<tr>
<td>Rice straw</td>
<td>32.1</td>
<td>24</td>
<td>18</td>
<td>[21]</td>
</tr>
<tr>
<td>Barley straw</td>
<td>31-34</td>
<td>24-29</td>
<td>14-15</td>
<td>[22]</td>
</tr>
<tr>
<td>Sunflower stalks</td>
<td>33.8</td>
<td>20.2</td>
<td>17.3</td>
<td>[23]</td>
</tr>
<tr>
<td>Leaves</td>
<td>15-20</td>
<td>80-85</td>
<td>0</td>
<td>[18]</td>
</tr>
<tr>
<td>Office paper</td>
<td>68.6</td>
<td>12.4</td>
<td>11.3</td>
<td>[24]</td>
</tr>
<tr>
<td>Corn cobs</td>
<td>42.7</td>
<td>34.3</td>
<td>18.4</td>
<td>[25]</td>
</tr>
<tr>
<td>Bamboo</td>
<td>26-43</td>
<td>15-26</td>
<td>21-31</td>
<td>[26]</td>
</tr>
<tr>
<td>Coconut fiber</td>
<td>17.7</td>
<td>22</td>
<td>34</td>
<td>[27]</td>
</tr>
<tr>
<td>Popular</td>
<td>49.9</td>
<td>17.4</td>
<td>18.1</td>
<td>[24]</td>
</tr>
<tr>
<td>Primary wastewater solids</td>
<td>8-15</td>
<td>NA</td>
<td>24-29</td>
<td>[18]</td>
</tr>
<tr>
<td>Sorghum</td>
<td>35.1</td>
<td>24.0</td>
<td>25.4</td>
<td>[28]</td>
</tr>
</tbody>
</table>

NA = Not available

White and brown rot fungi are two main groups, which decompose lignocellulose effectively. White rot fungi degrade more quickly than any other microorganisms [38,39]. Because of insolubility, fungal degradation occurs either exocellular in association with outer cell envelope layer or extracellular. Two enzyme systems are operated for lignocellulose degradation; a hydrolytic system in which hydrolases degrade polysaccharide and a distinctive oxidative and extracellular ligninolytic system, which degrades lignin and unlocks phenyl rings [40,41]. Despite a large number of microorganisms producing the lignocellulolytic enzyme, only a few studied broadly. *Trichoderma reesei* and its mutants are extensively employed in the commercial production of cellulases and hemicellulases [42]. Most of the microorganism used in enzymes production is acting mainly on either cellulose or hemicellulose. Only a few group of microorganisms has evolved with the ability to degrade lignin. It has been reported that *T. reesei* produces hemi and cellulytic enzymes significantly but unable to degrade lignin. The most efficient lignin degrading microbes are basidiomycetes, white rot fungi *Phanerochaete*
chrysosporium, producing plentiful amounts of a unique set of lignocellulytic enzymes which efficiently degrade lignin into CO₂ [43,44]. Other white-rot fungi such as Daedalea flavida, Phlebia fascicularia, P. floridensis and P. radiate have been established to degrade lignin selectively in wheat straw. So these fungi are used for selective removal of lignin leaving other components intact [45]. Some lignocellulose degrading brown-rot fungi rapidly depolymerize cellulose while only modifying lignin. The strong oxidative capacity and low substrate specificity make some white-rot fungi distinctive as they can degrade several environmental pollutants such as such as industrial dyes, chlorinated/heterocyclic aromatic compounds and synthetic polymers [46].

3. Enzymatic Degradation of Lignocellulosic Biomass

The conversion of lignocellulose into fermentable sugars is divided into two categories. First and primitive one used acids as a catalyst, while the second used an enzyme. Effective pretreatment is a key step in the success of hydrolysis where polymer sugars from cellulose and hemicellulose hydrolyzed into free monomer further undergo fermentation to produce bioethanol. Enzyme hydrolysis is more effective than inorganic catalysts because of high specificity and mild operating conditions. Although enzymatic hydrolysis offers several advantages but mechanism and relationship between substrate structure and function of various glycosyl hydrolases are still not well known. Enzymatic hydrolysis of lignocellulose biomass is a complex process since various enzymes with different specificities are required to degrade all components [47,48].

When enzymatic hydrolysis occurs sequentially, the first hydrolysis followed by fermentation, named separate hydrolysis and fermentation (SHF) but when hydrolysis carried out in presence of fermenting microorganisms, then it is called simultaneous saccharification and fermentation (SSF) [49]. In SHF, lignocellulose first hydrolyzes to produce glucose and then fermented to ethanol in separate reactors. Thus, both the hydrolysis and fermentation take place at optimum temperatures, 50°C for hydrolysis and 37°C for yeast fermentation. Accumulation of hydrolysis products is a foremost drawback because it acts as feedback inhibitor to enzymes. It has been reported that cellulase activity is inhibited mainly by cellobiose and glucose and the effect of cellobiose, a dimer of glucose is higher than the glucose. Cellobiose reduced cellulase activity by 60% at a concentration of 6 g/l [50-52]. In SSF both hydrolysis and fermentation operated in a single reactor so the optimum temperature maintained around 38°C which is between the optimum temperature for hydrolysis (45-50°C) and fermentation (30°C). Glucose released in hydrolysis is directly consumed by fermenting microorganism present in the culture, thus minimized end product inhibition. SSF is preferred over SHF because of low processing costs. Trichoderma reesei and Saccharomyces cerevisiae are the most preferred microorganisms in SSF [53].
3.1. Degradation of cellulose

Cellulose is the main component of plant cell wall, constitutes approximately 40-50% dry weight of wood. In terms of production cost and availability, it is one of the most promising raw materials for the preparation of biofuels and several value-added products [54,55]. Cellulose can be hydrolyzed by a series of enzymes with different specificities, working together called cellulosome. It is associated with the cell wall of bacteria and some fungi. Hydrolysis can be operated by the synergistic action of three distinct class of enzymes namely cellobiohydrolases (EC 3.2.1.91), endo-β-1,4-glucanases (EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21) [56,57]. According to CAZy (Carbohydrate-active enzymes) classification system, all these enzymes are grouped into glycosyl hydrolase family. These enzymes display structural resemblance in sequence homology and hydrophobic cluster analysis [58]. Cellobiohydrolases acting at the end of cellulose chains while endoglucanases hydrolyze internal β-1,4-glucosidic linkages randomly. The third enzyme, β-glucosidases acts on the hydrolyzed products called cellobiose and cello-oligosaccharides [59]. Structurally, cellobiohydrolases and endoglucanases have two domain: a carbohydrate binding module (CBM) and a catalytic domain (CD). These two domains connected together by a linker region [60]. Molecular weight ranges between 25 to 50 kDa and optimal activity is acidic pH. Endoglucanases have an open active site which enables its action in the middle of glucan chain while exoglucanases have tunnel shape active site, hydrolyze only ends and side chains [61]. CBM works to bring enzyme catalytic module close contact to a substrate in a proper orientation. It has been reported that in the absence of CBM, an activity of cellobiohydrolases on crystalline cellulose decreased remarkably but no changes occurred for soluble and amorphous substrates. So CBM increases a concentration of enzyme on the surface of the solid substrate [62,63]. The synergistic degradation of lignocellulose does not follow Michaelis-Menten kinetics. Additionally, heterogeneous nature of lignocellulose makes hydrolysis mechanisms complex [64].

To date number of fungi are discovered, producing a remarkable amount of cellulolytic enzymes and these number increasing continuously. Fungal species like brown-rot fungi (*Fomitopsis palustris, Fomitopsis palustris*), ascomycetes (*Trichoderma reesei*) and few anaerobic species (*Orpinomyces sp.*) show great potential in lignocellulose degradation at industrial scale [65-67]. Apart from fungi, many bacterial strains such as *Cellulomonas fimi* and *Thermomonomosporafusca* produce cellulolytic enzymes and grouped into aerobic and anaerobic bacteria as well as actinomycetes [68-70]. Recently, *Clostridium thermocellum* and other related microorganisms are largely exploited for single-step conversion of biomass into desired products [71,72].

3.2. Degradation of hemicellulose

Hemicelluloses in wood are made up of xylan and glucomannans. Xylan is a major
carbohydrate and its composition varies. Degradation of glucomannans and xylans require several synergistic enzymes, endoxylanases and endomannanases hydrolyse main backbone of xylans and glucomannan, respectively. Xylanases are placed in glycosyl hydrolase families 10 and 11 and differ from each other with respect to their catalytic properties. The catalytic domains of these two families are different in their molecular masses, net charges and isoelectric point. These properties might play some role in specificity and activity [73]. Complete hydrolysis of xylans into free monomers requires numerous enzymes like endo-1,4-β-xylanase, acetylesterase, α-glucuronidase and β-xylosidase. The major difference between endo-1,4-β-xylanase and 1,4-β-xylosidase are; former generate xylan oligosaccharides while later works on oligosaccharides generated by endo-1,4-β-xylanase to produce xylose [74]. Tenkanen and co-workers stated that enzymes from *Trichoderma reesei* synergistically hydrolyze beechwood xylan. Later it was perceived that endoxylanases produced by single fungi show different specificities towards xylans, showing complex nature of the substrate. It has been demonstrated that the α-glucuronidases, α-arabinosidases, and acetyl esterases are varying in specificities with respect to neighboring substituents and xylan chain length [75]. In addition, *Clostridium stercorarium* produced eight different enzymes to degrade arabinoxylan, however, only three of them required for hydrolysis. Therefore, the efficient hydrolysis of native xylan appears to comprise not only four different enzymes but also multiple isoenzyme systems [76].

Xylanases are produced by many species of bacteria, fungi and plants. The optimum temperature from the bacterial and fungal origin are ranging between 40 to 60°C but thermostability of bacterial xylanases are higher than fungal enzymes. A tadpole-shaped endoglucanases from *T. reesei* of almost 5 nm in diameter and 20 nm long are perceived showing acidic pH optima [64]. Two glycoproteins of 38 and 62 kDa with acidic pH optima were purified from *Irpex lacteus* which depolymerizes larch xylan [77]. The pH optima of fungal xylanases range between pH 4.5-5.5 while bacterial enzymes displayed maximum activity at pH 6.0-7.0. Xylanases from *Bacillus sp.* and *Streptomyces viridosporus* are active at alkaline pH [78,79].

Mannanases are the heterogeneous group of enzymes similar to xylanases. The complete hydrolysis of O-acetylgalactoglucomanann required many enzymes such as endomannanases, α-galactosidases, acetylglucomannan esterases and β-mannisidases. Degradation opens with rupturing of a polymer by endomannanases; acetylglucomannan esterase removes acetyl groups, similar to xylan esterase in xylans. After that α-Galactosidases remove substituted galactose residues and finally β-mannosidase and β-glycosidase breakdown β-1,4 bonds and release oligomers. Mannanases are larger proteins than xylanases with acidic isoelectric points. The molecular weight ranges between 30-90 kDa. Similar to a cellulolytic enzyme, multi-domain structure is reported in mannanase of *Trichoderma reesei*; a catalytic core domain and a cellulose binding domain, separated by a linker. In addition to these groups of enzymes, hemicellulose degradation required some supplementary enzymes like xylanesterases, ferulic
and p-coumaric esterases, α-l-arabinofuranosidases and α-4-O-methyl glucuronosidases for the efficient hydrolysis of xylans and mannans [80].

Endomannanases usually found in white-rot fungi like *Irpex lacteus*, *Haematostereum sanguinolentum* and *Coriolus versicolor* as well as gram-positive and gram-negative bacteria. They are extensively studied in several nonwood decaying ascomycetes such as *Sporotrichum cellulophilum*, *Trichoderma reesei*, and *Sclerotium rolfsii*. Additionally, α-galactosidases, acetylglucuronamidases and β-mannosidases are explored in *Aspergillus niger* and *P. polvorus sulfureus* [81,82].

3.3. Degradation of lignin

Degradation of lignin is challenging due to structural complexity. High molecular weight, insolubility and heterogeneous nature make less accessibility for enzymes. Lignin has inter-unit carbon-carbon and ether bonds, therefore, degradation mechanism is oxidative rather than hydrolytic. Degradation of lignin required nonspecific oxidative enzymes since the polymer is stereo-irregular [83]. Enzymes employed are lignin peroxidase (LiP, ligninase, EC 1.11.1.14), manganese peroxidase (MnP, Mn-dependent peroxidases, EC 1.11.1.13) and laccase (benzenediol, oxygen oxidoreductase, EC 1.10.3.2). It has been evidenced that these enzymes act on lower molecular weight intermediaries. Besides these, some additional enzymes like glyoxal oxidase and aryl alcohol oxidase (EC 1.1.3.7) are also taking part in hydrogen peroxide production [84]. Some white-rot fungi produce all three enzymes while others produce either two or even only one [85]. Several isoenzymes of LiP and MnP but not for laccase were produced by *Phanerochaete chrysosporium* while their genome contains ten LiP and five MnP genes [86,87]. Among several lignin degrading microorganism, white-rot basidiomycetes such as *Coriolus versicolor*, *Phanerochaete chrysosporium* and *Trametes versicolor* are widely studied [88,89].

Lignin peroxidases (LiPs) are heme-containing glycoproteins, catalytic properties are similar to other peroxidases [90]. Molecular mass ranges between 38 to 43 kDa with acidic pH optima and pI. LiPs and a series of their isoenzymes are found in fungi, encoded by different genes. LiPs are most effective peroxidases so far studied. Besides natural substrates like phenols and aromatic amines it oxidizes variety of other aromatic ethers, amines and polycyclic aromatics [91]. Catalysis of LiPs is H₂O₂ dependent oxidative de-polymerization, where oxidation begins with an abstraction of one electron from the aromatic ring of donor substrate resulted in aryl cation formation which acts as both cations and free radical thus generates variety of degradation fragments. LiPs catalyze Cα-Cβ bond cleavage, ring opening and many other reactions [82]. Piontek and coworkers reported that, heme group in LiPs is buried inside protein and acted on substrates through a channel, therefore, it catalyze only small substrates because the size of channel is not appropriate for larger polymer [92].
Similar to LiPs, MnPs are extracellular glycoproteins with slightly higher molecular masses (45-60 kDa). These enzymes are secreted in multiple isoforms having a heme molecule as iron protoporphyrin IX. Catalytic mechanism of MnPs is very similar to conventional peroxidase with a slight difference by means of Mn (II) acting as a substrate. During the catalytic reaction, Mn(II) is converted into Mn(III) and oxidizes phenolic rings to phenoxy radicals leading to decomposition of substrate. It has been reported that Mn(II) must be chelated via bidentate organic acid chelators so that, product Mn(III) stabilized and released easily. Chelated Mn(III) complex is a diffusible low molecular weight redox-mediator that can act at some distance from the enzyme. LiPs can only act on phenolic substrates such as simple phenols, amines, dyes and phenolic dimers because of weak oxidation nature of these substrates [93-95]. White-rot fungi produce MnPs but lacking LiPs, can also degrade nonphenolic lignin substructures, directing towards other ligninolytic mechanisms [96]. Wesenberg et al reported that oxidation of non-phenolic lignin occurs in presence of Mn(II) through peroxidation of unsaturated lipids. These MnP/lipid peroxidation systems strongly depolymerize phenolic and non-phenolic lignins more efficiently [97]. Camarero et al described a novel versatile peroxidase having activities of both manganese peroxidase and lignin peroxidase and degrades natural lignin more effectively. Versatile peroxidase can oxidize hydroquinone even in the absence of exogenous $H_2O_2$ but require Mn(II) thus, it promoting chemical oxidation of hydroquinones [98].

Laccases are a blue-copper oxidoreductase, utilizes molecular oxygen as oxidant. They oxidize a number of phenolics, aromatic amines and other electron-rich substrates [99]. The reaction starts with a reduction of molecular oxygen into the water with one-electron oxidation mechanism. These enzymes oxidize phenolic unit into phenoxy radicals which cause aryl-Ca cleavage. In this reaction, free radicals acting as an intermediate substrate for enzyme. The catalytic center of a molecule has four copper atoms which can be differentiated by UV-vis spectroscopy. Usually, laccases oxidize phenolics but in presence of redox mediators like ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid). They effectively oxidize non-phenolic compounds also [100].

Wood-rotting fungi are the main producers of laccases but most of the enzymes isolated and characterized belong to white-rot fungi. The common laccase producing white-rot fungi are *Lentinustigrinus, Pleurotusostreatus* D1, *Tramentesversicolor, Trametes sp. strain AH28-2, Tramentespubescens* and *Cyathusbulleri*. Laccases from different organisms display extensive diversity in substrate specificity, pH optima, molecular weight and other properties. The molecular mass of laccases in white-rot fungi ranges between 60 to 80 kDa with acidic pls and pH optima. Laccases have significant biotechnological applications, used in biosensors, soil bioremediation, food and textile industries and synthetic chemistry [101-103].
4. Conclusions

The important socioeconomic issues today are energy and environmental crises, food security and agro-processing. Some of these issues in developing countries can be addressed by lignocellulose biotechnology where most of the radially available biomass waste can utilize and converted into numerous value-added products. Additionally, lignocellulose biomass can be used to produce bioenergy to replace exhausting fossil fuels. The major hurdles in enzymatic bioconversion of lignocelluloses are the crystalline nature of cellulose, protection of accessible surface area by lignin and sheathing by hemicellulose. This study presented an overview of current knowledge on lignocellulose degradation by a variety of microbial enzyme systems. Cellulosic degradation is multi-step process require complex enzyme system for conversion of biomass into fermentable sugars. Although synergy and interaction between cellulases have been well-established, lignin and hemicellulose are more diverse, therefore, further research is required towards enzymatic degradation of hemicellulases and lignin.

5. Acknowledgements

MJ Khan gratefully acknowledge to University Grant Commission (UGC), India for Dr. DS Kothari Fellowship.

6. References


10. Champagne PP, Ramsay JA. Reactive blue 19 decolouration by laccase immobilized on silica beads. Appl Microbiol


29. Elisashvili V, Penninckx M, Kachlishvili E, Asatiani M, Kvestiadze G. Use of Pleurotusdryinus for lignocellulolyt-


31. Xia L, Len P. Cellulose production by solid-state fermentation on lignocellulosic waste from the xylose industry. Process Biochemistry, 199; 34: 909e912.


34. Trigo C, Ball AS. Is the solubilized product from the degradation of lignocellulose by actinomycetes a precursor of humic substances? Microbiology. 1994; 140: 3145-3152.


68. Das M, Royer TV, Leff LG. Diversity of fungi, bacteria, and actinomycetes on leaves decomposing in a stream. Appl


The Emerging Prospects of Global Anti Microbial Resistance: Pros and Cons

Mohammad Kashif; Abrar Ahmad; Mohd Jahir Khan; Somnath Rahangdale; Bhupendra Kumar; Anjum Bee; Mahmood Ahmad Khan and Md. Salman Akhtar

1Plant Molecular Biology and Genetic Engineering Division, CSIR-NBRI, Lucknow, India.
2Environmental Biotechnology Division, CSIR-IITR, Lucknow, India.
3School of Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India.
4Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow.
5Department of Biochemistry, University College of Medical Sciences & GTB Hospital, Dilshad Garden, Delhi 110095, India.
6Department of Biotechnology, Jamia Millia Islamia (Central University), New Delhi -110025, India.

*Correspondence to: Mohammad Kashif, CPMB Division, CSIR-NBRI, Lucknow, India.
Email: kashifibu@gmail.com

Keywords: Antimicrobial Resistance; MDR; XDR; PDR; Antibiotic; Transposons; Integron; Plasmids

Abstract

In the past years infections caused by multidrug-resistant (MDR) microorganism have dramatically increased in all parts of the world. Novel resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases, resulting in prolonged illness, disability, and death. Although MDR is typically credited to chromosomal mutations, resistance is most commonly associated with extrachromosomal elements acquired from other microorganism in the environment. These include altered types of mobile DNA segments, such as plasmids, deletion and insertion sequences, transposons, and integrons. However, inherent mechanisms includes decreased cell wall permeability to antibiotics, alternative relying on a glycoprotein cell wall, altered target sites of antibiotic, enzymatic deactivation of antibiotics, efflux pumps that expel multiple kinds of antibiotics are now recognized as major contributors to resistance in microorganisms. In present scenario, combating with emergence and spread of antibiotic-resistant microorganism is one of the major global issues.
1. Introduction

In the last decade we have witnessed a dramatic increase both in the proportion and absolute number of bacterial pathogens presenting multidrug resistance to antibacterial agents [1]. Organizations such as the US Centers for Disease Control and Prevention (CDC), the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO) are considering infections caused by multidrug-resistant (MDR) bacteria as an emergent global disease and a major public health problem [2].

“There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring ‘fastness’ [resistance].”

Alexander Fleming, 1946

2. Antimicrobial Resistance (AMR)

Antimicrobial resistance (AMR) is recognized as a growing global threat. AMR develops when micro-organisms – bacteria, parasites or viruses – no longer respond to the drug or drugs designed to treat them. AMR is a way for any bacteria that has been exposed to an antibiotic to develop resistance or modify its genetic footprint in order to survive [3]. Antimicrobial resistance occurs everywhere in the world today, compromising our ability to combat infectious diseases, as well as undermining many other advances in health and medicine. AMR also increases the costs of health care. When infections become resistant to first-line drugs, more expensive therapies must be used to treat them. Lengthier treatment, often in hospitals, substantially increases health care costs as well as the economic burden on families and societies [4].

2.1. Antibiotic resistance

Microbes are small organisms which can not see by neked eye. There are various types of microbes as, bacteria, viruses, fungi, and parasites. Although most microbes are harmless and even useful to living organisms, some can cause disease. These disease-causing microbes are called pathogens. Microbes have the ability to develop resistance to the drugs becoming drug-resistant organisms. An antimicrobial is a kind of drug that destroys or rests the growth of microbes, as bacteria, viruses, fungi, and parasites [5]. Antibiotic resistance is the ability of bacteria to resistance the effects of an antibiotic, so the bacteria are not destroyed and their growth still occur. Resistant bacteria to the antibiotic lead to rapid growth of microorganisms and spread them in to other organs. Furthermost infection-causing bacteria can become resistan to at least some antibiotics. Bacteria that are resistant to numerous antibiotics are known as multi-resistant organisms (MRO). A number of bacteria are naturally resistant to some antibiotics such as bacteria in gut [6,7].
3. Terminology Related to Antimicrobial Resistance

3.1. Multiple drug resistance (MDR)

Multidrug resistance or multiresistance is antimicrobial resistance shown by a species of microorganism to multiple antimicrobial drugs. In literal terms, MDR means ‘resistant to more than one antimicrobial agent. Many definitions are being used in order to characterize patterns of multidrug resistance in Gram-positive and Gram-negative organisms. The definition most frequently used for Gram-positive and Gram-negative bacteria are ‘resistant to three or more antimicrobial classes of antibiotics’ [8,9] (Figure-1).

3.2. Extensively drug-resistant (XDR)

XDR microbes that are classified as XDR are epidemiologically significant due not only to their resistance to multiple antimicrobial agents, but also to their ominous likelihood of being resistant to all, or almost all, approved antimicrobial agents. In the medical literature XDR has been used as an acronym for several different terms such as ‘extreme drug resistance’, ‘extensive drug resistance’, ‘extremely drug resistant’ and ‘extensively drug resistant’. Initially, the term XDR was created to describe extensively drug-resistant Mycobacterium tuberculosis (XDR MTB) and was defined as ‘resistance to the first-line agents isoniazid and rifampicin, to a fluoroquinolone and to at least one of the three-second-line parenteral drugs (i.e. amikacin, kanamycin or capreomycin)’ [10]. Subsequent to this, definitions for strains of non-mycobacterial bacteria that were XDR were constructed according to the principle underlying this definition for XDR MTB (i.e. describing a resistance profile that compromised most standard antimicrobial regimens) [11] (Figure-1).

3.3. Pandrug resistant (PDR)

PDR From the Greek prefix ‘pan’, meaning ‘all’, pandrug resistant (PDR) means ‘resistant to all antimicrobial agents’ [12]. Definitions in the literature for PDR vary even though this term is etymologically exact and means that, in order for a particular species and a microbes isolate of this species to be characterized as PDR, it must be tested and found to be resistant to all approved and useful agents. Examples of current definitions are: ‘resistant to almost all commercially available antimicrobials’, ‘resistant to all antimicrobials routinely tested’ and ‘resistant to all antibiotic classes available for empirical treatment’, making the definition of PDR subject to inconsistent use and liable to potential misinterpretation of data [13] (Figure-1).
4. Diversified Microbial Resistance

4.1. Resistance in bacteria

Various microorganisms have survived for thousands of years by their ability to adapt to antimicrobial agents. They do so via spontaneous mutation or by DNA transfer. This process enables some bacteria to oppose the action of certain antibiotics, rendering the antibiotics ineffective [14].

**Commonest multidrug-resistant organisms (MDROs)**

- Multi-drug-resistant Tuberculosis(15).
- Methicillin-Resistant Staphylococcus aureus (MRSA)(16).
- Vancomycin-Resistant Enterococci (VRE).
- Extended-spectrum β-lactamase (ESBLs) producing Gram-negative bacteria.
- Klebsiella pneumoniae carbapenemase (KPC) producing Gram-negatives
- Multidrug-Resistant gram negative rods (MDR GNR) MDRGN bacteria such as Enterobacter species, E.coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa (17).
- A group of gram-positive and gram-negative bacteria of particular recent importance have been dubbed as the ESKAPE group (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter species) (18).

4.2. Resistance in fungi

Some yeasts species like Candida can become resistant under long term treatment withazole preparations, requiring treatment with a different drug class. Scedosporium prolificans
infections are almost uniformly fatal because of their resistance to multiple antifungal agents [19,20].

4.3. Resistance in viruses

In 2010, an approximate 7% of people starting antiretroviral therapy (ART) in developing countries had drug-resistant HIV. In developed countries, the same figure was 10–20%. Some countries have recently reported levels at or above 15% amongst those starting HIV treatment, and up to 40% among people re-starting treatment [21]. HIV is the prime example of MDR against antivirals, as it mutates rapidly under monotherapy [22]. Influenza virus has become increasingly MDR; first to amantadines, then to neuraminidase inhibitors such as oseltamivir, (2008-2009: 98.5% of Influenza A tested resistant), also more commonly in people with weak immune systems [23,24]. Cytomegalovirus can become resistant to ganciclovir and foscarnet under treatment, especially in immunosuppressed patients [25]. Herpes simplex virus rarely becomes resistant to acyclovir preparations, mostly in the form of cross-resistance to famciclovir and valacyclovir, usually in immunosuppressed patients [26].

4.4. Resistance in parasites

In July 2016, resistance to the first-line treatment for P. Falciparum malaria (artemisinin-based combination therapies) has been confirmed in 5 countries Cambodia, the Lao People’s Democratic Republic, Myanmar, Thailand and Viet Nam) [27]. The prime example for MDR against antiparasitic drugs is malaria. Plasmodium vivax has become chloroquine and sulfadoxinepyrimethamine resistant a few decades ago, and as of 2012 artemisinin-resistant Plasmodium falciparum has emerged in western Cambodia and western Thailand. Toxoplasma gondii can also become resistant to artemisinin, as well as atovaquone and sulfadiazine, but is not usually MDR. Antihelminthic resistance is mainly reported in the veterinary literature, for example in connection with the practice of livestock drenching and has been recent focus of FDA regulation [28].

5. Genetics of Multidrug Resistance

Bacterial antibiotic resistance can be attained through intrinsic or acquired mechanisms. Intrinsic mechanisms are those specified by naturally occurring genes found on the host’s chromosome, such as, AmpC β-lactamase of gram-negative bacteria and many MDR efflux systems [29]. Acquired mechanisms involve mutations in genes targeted by the antibiotic and the transfer of resistance determinants borne on plasmids, bacteriophages, transposons, and other mobile genetic material. In general, this exchange is accomplished through the processes of transduction (via bacteriophages), conjugation (via plasmids and conjugative transposons), and transformation (via incorporation into the chromosome of chromosomal DNA, plasmids, and other DNAs from dying organisms). Although gene transfer among organisms within the
same genus is common, this process has also been observed between very different genera, including transfer between such evolutionarily distant organisms as gram-positive and gram-negative bacteria [30]. Plasmids contain genes for resistance and many other traits; they replicate independently of the host chromosome and can be distinguished by their origins of replication [31]. Multiple plasmids can exist within a single bacterium, where their genes add to the total genetics of the organism. Transposons are mobile genetic elements that can exist on plasmids or integrate into other transposons or the host’s chromosome. In general, these pieces of DNA contain terminal regions that participate in recombination and specify a protein(s) (e.g., transposase or recombinase) that facilitates incorporation into and from specific genomic regions [31,32]. Conjugative transposons are unique in having qualities of plasmids and can facilitate the transfer of endogenous plasmids from one organism to another. Integrons contain collections of genes (gene cassettes) that are generally classified according to the sequence of the protein (integrase) that imparts the recombination function. They have the ability to integrate stably into regions of other DNAs where they deliver, in a single exchange, multiple new genes, particularly for drug resistance. The super-integron, one which contains hundreds of gene cassettes), is distinct from other integrons; it was first identified in Vibrio cholera [33,34].

6. Mechanism of Action of Multidrug Resistance

Once exposure to bacteria occurs, infection and bacteria spread occur, so, treatment with suitable drugs as antibiotics must begin. Antibiotics responsible for stop the growth of bacteria and prevent bacteria multiply, so kill them, hence use in treatment of disease. While in the other cases antibiotics loss their ability to stop growth of bacteria, hence multiplication of bacteria increase and this lead to spread antibiotics resistance bacteria and development of disease. Antibiotic resistance can be occurring through various types of mechanisms as shown in Figure-2.

6.1. Drug inactivation or modification: for example, enzymatic deactivation as in penicillin G in some penicillin-resistant bacteria through the production of β-lactamases. Protecting enzymes manufactured by the bacterial cell will add an acetyl or phosphate group to a specific site on the antibiotic, which will diminish its capacity to bind to the bacterial ribosomes and disrupt protein synthesis [35,36] (Figure-2).

6.2. Modification of target or binding site: for example, alteration of PBP-the binding target site of penicillin's-in MRSA and other penicillin-resistant bacteria, or modification in structure of ribosomal protection proteins. These proteins guard the bacterial cell from antibiotics through changes its conformational shape. Change of proteins conformational shape allows these proteins to loss their activity so, prevent inhibit protein synthesis, and this help in grow of bacteria and spread it [37,38] (Figure-2).
6.3. **Alteration of metabolic pathway**: for example, absence of paraaminobenzoic acid (PABA), this is precursor for the synthesis of folic acid and nucleic acids [39].

6.4. **Reduced drug accumulation**: By decreasing drug permeability or increasing active pumping out of drugs through cell membrane.

6.5. **Efflux Systems**: Altered Membranes mechanism also operates in antibiotic resistance for example Porins [40] (Figure-2).

6.6. **Mutation rate**: Increased mutation rate as a stress response leads to evasion of antibiotics.

![Figure 2: Schematic representation of various aspects of action mechanism of multidrug resistance.](image)

7. **Prevention and Control Measures of Microbial Resistance**

Antibiotic resistant microbes can be transfer from person to person inside the community. This is becoming more common. With the emergence and spread of antimicrobial resistant pathogens, antimicrobial resistance surveillance is becoming an important task of the Microbiology Laboratory. Antimicrobial resistance surveillance is a ongoing (and organized) data collection that after being analyzed and reported provides useful information for empirical antimicrobial therapy. The following measures can be taken to prevent the emergence and spread of antibiotic resistance worldwide [42,43].

7.1. **Prevention and Control: Manual Level**

Antimicrobial resistance is a complex problem that affects all of society and is driven by many interconnected factors. Single, isolated interventions have limited impact. Coordinated action is required to minimize the emergence and spread of antimicrobial resistance [44].
WHO is providing technical assistance to help countries develop their national action plans, and strengthen their health and surveillance systems so that they can prevent and manage antimicrobial resistance [45]. The following manual way should be taken to combat the emergence and spread of antibiotic resistance worldwide described in Figure-3.

![Diagram Figure 3](image)

**Figure 3:** Diagrammatic representation of manual strategies to combat multidrug resistance.

### 7.2. Prevention and Control: Molecular Level

Greater innovation and investment are required in molecular research and development of new antimicrobial medicines, vaccines, and diagnostic tools. A better understanding of the molecular basis of antimicrobial resistance has facilitated the development of bioinformatic tools to identify antibiotic resistance genes in bacterial genomes [46,47]. It has defined a strategic research agenda under the assumption that only a collaborative effort will provide the necessary critical mass and scientific expertise to answer the most important and urgent research questions related to antimicrobial resistance [48,49,50]. The following molecular strategies should be taken to combat the emergence and spread of antibiotic resistance worldwide described in Figure-4 [51].

![Diagram Figure 4](image)

**Figure 4:** Diagrammatic representation of molecular strategies to combat multidrug resistance.
8. Future Prospects and Concluding Remark

The presence of multiple drug-resistant bacteria is responsible for spreading various diseases in the world. Traditional technique fails to solve this problem. The prompt identification of the antimicrobial susceptibility of a microorganism, on the other hand, ensures the administration of the correct treatment and reduces the need for broad-spectrum drugs, limiting the emergence of antimicrobial resistance. Molecular technique like mass spectrometry, Crystallography, NMR, 2 Dimensional electrophoresis have shortened the time to detect specific resistance mechanisms and the development of next generation sequencing technologies has increased the number of sequenced bacterial genomes at an exponential rate. A better understanding of the molecular basis of antimicrobial resistance has facilitated the development of bioinformatic tools to identify antibiotic resistance genes in bacterial genomes. Similarly, advanced applications of nanoparticles and bacterial microencapsulation to clinical are very promising and might be fully developed in the years to come. Phage therapy is an important alternative to antibiotics in the current era of drug-resistant pathogens. Bacteriophages have played an important role in the expansion of molecular biology, not only, but also play important role in overcome antibiotic resistance.

A global and coordinated initiative to tackle antibiotic resistance will be needed to persuade the general population and policy makers of the advantages, both medical and economic, of combating the threat of antimicrobial resistance.

9. References


