
Current Research in
Microbiology
OPEN ACCESS
eBooks

**INDEX**




**Published in:** June 2018

**Online Edition available at:** http://openaccessebooks.com/

**Reprints request:** info@openaccessebooks.com

Current Research in Microbiology

Chapter 1

# Microsporidian Parasite Impact on Humans Health

***Dipti Kashyap*[1]; Abrar Ahmad***[2]

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

*[2]Environmental 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 Microsporidiasis infection in Humans

| Microsporidial species | Detection of parasite | Clinical Manifestation | Reference No. |
|---|---|---|---|
| *Nosema connori* | Autopsy | Disseminated infection | Margileth *et al*, (1973) [18] |
| *Pleistophora* spp. | Histological examination | Myositis | Ledford *et al*, (1985) [19] |
| *Enterocytozoon bieneusi* | Stool specimen | Diarrheoa | Sandfort *et al*, (In press) [20] |
| *Nosema corneum* | Histological examination | Keratitis | Arison *et al*, (1966) [21] |
| *Encephalitozoon cuniculi* | Cerebrospinal fluid | Seizures | Matsubayashi *et al*,(1959) [3] |

## 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. Sero-epidemiologic 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

1. Canning, E. U., J. Lom, and I. Dykova. 1986. The microsporidia of vertebrates. Academic Press, Inc., New York.

2. Sprague, V. 1982. Microspora, p. 589-594. In S. B. Parker (ed.), Synopsis and classification of living organisms, vol. 1. McGraw Hill, New York.

3. Matsubayashi, H., T. Koike, T. Mikata, and S. Hagiwara. 1959. A case of Encephalitozoon-like body infection in man. Arch. Pathol. 67: 181-187.

4. Bryan, R T. Microsporidia. In G. L. Mandell, J. E. Bennett, and R. Dolan (ed.), Principles and practice of infectious diseases, 4th ed., in press. Churchill Livingstone, New York. .

5. McCully, R. M., A. F. Van Dellen, P. A. Basson, and J. Lawrence. 1978. Observations on the pathology of canine microsporidiosis. Onderstepoort J. Vet. Res. 45: 75-92.

6. Weiss LM, Edlind TD, Vossbrinck CR, Hashimoto T. Microsporidian molecular phylogeny: the fungal connection. J Eukaryot Microbiol. 1999; 46: 17S–18S.

7. Canning, E. U. 1993. Microsporidia, p. 299-385. In J. P. Kreier and J. R. Baker (ed.), Parasitic protozoa, 2nd ed., vol. 6. Academic Press, Inc., New York.

8. Katinka MD, Duprat S, Cornillot E, Metenier G, Thomarat F, Prensier G, Barbe V, Peyretaillade E, Brottier P, Wincker P, Delbac F, El Alaoui H, Peyret P, Saurin W, Gouy M, Weissenbach J, Vivares CP. Genome sequence and gene compaction of the eukaryote parasite Encephalitozoon cuniculi. Nature. 2001; 414: 450–453.

9. Ishihara, R., and Y. Hyashi. 1968. Some observations on the fine structure of sporoplasm discharged from spores of a microsporidium, Nosema bombycis. J. Invert. Pathol. 11: 377-385.

10. Modigliani, R., C. Bories, Y. Le Charpentier, M. Salmeron, B. Messing, A. Galian, J. C. Rambaud, A. Lavergne, B. CochandPriollet, and I. Desportes. 1985. Diarrhea and malabsorption in acquired immune deficiency syndrome: a study of four cases with special emphasis on opportunistic protozoan infestation. Gut 26: 179-181.

11. Cali, A. 1991. General inicrosporidian features and recent findings on AIDS isolates. J. Protozool. 38: 625-630.

12. Cali, A., and R L. Owen. 1988. Microsporidiosis, p. 928-949. In A. Balows, W. Hausler, Jr., and E. H. Lennette (ed.), The laboratory diagnosis of infectious diseases: principles and practice, vol. 1. Springer-Verlag, New York.

13. Bryan, R T. Microsporidia. In G. L. Mandell, J. E. Bennett, and R. Dolan (ed.), Principles and practice of infectious diseases, 4th ed., in press. Churchill Livingstone, New York.

14. Canning, E. U., and W. S. Hollister. 1992. Human infections with microsporidia. Rev. Med. Microbiol. 3:35-42.

15. Bergquist, N. R., G. Stintzing, L. Smedman, T. Waller, and T. Andersson. 1984. Diagnosis of encephalitozoonosis in man by serological tests. Br. Med. J. 288: 902.

16. Bergquist, R., L Morfeldt-Mansson, P. 0. Pehrson, B. Petrini, and J. Wasserman. 1984. Antibody against Encephalitozoon cuniculi in Swedish homosexual men. Scand. J. Infect. Dis. 16:389-391

17. Shadduck, J. A., R A. Meccoli, R. Davis, and R L. Font. 1990. First isolation of a microsporidian from a human patient. J. Infect. Dis. 162: 773-776.

18. Margileth, A. M., A. J. Strano, R. Chandra, R. Neafie, M. Blum, and R. M. McCully. 1973. Disseminated nosematosis in an immunologically compromised infant. Arch. Pathol. 95: 145-150.

19. Ledford, D. K., M. D. Overman, A. Gonzalo, A. Cali, W. Mester, and R. F. Lockey. 1985. Microsporidiosis myositis in a patient with acquired immunodeficiency syndrome. Ann. Intern. Med. 102: 628-630.

20. Sandfort, J., A. Hannemann, D. Stark, R L. Owen, and B. Ruf. Enterocytozoon bieneusi infection in an immunocompetent HIVnegative patient with acute diarrhea. Clin. Infect. Dis., in press.

21. Arison, R. N., J. A. Cassaro, and M. P. Pruss. 1966. Studies on a murine ascites-producing agent and its effect on tumor development. Cancer Res. 26: 1915-1920.

22. Weber R, Bryan RT. Microsporidial infections in immunodeficient and immunocompetent patients. Clin Infect Dis. 1994; 19:517–521.

23. Weber R, Deplazes P, Flepp M, Mathis A, Baumann R, Sauer B, Kuster H, Luthy R. Cerebral microsporidiosis due to Encephalitozoon cuniculi in a patient with human immunodeficiency virus infection. N Engl J Med. 1997; 336: 474–478.

24. Weber R, Deplazes P, Schwartz D. Diagnosis and clinical aspects of human microsporidiosis. Contrib Microbiol. 2000; 6: 166–192.

Current Research in Microbiology

Chapter 2

# Microbial Production of Nanoparticles and their Applications

***Pragati Sahai[1]; Nabeel Ahmad[2]; Vimlendu B Sinha[1]; Rajiv Dutta****

*[1]Department of Biotechnology, School of Engineering & Technology, Sharda University, Greater Noida-201306 (U.P.) India.*

*[2]Department of Biotechnology, School of Engineering & Technology, IFTM University, NH-24, Delhi Road, Moradabad-244001 (U.P.), India.*

***Correspondence to**: **Rajiv Dutta,** Department of Biotechnology, College of Engineering & Technology, IILM-AHL, Greater Noida-201306 (U.P.), India.*

*Email: director.sbt@gmail.com*

## 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**)

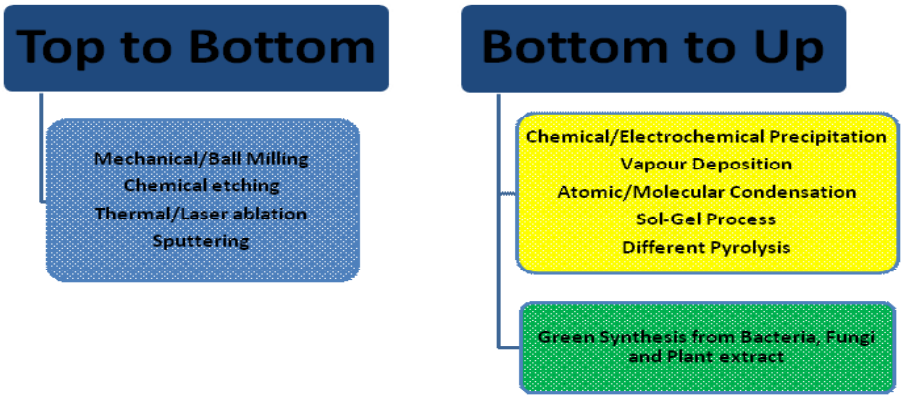



**Figure 1**: Synthesis of Nanoparticles

## 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**).

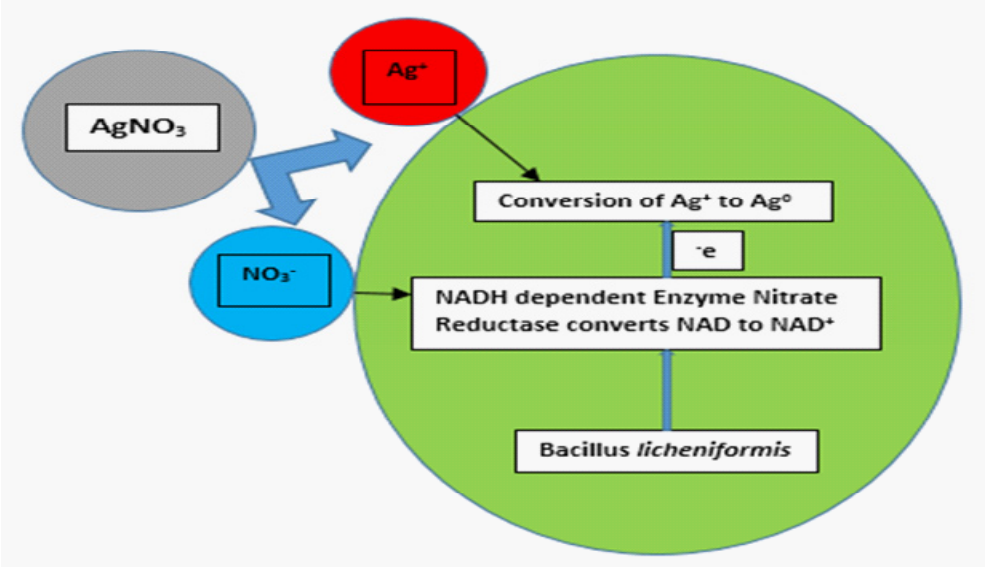



**Figure 2**: Example of Microbial Synthesis of Nanoparticles

## 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 $M^0$ 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.

| Microorganism | Nanoparticle | Culture Temperature (oC) | Size (nm) | Shape and Location |
|---|---|---|---|---|
| *Shewanella oneidensis* | $Fe_3O_4$ | 28 | 40–50 | **Rectangular and Extracellular** |
| *Saccharomyces cerevisiae* | $Sb_2O_3$ | 25–60 | 2–10 | **Spherical and Intracellular** |
| *Lactobacillus sp.* | $TiO_2$ | 25 | 8–35 | **Spherical and Extracellular** |
| *Fusarium oxysporum* | $BaTiO_3$ | 25 | 4-5 | **Spherical and Extracellular** |
| *Brevibacterium casei* | PHB (Poly hydroxybutyrate) | 37 | 100–125 | **Intracellular** |
| *E. coli* | CdS | 25 | 2–5 | **Wurtzite crystal and Intracellular** |
| *Rhodobacter sphaeroides* | ZnS | Unknown | 10.5+/-0.15 | **Spherical and Extracellular** |
| *Desulfobacteraceae* | FeS | Unknown | 2 | **Spherical and Extracellular** |
| *Brevibacterium casei* | Au, Ag | 37 | 10–50 | **Spherical and Intracellular** |

**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-

crobes 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 mineof Pseudomon *asstutzeri* 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 afilm 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 $p^H$ within an hour in presence of Lactate as electron donor [38]. Similarly Mercury nanoparticles of size 2-5 nm were prepared by Enterobactersp. at slightly alkaline $p^H$ of 8 and lower concentration of mercury lead to increase in chemical detoxification [39]. The palladium nanoparticles could be synthesized 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 type 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 $Fe_3O_4$ (magnetite) and $Fe_2O_3$ (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$^{o}$C however uncultured magnetotactic bacteria were mostly at or below 30$^{o}$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 $Fe_3O_4$ 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.

Beside 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 $Sb_2O_3$ nanoparticles can be mediated by Saccharomycescerevisiae and this green process is economical and reproducible [48]. Similarly Bansal et al. used *F. oxysporum* (Fungus) to produce $SiO_2$ and $TiO_2$ nanoparticles from aqueous anionic complexes $SiF_6^{2-}$and $TiF_6^{2-}$, 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 $Fe_3S_4$ 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 $PbCO_3$, $CdCO_3$, $SrCO_3$, 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 crystalsor 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 ($Cd–S–CH_2COOH$) 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 atomand $Cd^{2+}$ 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 $p^H$, 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.

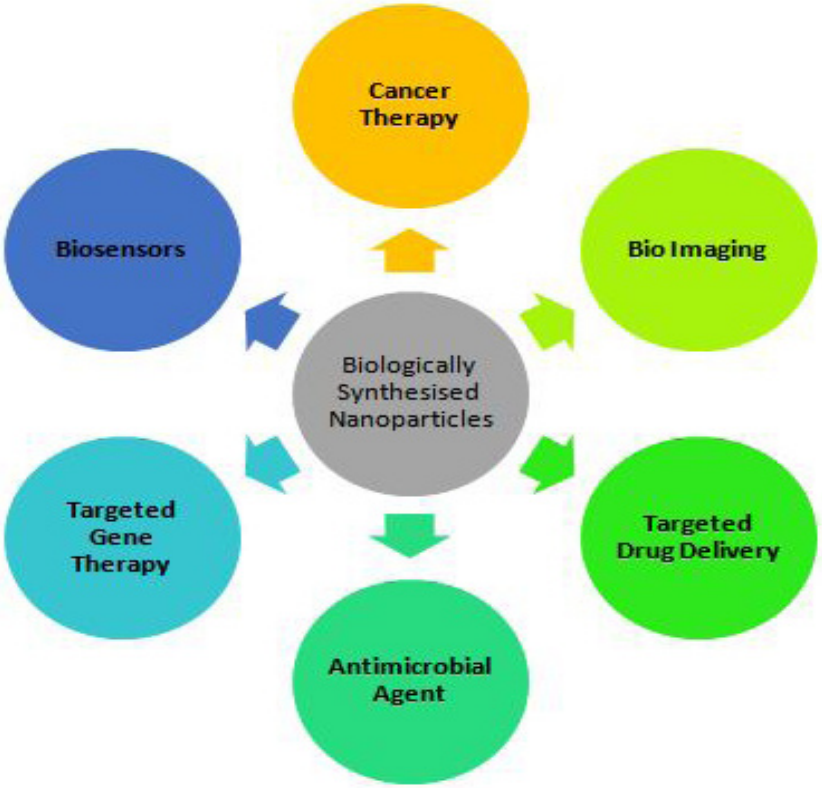



**Figure 4**: Application of biologically synthesised nanoparticles

**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 $H_2O_2$ biosensors had high sensitivity and affinity for $H_2O_2$. 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 $H_2O_2$ 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 particlesize 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, $p^H$, 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 environmentfurther 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

1. Daniel MC, Astruc D. Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology. Chemical reviews. 2004; 104(1): 293-346.

2. Kato H. In vitro assays: tracking nanoparticles inside cells. Nature Nanotechnology. 2011; 6(3): 139-140.

3. Liu J, Qiao SZ, Hu QH. Magnetic nanocomposites with mesoporous structures: synthesis and applications small. 2011; 7(4): 425-443.

4. Luechinger NA, Grass RN, Athanassiou EK, Stark WJ. Bottom-up fabrication of metal/metal nanocomposites from nanoparticles of immiscible metals. Chemistry of Materials. 2009; 22(1): 155-160.

5. Tiwari DK, Behari J, Sen P. Time and dose-dependent antimicrobial potential of Ag nanoparticles synthesized by top-down approach. Current Science. 2008; 647-655.

6. Mohanpuria P, Rana NK, Yadav SK. Biosynthesis of nanoparticles: technological concepts and future applications. Journal of Nanoparticle Research. 2008; 10(3): 507-517.

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

8. Gottschalk F, Nowack B. The release of engineered nanomaterials to the environment. Journal of Environmental Monitoring. 2011; 13(5): 1145-1155.

9. Wiesner MR, Lowry GV, Jones KL, Hochella, Jr MF, Di Giulio RT, Casman E, Bernhardt ES. Decreasing uncertainties in assessing environmental exposure, risk, and ecological implications of nanomaterials.

10. Pearce CI, Coker VS, Charnock JM, Pattrick RA, Mosselmans JF, Law N, Beveridge TJ, Lloyd JR. Microbial manufacture of chalcogenide-based nanoparticles via the reduction of selenite using Veillonella atypica: an in situ EXAFS study. Nanotechnology. 2008; 19(15): 155603.

11. Oremland RS, Herbel MJ, Blum JS, Langley S, Beveridge TJ, Ajayan PM, Sutto T, Ellis AV, Curran S. Structural and spectral features of selenium nanospheres produced by Se-respiring bacteria. Applied and environmental microbiology. 2004; 70(1): 52-60.

12. Baesman SM, Bullen TD, Dewald J, Zhang D, Curran S, Islam FS, Beveridge TJ, Oremland RS. Formation of tellurium nanocrystals during anaerobic growth of bacteria that use Te oxyanions as respiratory electron acceptors. Applied and environmental microbiology. 2007; 73(7): 2135-2143.

13. Klonowska A, Heulin T, Vermeglio A. Selenite and tellurite reduction by Shewanella oneidensis. Applied and environmental microbiology. 2005; 71(9): 5607-5609.

14. Lang C, Schüler D. Biogenic nanoparticles: production, characterization, and application of bacterial magnetosomes. Journal of Physics: Condensed Matter. 2006; 18(38): S2815.

15. Narayanan KB, Sakthivel N. Biological synthesis of metal nanoparticles by microbes. Advances in colloid and interface science. 2010; 156(1): 1-3.

16. Chen JF, Wang YH, Guo F, Wang XM, Zheng C. Synthesis of nanoparticles with novel technology: high-gravity reactive precipitation. Industrial & engineering chemistry research. 2000; 39(4): 948-954.

17. Simkiss K, Wilbur KM. Biomineralization. Elsevier; 2012.

18. Mann S. Biomineralization: principles and concepts in bioinorganic materials chemistry. Oxford University Press on Demand; 2001.

19. Zhang X, Yan S, Tyagi RD, Surampalli RY. Synthesis of nanoparticles by microorganisms and their application in enhancing microbiological reaction rates. Chemosphere. 2011; 82(4): 489-494.

20. Mukherjee P, Senapati S, Mandal D, Ahmad A, Khan MI, Kumar R, Sastry M. Extracellular synthesis of gold nanoparticles by the fungus Fusarium oxysporum. Chem Bio Chem. 2002 May 3; 3(5): 461-463.

21. Ahmad A, Senapati S, Khan MI, Kumar R, Sastry M. Extracellular biosynthesis of monodisperse gold nanoparticles by a novel extremophilic actinomycete, Thermomonospora sp. Langmuir. 2003; 19(8): 3550-3553.

22. Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Ramani R, Parischa R, Ajayakumar PV, Alam M, Sastry M. Bioreduction of AuCl4− ions by the fungus, Verticillium sp. and surface trapping of the gold nanoparticles formed. Angewandte Chemie International Edition. 2001; 40(19): 3585-3588.

23. Southam G, Beveridge TJ. The occurrence of sulfur and phosphorus within bacterially derived crystalline and pseudocrystalline octahedral gold formed in vitro. Geochimica et Cosmochimica Acta. 1996; 60(22): 4369-4376.

24. Ahmad A, Senapati S, Khan MI, Kumar R, Ramani R, Srinivas V, Sastry M. Intracellular synthesis of gold nanoparticles by a novel alkalotolerant actinomycete, Rhodococcus species. Nanotechnology. 2003; 14(7): 824.

25. Lengke MF, Fleet ME, Southam G. Morphology of gold nanoparticles synthesized by filamentous cyanobacteria from gold (I)− thiosulfate and gold (III)− chloride complexes. Langmuir. 2006; 22(6): 2780-2787.

26. Lengke MF, Ravel B, Fleet ME, Wanger G, Gordon RA, Southam G. Mechanisms of gold bioaccumulation by filamentous cyanobacteria from gold (III)− chloride complex. Environmental science & technology. 2006; 40(20): 6304-6309.

27. Nair B, Pradeep T. Coalescence of nanoclusters and formation of submicron crystallites assisted by Lactobacillus strains. Crystal Growth & Design. 2002; 2(4): 293-298.

28. Panáček A, Kvítek L, Prucek R, Kolář M, Večeřová R, Pizúrová N, Sharma VK, Nevěčná TJ, Zbořil R. Silver colloid nanoparticles: synthesis, characterization, and their antibacterial activity. J. Phys. Chem. B. 2006; 110(33): 16248-16253.

29. Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Parishcha R, Ajaykumar PV, Alam M, Kumar R, Sastry M. Fungus-mediated synthesis of silver nanoparticles and their immobilization in the mycelial matrix: a novel biological approach to nanoparticle synthesis. Nano Letters. 2001; 1(10): 515-519.

30. Syed A, Ahmad A. Extracellular biosynthesis of platinum nanoparticles using the fungus Fusarium oxysporum. Colloids and Surfaces B: Biointerfaces. 2012; 97: 27-31.

31. Krishna GU, Kumar SS, Pranitha VA, Alha MA, Charaya S. Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: A study against salmonella SP. International journal of pharmacy and pharmaceutical Sciences. 2015; 7(11): 84-88.

32. Klaus T, Joerger R, Olsson E, Granqvist CG. Silver-based crystalline nanoparticles, microbially fabricated. Proceedings of the National Academy of Sciences. 1999; 96(24): 13611-13614.

33. Jain N, Bhargava A, Majumdar S, Tarafdar JC, Panwar J. Extracellular biosynthesis and characterization of silver nanoparticles using Aspergillus flavus NJP08: a mechanism perspective. Nanoscale. 2011; 3(2): 635-641.

34. Babu MG, Gunasekaran P. Production and structural characterization of crystalline silver nanoparticles from Bacillus cereus isolate. Colloids and surfaces B: Biointerfaces. 2009; 74(1): 191-195.

35. Senapati S, Ahmad A, Khan MI, Sastry M, Kumar R. Extracellular biosynthesis of bimetallic Au–Ag alloy nanoparticles. Small. 2005; 1(5): 517-520.

36. Zheng D, Hu C, Gan T, Dang X, Hu S. Preparation and application of a novel vanillin sensor based on biosynthesis of Au–Ag alloy nanoparticles. Sensors and Actuators B: Chemical. 2010; 148(1): 247-252.

37. Sawle BD, Salimath B, Deshpande R, Bedre MD, Prabhakar BK, Venkataraman A. Biosynthesis and stabilization of Au and Au–Ag alloy nanoparticles by fungus, Fusarium semitectum. Science and technology of advanced materials. 2008; 9(3): 035012.

38. Konishi Y, Ohno K, Saitoh N, Nomura T, Nagamine S, Hishida H, Takahashi Y, Uruga T. Bioreductive deposition of platinum nanoparticles on the bacterium Shewanella algae. Journal of biotechnology. 2007; 128(3): 648-653.

39. Sinha A, Khare SK. Mercury bioaccumulation and simultaneous nanoparticle synthesis by Enterobacter sp. cells. Bioresource Technology. 2011; 102(5): 4281-4284.

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.

41. Windt WD, Aelterman P, Verstraete W. Bioreductive deposition of palladium (0) nanoparticles on Shewanella oneidensis with catalytic activity towards reductive dechlorination of polychlorinated biphenyls. Environmental Microbiology. 2005; 7(3): 314-325.

42. Bazylinski DA, Garratt-Reed AJ, Frankel RB. Electron microscopic studies of magnetosomes in magnetotactic bacteria. Microscopy research and technique. 1994; 27(5): 389-401.

43. 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.

44. Thornhill RH, Burgess JG, Matsunaga T. PCR for direct detection of indigenous uncultured magnetic cocci in sediment and phylogenetic analysis of amplified 16S ribosomal DNA. Applied and environmental microbiology. 1995; 61(2): 495-500.

45. Spring S, Schleifer KH. Diversity of magnetotactic bacteria. Systematic and Applied Microbiology. 1995; 18(2): 147-53.

46. Zhou W, He W, Zhong S, Wang Y, Zhao H, Li Z, Yan S. Biosynthesis and magnetic properties of mesoporous Fe 3 O 4 composites. Journal of Magnetism and Magnetic Materials. 2009; 321(8): 1025-1028.

47. Zhou W, He W, Zhang X, Yan S, Sun X, Tian X, Han X. Biosynthesis of iron phosphate nanopowders. Powder Technology. 2009; 194(1): 106-108.

48. Jha AK, Prasad K, Prasad K. A green low-cost biosynthesis of Sb 2 O 3 nanoparticles. Biochemical engineering journal. 2009; 43(3): 303-306.

49. Bansal V, Rautaray D, Bharde A, Ahire K, Sanyal A, Ahmad A, Sastry M. Fungus-mediated biosynthesis of silica and titania particles. Journal of Materials Chemistry. 2005; 15(26): 2583-2589.

50. Yang H, Sentra S, Holloway PH. Syntheses and applications of MN-doped II-VI semiconductor Nano crystals. Journal of nanosciences and nanotechnology. 2005; 5(9): 1364-1375.

51. Cunningham DP, Lundie LL. Precipitation of cadmium by Clostridium thermoaceticum. Applied and Environmental Microbiology. 1993; 59(1): 7-14.

52. Holmes JD, Richardson DJ, Saed S, Evans-Gowing R, Russell DA, Sodeau JR. Cadmium-specific formation of metal sulfide 'Q-particles' by Klebsiella pneumoniae. Microbiology. 1997; 143(8): 2521-2530.

53. Bai HJ, Zhang ZM, Gong J. Biological synthesis of semiconductor zinc sulfide nanoparticles by immobilized Rhodobacter sphaeroides. Biotechnology letters. 2006; 28(14): 1135-1139.

54. Labrenz M, Druschel GK, Thomsen-Ebert T, Gilbert B, Welch SA, Kemner KM, Logan GA, Summons RE, De Stasio G, Bond PL, Lai B. Formation of sphalerite (ZnS) deposits in natural biofilms of sulfate-reducing bacteria. Science. 2000; 290(5497): 1744-1747.

55. Bai HJ, Zhang ZM. Microbial synthesis of semiconductor lead sulfide nanoparticles using immobilized Rhodobacter sphaeroides. Materials Letters. 2009; 63(9): 764-766.

56. Watson JH, Ellwood DC, Soper AK, Charnock J. Nanosized strongly-magnetic bacterially-produced iron sulfide materials. Journal of Magnetism and Magnetic Materials. 1999; 203(1): 69-72.

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.

58. Mukherjee P, Senapati S, Mandal D, Ahmad A, Khan MI, Kumar R, Sastry M. Extracellular synthesis of gold nanoparticles by the fungus Fusarium oxysporum. ChemBioChem. 2002; 3(5): 461-463.

59. Rautaray D, Sanyal A, Adyanthaya SD, Ahmad A, Sastry M. Biological synthesis of strontium carbonate crystals using the fungus Fusarium oxysporum. Langmuir. 2004; 20(16): 6827-6833.

60. Kumar SA, Ansary AA, Ahmad A, Khan MI. Extracellular biosynthesis of CdSe quantum dots by the fungus, Fusarium oxysporum. Journal of Biomedical Nanotechnology. 2007; 3(2): 190-194.

61. Yan S, He W, Sun C, Zhang X, Zhao H, Li Z, Zhou W, Tian X, Sun X, Han X. The biomimetic synthesis of zinc phosphate nanoparticles. Dyes and Pigments. 2009; 80(2): 254-258.

62. Kalishwaralal K, Deepak V, Ramkumarpandian S, Nellaiah H, Sangiliyandi G. Extracellular biosynthesis of silver nanoparticles by the culture supernatant of Bacillus licheniformis. Materials letters. 2008; 62(29): 4411-4413.

63. Reith F, Lengke MF, Falconer D, Craw D, Southam G. The geomicrobiology of gold. The ISME Journal. 2007; 1(7): 567.

64. Nies DH. Microbial heavy-metal resistance. Applied microbiology and biotechnology. 1999; 51(6): 730-50.

65. Mergeay M, Monchy S, Vallaeys T, Auquier V, Benotmane A, Bertin P, Taghavi S, Dunn J, van der Lelie D, Wattiez R. Ralstonia metallidurans, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. FEMS microbiology reviews. 2003; 27(2-3): 385-410.

66. Tang H, Yan M, Zhang H, Xia M, Yang D. Preparation and characterization of water-soluble CdS nanocrystals by surface modification of ethylene diamine. Materials letters. 2005; 59(8): 1024-1027.

67. Løver T, Henderson W, Bowmaker GA, Seakins JM, Cooney RP. Functionalization and capping of a cds nanocluster: a study of ligand exchange by electrospray mass spectrometry. Chemistry of materials. 1997; 9(8): 1878-1886.

68. Meng C, Tian J, Li Y, Zheng S. Influence of native bacterial magnetic particles on mouse immune response. Wei sheng wu xue bao= Acta microbiologica Sinica. 2010; 50(6): 817-821.

69. Sun JB, Duan JH, Dai SL, Ren J, Zhang YD, Tian JS, Li Y. In vitro and in vivo antitumor effects of doxorubicin loaded with bacterial magnetosomes (DBMs) on H22 cells: the magnetic bio-nanoparticles as drug carriers. Cancer letters. 2007; 258(1): 109-117.

70. Felfoul O, Mohammadi M, Martel S. Magnetic resonance imaging of Fe 3 O 4 nanoparticles embedded in living magnetotactic bacteria for potential use as carriers for in vivo applications. InEngineering in Medicine and Biology Society, 2007. EMBS 2007. 29th Annual International Conference of the IEEE 2007 Aug 22 (pp. 1463-1466). IEEE.

71. Durán N, Marcato PD, De Souza GI, Alves OL, Esposito E. Antibacterial effect of silver nanoparticles produced by fungal process on textile fabrics and their effluent treatment. Journal of biomedical nanotechnology. 2007; 3(2): 203-208.

72. Zheng B, Qian L, Yuan H, Xiao D, Yang X, Paau MC, Choi MM. Preparation of gold nanoparticles on eggshell membrane and their biosensing application. Talanta. 2010; 82(1): 177-183.

73. Zheng D, Hu C, Gan T, Dang X, Hu S. Preparation and application of a novel vanillin sensor based on biosynthesis of Au–Ag alloy nanoparticles. Sensors and Actuators B: Chemical. 2010; 148(1): 247-52.

74. Wang T, Yang L, Zhang B, Liu J. Extracellular biosynthesis and transformation of selenium nanoparticles and application in H 2 O 2 biosensor. Colloids and Surfaces B: Biointerfaces. 2010; 80(1): 94-102.

75. El-Sayed IH, Huang X, El-Sayed MA. Surface plasmon resonance scattering and absorption of anti-EGFR antibody conjugated gold nanoparticles in cancer diagnostics: applications in oral cancer. Nano letters. 2005; 5(5): 829-834.

76. Shan G, Xing J, Zhang H, Liu H. Biodesulfurization of dibenzothiophene by microbial cells coated with magnetite nanoparticles. Applied and environmental microbiology. 2005; 71(8): 4497-4502.

77. Raschke G, Kowarik S, Franzl T, Sönnichsen C, Klar TA, Feldmann J, Nichtl A, Kürzinger K. Biomolecular recognition based on single gold nanoparticle light scattering. Nano letters. 2003; 3(7): 935-938.

78. Tanaka T, Takeda H, Ueki F, Obata K, Tajima H, Takeyama H, Goda Y, Fujimoto S, Matsunaga T. Rapid and sensitive detection of 17β-estradiol in environmental water using automated immunoassay system with bacterial magnetic particles. Journal of biotechnology. 2004; 108(2): 153-159.

79. Pumera M, Castaneda MT, Pividori MI, Eritja R, Merkoçi A, Alegret S. Magnetically trigged direct electrochemical detection of DNA hybridization using Au67 quantum dot as electrical tracer. Langmuir. 2005; 21(21): 9625-9629.

80. Wang J, Liu G, Polsky R, Merkoçi A. Electrochemical stripping detection of DNA hybridization based on cadmium sulfide nanoparticle tags. Electrochemistry communications. 2002; 4(9): 722-726.

81. Li YV, Cathles LM, Archer LA. Nanoparticle tracers in calcium carbonate porous media. Journal of nanoparticle research. 2014; 16(8): 2541.

Current Research in Microbiology

Chapter 3

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

***Abrar Ahmad[1*]; Mohd Jahir Khan[2]; Satya Prakash Gupta[3]; Mohd Mabood Khan[4]; Mohammad Kashif[5]; Mahmood Ahmad Khan[6] and Anjum Bee[7]***

*[1]Environmental Biotechnology Division, CSIR-Indian Institute of Toxicology Research, Mahatma Gandhi Marg, Lucknow 226001, India.*

*[2] School of Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India.*

*[3] Department of Biotechnology, DDU, Gorakhpur University, Gorakhpur, India.*

*[4] Department of Pathology, King George Medical University, Lucknow, India.*

*[5] Plant Molecular Biology and Genetic Engineering Division, CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, India.*

*[6] Department of Biochemistry, UCMS & GTB Hospital, Dilshad Garden, Delhi 110095, India.*

*[7] Department 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 degardative 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, Benzonaphthothiopphene.

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.

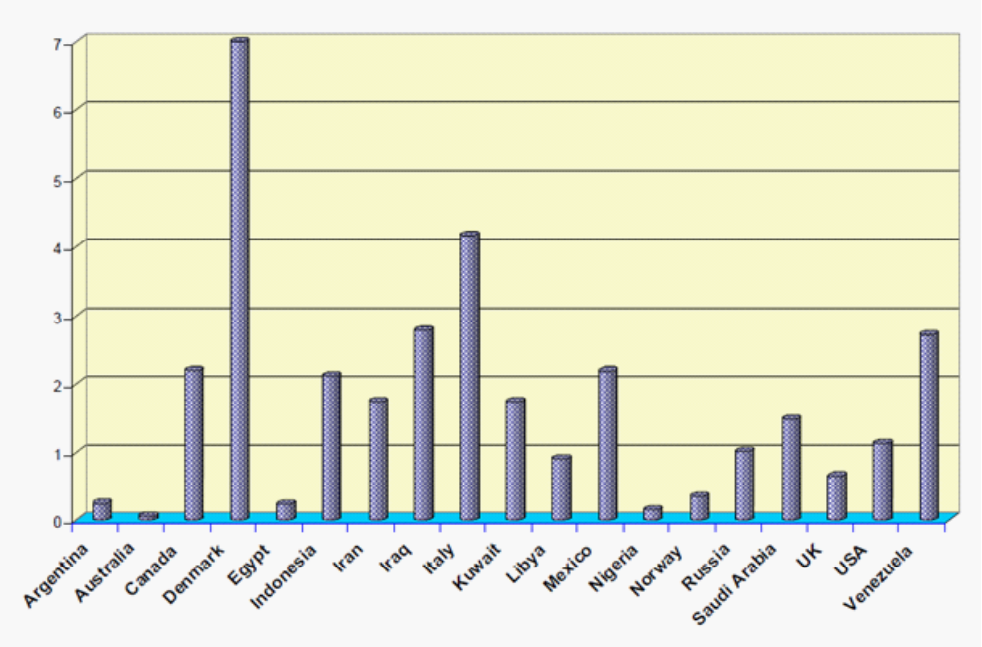
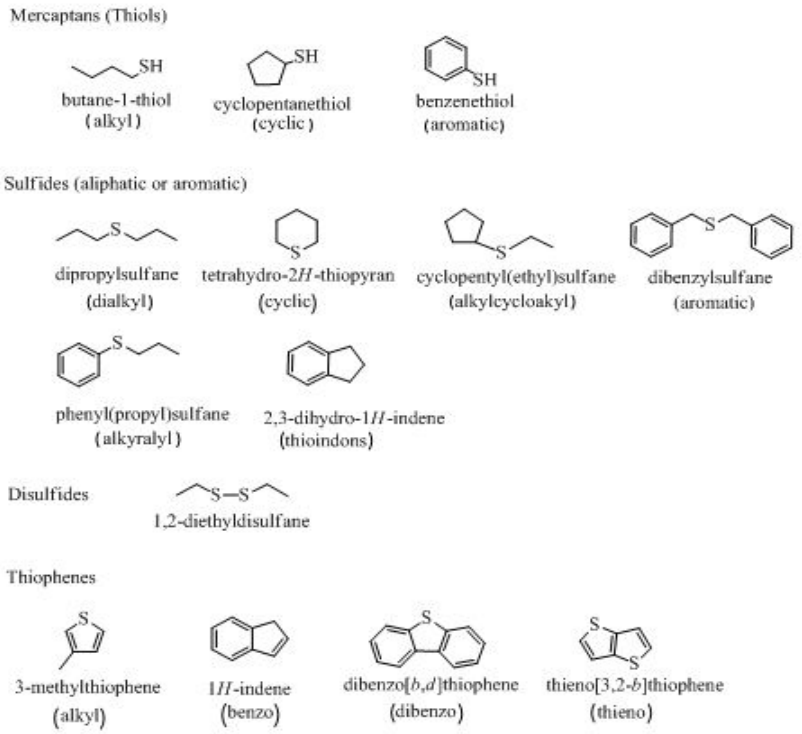



**Figure 1.1**: Average organic sulfur contents in crude oils [11].




**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].

$SO_2$ is also responsible for various health hazards, such as respiratory tract cancer and cardio-respiratory diseases [16]. $SO_2$ 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 mixting of $SO_2$ with dust in the atmosphere or with fog increases the noxious effect. The plant kingdom is also very sensitive to the $SO_2$ concentration; exposure to 1-2 ppm $SO_2$ 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 ($SO_2$) 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 bioacumulated 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 organisations 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 sufur 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-

odology [46].

## 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].

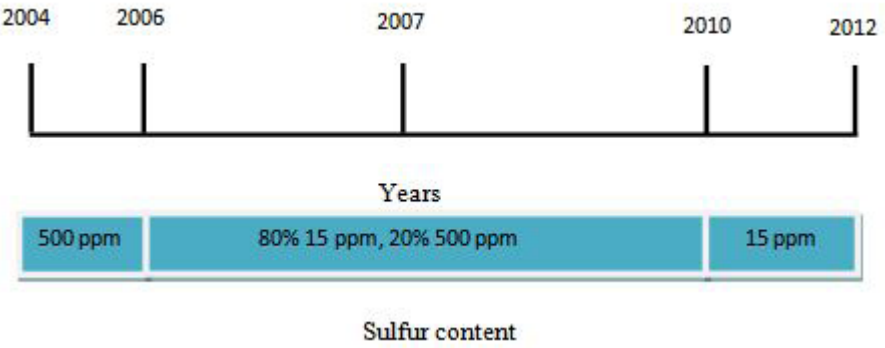
## 8. The need for Enhancement of Biodesulfurization

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) $SO_x$ 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.




Evolution of permissible sulfur content of diesel fuel [64].

## 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].

| Bacterium | Reference |
|---|---|
| *Agrobacterium* sp. strain MC501 | Constanti *et al.* (1994) |
| *Arthrobacter* sp. strain ECRD-1 | Lee *et al.* (1995) |
| *Arthrobacter sulfureus* | Labana *et al.* (2005) |
| *Bacillus subtilis* strain WU-S2B | Kirimura *et al.* (2001) |
| *Bacillus brevis* strain R-6 | Jiang *et al.* (2002) |
| *Bacillus sphaericus* strain R-16 | Jiang *et al.* (2002) |
| *Bacillus subtilis* strain Fds-1 | Ma *et al.* (2006c) |
| *Corynebacterium* sp. strain SY1 | Omori *et al.* (1992) |
| *Corynebacterium* sp. strain P32C1 | Maghsoudi *et al.* (2000) |
| *Corynebacterium* sp. strain ZD-1 | Wang *et al.* (2006) |
| *Desulfovibrio desulfuricans* | Yamada *et al.* (1968) |
| *Gordona* sp. strain CYKS1 | Rhee *et al.* (1998) |
| *Gordona* sp. strain WQ-01 | Jia *et al.* (2006) |
| *Gordonia* alkanivorans strain 1B | Alves *et al.* (2005) |
| *Gordonia* sp. strain F.5.25.8 | Duarte *et al*. (2001) |
| *Gordonia* sp. strain ZD-7 | Li *et al.* (2006) |
| *Gordonia* alkanivorans RIPI90A | Mohebali *et al.* (2007) |
| *Klebsiella* sp. | Dudley & Frost (1994) |
| *Mycobacterium* sp. strain G3 | Nekodzuka *et al.* (1997) |
| *Mycobacterium* sp. strain X7B | Nekodzuka *et al.* (1997) |
| *Mycobacterium* sp. strain ZD-19 | Li *et al*. (2003) |
| *Mycobacterium* goodii strain X7B | Chen *et al.* (2008) |
| *Mycobacterium phlei* strain SM120-1 | Srinivasaraghavan *et al.* (2006) |
| *Mycobacterium phlei* strain GTIS10 | Kayser *et al.* (2002) |
| *Nocardia globelula* | Wang & Krawiec (1994) |
| *Nocardia globerula* strain R-9 | Jiang *et al*. (2002) |
| *Nocardia asteroids* | Olson (2000) |
| *Paenibacillus* sp. strain A11-2 | Konishi *et al.* (1997) |
| *Pseudomonas abikonensis* strain DDA109 | Yamada *et al.* (1968) |
| *Pseudomonas jianii* strain DDC279 | Yamada *et al.* (1968) |
| *Pseudomonas jianii* strain DDE 27 | Yamada *et al.* (1968) |
| *Pseudomonas delafieldii* strain R-8 | Jiang *et al.* (2002) |
| *Pseudomonas* sp. strain ARK | Honda *et al.* (1998) |
| *Pseudomonas putida* strain CECT5279 | Alcon *et al*. (2005) |
| *Rhodococcus erythropolis* strain IGTS8 | Kilbane (1992) |
| *Rhodococcus* sp. strain UM3 | Purdy *et al.* (1993) |

| | |
|---|---|
| *Rhodococcus* sp. strain UM9 | Purdy *et al.* (1993) |
| *Rhodococcus erythropolis* strain D-1 | Izumi *et al.* (1994) |
| *Rhodococcus* sp. strain ECRD-1 | Lee *et al.* (1995) |
| *Rhodococcus erythropolis* strain H-2 | Ohshiro *et al*. (1995) |
| *Rhodococcus* sp. strain X309 | Omori *et al.* (1995) |
| *Rhodococcus* sp. strain B1 | Denis-Larose *et al*. (1997) |
| *Rhodococcus erythropolis* strain I-19 | Denis-Larose *et al.* (1997) |
| *Rhodococcus erythropolis* strain KA2-5-1 | Folsom *et al.* (1999) |
| *Rhodococcus* sp. strain P32C1 | Kobayashi *et al.* (2000) |
| *Rhodococcus* sp. strain T09 | Maghsoudi *et al.* (2000) |
| *Rhodococcus* sp. strain IMP-S02 | Matsui *et al.* (2001) |
| *Rhodococcus* sp. strain DS-3 | Akbarzadeh *et al.* (2003) |
| *Rhodococcus* sp. | **Ma** *et al.* **(2006d)** |
| *Rhodococcus erythropolis* strain XP | Labana *et al.* (2005) |
| *Rhodococcus* sp. strain 1awq | Yu *et al.* (2006) |
| *Rhodococcus erythropolis* strain XP | Ma *et al.* (2006a) |
| *Rhodococcus erythropolis* strain DS-3 | Yu *et al.* (2006) |
| *Rhodococcus erythropolis* strain DR-1 | Ma *et al.* (2006b) |
| *Rhodococcus erythropolis* strain NCC-1 | Li *et al.* (2007a) |
| *Rhodococcus erythropolis* strain LSSE8-1 | Li *et al*. (2007b) |
| *Sphingomonas* sp. strain AD109 | Zhang *et al.* (2007) |
| *Sphingomonas subarctica* strain T7b | Darzins & Mrachko (1998) |
| *Xanthomonas* sp. | *Gunam et al. (2006)* |
| *Gordonia* sp. IITR100 | Ahmad *et al*. (2014) |

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

Heterocyclic compounds such as dibenzothiophene, 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 dibenzothiophene, 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 dibenzothiophene. The reaction is accompanied by the production of sulfite ions and 2-hydroxybiphenyl. In nature, many bacterial strains capable of desulfurizing dibenzothiophene 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 dibenzothiophene [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 dibenzothiophene (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

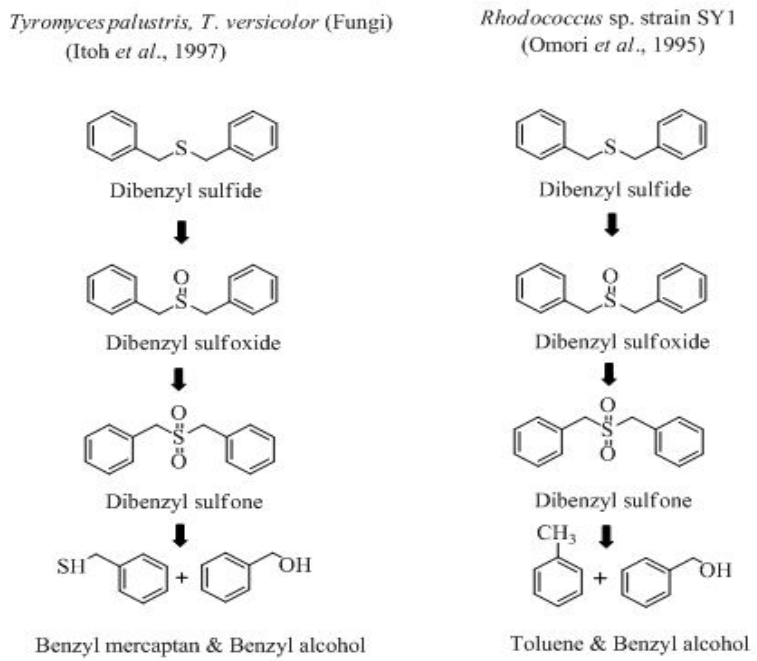
Large reserves of different heavy crude oils which have high viscosity, correlated to the average molecular weight of the material and to the asphaltene content have been reported [86, 87]. Transportation of such heavy and viscous oils by pipeline requires the addition of low molecular weight hydrocarbon 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 easiear. 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. KIbD 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 *Dsz*D 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 *dsz*ABC genes and one *dsz*D 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 *dsz*B 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 *dsz*ABC to *dsz*BCA 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 organosulfur 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

1. Kilbane II JJ (2006) Microbial biocatalysts development to upgrade fossil fuels. Curr Opin Biotechnol. 17: 305-314.

2. Lu J, Nakajima-Kambe T, Shigeno T, Ohbo A, Nomura N, Nakahara T (1999) Biodegradation of dibenzothiophene and 4, 6-dimethyldibenzothiophene by Sphingomonas paucimobilis strain TZS-7. J Biosci Bioeng. 88: 293-299.

3. Kropp K, Fedorak P (1998) A review of the occurrence, toxicity, and biodegradation of condensed thiophenes found in petroleum. Can J Microbiol. 44: 605-622.

4. Van Hamme JD, Singh A, Ward OP (2003a) Recent advances in petroleum microbiology. Microbiol Mol Biol Rev. 67: 503–549.

5. Kropp K G, Andersson JT, Fedorak PM (1997) Bacterial transformations of 1,2,3,4 tetrahidrodibenzothiophene and dibenzothiophene. Appl Environ Microbiol. 63: 3032-3042.

6. Oldfield C, Wood NT, Gilbert SC, Murray FD, Faure FR (1998) Desulfurisation of benzothiophene and dibenzothiophene by actinomycete organisms belonging to the genus Rhodococcus, and related taxa. Antonie van Leeuwenhoek. 74: 119-132.

7. Wang P, Krawiec S (1994) Desulfurization of dibenzothiophene to 2-hydroxybiphenyl by some newly isolated bacterium strain. Arch Microbiol. 161: 266-271.

8. Fairbairn DJ, Bushell ME (1992) Evaluation of continous enrichment as a method for isolating microbial cultures of coal desulfurization. Enzyme Microb Technol. 14: 48-52.

9. Blumberg KO, Walsh MP (2003) Pera. Ch. Low-sulfur gasoline and diesel: The key to lower vehicle emission. International council on clean transportation (ICCT).

10. Rall HT, Thompson CJ, Coleman HJ, Hopkins RL (1972) Sulfur compounds in crude oil. Bulletin 659, US, Dept Interior, Bureau of mines.

11. Marcelis CLM (2003) Anaerobic biodesulfurization of thiophene. PhD thesis.University of Wageningen.

12. Schulz H, Bohringer W, Ousmanov F, Waller P (1995) Referectory sulfur compounds in gas oils. Fuel Process. Technol. 6: 15-41.

13. Monticello DJ (2000) Biodesulfurization and the upgrading of petroleum distillates. Curr Opi Biotechnol. 11: 540-546.

14. Smith SJ, Aardenne Jv, Klimont Z, Andres RJ, Volke A, Delgado Arias S (2011) Anthropogenic sulfur dioxide emissions: 1850–2005. Atmos Chem Phy. 11: 1101-1116.

15. Oldfield C, Wood NT, Gilbert SC, Murray FD, Faure FR (1998) Desulfurisation of benzothiophene and dibenzothiophene by actinomycete organisms belonging to the genus Rhodococcus, and related taxa. Antonie van Leeuwenhoek. 74: 119-132.

16. Malik A, Dastidar MG, Roychoudhury PK (2001b) Biodesulfurization of coal: Mechanism and rate limiting factors. J Environ Sci Health A. 36: 1113-1128

17. Schmidt M, Siebert W, Bagnall KW (1973) The chemistry of sulfur, selenium, tellurium and polonium. Pergamon Texts in Inorganic Chemistry, vol.15, Pergamon Press, Oxford.

18. Monticello DJ (1998) Riding the fossil fuel biodesulfurization wave. Chemtech. 28: 38145.

19. Maliyantz AA (1935) Bacteriologische Methods der Entschwefelung Von erdel. Azerbaidzhanskoe Neftyanoe Khoz. 6: 89-93.

20. Shennan, J. L. (1996) Microbial attack on sulfur-containing hydrocarbons: implications for the biodesulfurisation of oils and coals. J Chem Technol Biotechnol. 67: 109-123.

21. Kodama K, Umehara K, Shimizua K, Nakatanni S, Minuda Y, Yamad K (1973) Identification of microbial products from dibenzothiophene and its proposed oxidation pathway. Agr Biol Chem. 37: 45-50.

22. Kilbane JJ, Bielaga BA (1990) Towards sulfur free fuels. Chemtech. 20: 747-751.

23. Campbell IM (1993) An orphaned child makes good. The story of USDOE/PETC's foray into fossil fuels biodesulfurization. Prepr Am Chem So Div Petroleum Chem. 38: 275-278.

24. Naser W (1999) CEO energy biosystem corporation the woodlands,texas before the senate environment and public works subcommittee on clean air,wetlands,private property and nuclear safety, http://epw.senate.gov/107th/nas_5-18.htm.

25. Arensdorf JJ, Loomis AK, Digrazia PM, Monticello DJ, Pienkos PT (2002) Chemostat approach for the directed evolution of biodesulfurization gain of function mutants. Appl Environ Microbiol. 68: 691-698.

26. Hirasawa K, Ishii Y, Kobayashi M, Koizumi K, Maruhashi K (2001) Improvement of desulfurization activity in Rhodococcus erythropolis KA2-5-1 by genetic engineering. Biosci Biotechnol Biochem 65:239-246.

27. Matsui T, Hirasawa K, Konishi J, Tanaka Y, Maruhashi K, Kurane R (2001) Microbial desulfurization of alkylated dibenzothiophene and alkylated benzothiophene by recombinant Rhodococcus sp. strain T09. Appl Microbiol Biotechnol. 56: 196-200.

28. Kerterz MA, Wietek C (2001) Desulfurization and desulfonation: Application of sulfur controlled gene expression in bacteria. App Microbiol Biotechnol. 57: 460-466.

29. Li MZ, Squires CH, Monticello DJ, Childs JD (1996) Genetic analysis of the dsz promoter and associated regulatory regions of Rhodococcus erythropolis IGTS8. J Bacteriol. 178: 6409-6418.

30. Kobayashi M, Horiuchi K,Yoshikawa O, Hirasawa K, Ishii P, Fujino K, Sugiyam H, Maruhashi K (2001) Kinetic analysis of microbial desulfurization of model and light gas oil containing multiple alkyl dibenzothiophenes. Biosci Biotech Biochem. 65: 298-304.

31. Folsom BR, Schieche DR., DiGrazia PM, Werner J, Palmer S (1999) Microbial desulfurization of alkylated dibenzothiophenes from a hydrodesulfurized middle distillate by Rhodococcus erythropolis I-19. Appl Environ Microbiol. 65: 4967-4972.

32. Grossman MJ, Lee MK, Prince RC, Minak-Bernero V, George GN, Pickering IJ (2001) Deep desulfurization of extensively hydrodesulfurized middle distillate oil by Rhodococcus sp. strain ECRD-1. Appl Environ Microbiol. 67: 1949-1952.

33. Rhee S, Chang JH, Chang YK, Chang HN (1998) Desulfurization of dibenzothiophene and diesel oils by a newly isolated Gordona strain, CYKS1. Appl Environ Microbiol. 64: 2327-2331.

34. Folsom BR, Schieche DR., DiGrazia PM, Werner J, Palmer S (1999) Microbial desulfurization of alkylated dibenzothiophenes from a hydrodesulfurized middle distillate by Rhodococcus erythropolis I-19. Appl Environ Microbiol. 65: 4967-4972.

35. Grossman MJ, Lee MK, Prince RC, Garrett KK, George GN, Pickering IJ (1999) Microbial desulfurization of a crude oil middle-distillate fraction: analysis of the extent of sulfur removal and the effect of removal on remaining sulfur. Appl Environ Microbiol 65:181-188.

36. Furuya T, Ishii Y, Noda K, Kino K, Kirimura K (2003) Thermophilic biodesulfurization of hydrodesulfurized light gas oils by Mycobacterium phlei WU-F1. FEMS Microbiol. Lett 221:137-142.

37. Guobin S, Jianmin X, Huaiying Z, Huizhou L (2005) Deep desulfurization of hydrodesulfurized diesel oil by Pseudomonas delafieldii R-8. J Chem Technol Biotechnol 80:420-424.

38. Lee IS, Bae H, Ryu HW, Cho K, Chang YK (2005) Biocatalytic desulfurization of diesel oil in an air-lift reactor with immobilized Gordonia nitida CYKS1 cells. Biotechnol Prog. 21: 781-785.

39. Gupta N, Roychoudhury PK, Deb JK (2005) Biotechnology of desulfurization of diesel: prospects and challenges. Appl Microbiol Biotechnol 66: 356-366.

40. Kaufman EN, Harkins JB, Barole AP (1998) Comparison of batch-stirred and electrospray reactors for biodesulfurization of dibenzothiophene in crude oil and hydrocarbon feedstocks. Appl Biochem Biotechnol. 73: 127-14.

41. Van Hamme JD, Wong ET, Dettman, H, Gray MR, Pickard MA (2003b) Dibenzyl sulfide metabolism by White Rot Fungi. Appl Environ Microbio. 69: 1320-1324.

42. Shan GB, Xing JM, Zhang HY, Liu HZ (2005) Biodesulfurization of dibenzothiophene by microbial cells coated with magnetite nanoparticles. Appl Environ Microbiol. 71: 4497-4502.

43. Hou Y, Kong Y, Yang J, Zhang J, Shi D, Xin W (2005) Biodesulfurization of dibenzothiophene by immobilized cells of Pseudomonas stutzeri UP-1. Fuel. 84: 1975-1979.

44. Kaufman EN, Harkins JB, Rodriguez M, Tsouris C, Selvaraj PT, Murphy SE (1997) Development of an electrospray bioreactor for crude oil processing. Fuel Process Technol. 52: 127-144.

45. Konishi M, Kishimoto M, Tamesui N, Omasa I, Shioya S, Ohtake H (2005b) The separation of oil from an oil-water-bacteria mixture using a hydrophobic tubular membrane. Biochem. Eng J. 24: 49-54.

46. Bordoloi NK, Rai SK, Chaudhuri MK, Mukherjee AK (2014) Deep-desulfurization of dibenzothiophene and its derivatives present in diesel oil by a newly isolated bacterium Achromobacter sp. to reduce the environmental pollution from fossil fuel combustion. Fuel Process Technol. 119: 236-244.

47. Ohshiro T, Izumi Y (1999) Microbial desulfurization of organic sulfur compounds in petroleum. Biosci Biotechnol Biochem. 63: 1-9.

48. Grossman MJ, Lee MK, Prince RC, Garrett KK, George GN, Pickering IJ (1999) Microbial desulfurization of a crude oil middle-distillate fraction: analysis of the extent of sulfur removal and the effect of removal on remaining sulfur. Appl Environ Microbiol. 65: 181-188.

49. Maghsoudi S, Vossoughi M, Kheirolomoom A, Tanaka E, Katoh S (2001) Biodesulfurization of hydrocarbons and diesel fuels by Rhodococcus sp. strain P32C1. Biochem Eng J. 8: 151-156.

50. Ma T, Li G, Li J, Liang F, Liu R (2006) Desulfurization of dibenzothiophene by Bacillus subtilis recombinants carrying dszABC and dszD genes. Biotechnol Lett. 28: 1095-1100.

51. Chang JH, Rhee SK, Chang YK, Chang HN (1998) Desulfurization of diesel oils by a newly isolated dibenzothiophene-degrading Nocardia sp. strain CYKS2. Biotechnol Prog. 14: 851-855.

52. Ishii Y, Kozaki S, Furuya T, Kino K, Kirimura K (2005) Thermophilic biodesulfurization of various heterocyclic sulfur compounds and crude straight-run light gas oil fraction by a newly isolated strain Mycobacterium phlei WU-0103. Curr Microbiol. 50: 63-70.

53. McFarland BL (1999) Biodesulfurization. Curr Opin Microbiol. 2: 257-264.

54. Konishi J, Ishii Y, Onaka T, Okumura K, Susuki M (1997) Thermofilic carbon-sulfur bond targeted biodesulfurization. Appl Environ Microbiol 63:3164-3169.

55. Onaka T, Konishi J, Ishii Y, Maruhashi K (2001b) Desulfurization characteristics of thermophilic Paenibacillus sp. strain A11-2 against asymmetrically alkylated dibenzothiophenes. J Biosci Bioeng. 92: 193-196.

56. Konishi J, Ishii Y, Onaka T, Ohta Y, Suzuki M, Maruhashi K (2000b) Purification and characterization of dibenzothiophene sulfone monooxygenase from the thermophilic bacterium Paenibacillus sp. strain A11-2. J Biosci Bioeng. 90: 607-613.

57. Kirimura K, Furuya T, Nishii Y, Ishii Y, Kino K, Usami S (2001) Biodesulfurization of dibenzothiophene and its derivatives through the selective cleavage of carbon-sulfur bonds by a moderately thermophilic bacterium Bacillus subtilis WU-S2B. J Biosci Bioeng. 91:262-266.

58. EPA, 2011. Sulfur regulation for cleaner environment and health.

59. Gary JH, Handwerk GE (2001) Petroleum Refining: Technology and Economics. 4th edn, New York: Marcel Dekker.

60. Shafi R, Hutchings GJ (2000) Hydrodesulfurization of hindered dibenzothiophene: An Overview. Cata Today. 59: 423-442.

61. Blumberg KO, Walsh MP (2003) Pera. Ch. Low-sulfur gasoline and diesel: The key to lower vehicle emission. International council on clean transportation (ICCT).

62. EPA, 2012. Sulfur limition for cleaner environment and health.

63. Exxon Mobil, 2007. The Outlook for Energy - A View to 2030.

64. Keaveney T (2008) Ultra low sulfur diesel update. http://www.northeastdiesel.org/ pdf/nhriworkshops08/ULSD-Update Sprague-Energy-pdf (accessed 10th july 2008).

65. Mohebali G, Ball AS (2008) Biocatalytic desulfurization (BDS) of petrodiesel fuels. Microbiology. 154: 2169-2183.

66. Yang JZ, Marison IW (2005) Two stage process design for the biodesulfurization of a model diesel by a newly isolated Rhodococcus globerulus DAQ3. Biochem Eng J. 27: 667-671.

67. Lee K, Levy EM (1991) Bioremediation: Waxy crude oils stranded on low energy shorelines. In Proceedings of the 1991 oil spill conference. American Petroleum Institute, Washington DC.

68. Kilbane JJ, Bielaga BA (1990) Towards sulfur free fuels. Chemtech. 20: 747-751.

69. Monticello DJ (1995) Continuous process for biological desulfurization of sulfur-bearing heterocyclic molecules. Energy Biosystem corporation, The woodlands, Texas: US patent. 5: 472-875.

70. Gallagher JR, Olson ES, Stanley DC (1993) Miccrobial desulfurization of dibenzothiophene: a sulfur specific pathway. FEMS Microbiol Lett. 107: 31-36.

71. Mohebali G, Ball AS (2008) Biocatalytic desulfurization (BDS) of petrodiesel fuels. Microbiology. 154: 2169-2183.

72. Kayser KJ, Bielaga-Jones BA, Jackowsky K, Odusan O, Kilbane JJ (1993) Utilization of organosulfur compounds by axenic and mixed cultures of Rhodococcus rhodochrous IGTS8. J Gen Microbiol. 139: 3123-3129.

73. Izumi Y,Ohshiro T,Ogino H, Hine Y,Shimao M (1994) Selective desulfurization of dibenzothiophene by Rhodococcus erythropolis D-1. Appl Environ Microbiol. 60: 223-226.

74. Wang P, Krawiec S (1994) Desulfurization of dibenzothiophene to 2-hydroxybiphenyl by some newly isolated bacterium strain. Arch Microbiol. 161: 266-271.

75. Kropp KG, Gonalves JA, Anderson JT, Fedorak FM (1994) Bacterial tansformation of benzothiophene and methylbenzothiophene. Env Sci Technol. 28: 1348-1356.

76. Gilbert SC, Morton J, Buchanan S, Oldfield C, McRoberts A (1998) Isolation of a unique benzothiophene desulfurizing bacterium Gordona sp. 213 E (NCIMB 40816) and characterization of the desulfurization pathway. Microbiol.144: 2543-2553.

77. Konishi J, Ishii Y, Onaka T, Okumura K, Susuki M (1997) Thermofilic carbon-sulfur bond targeted biodesulfurization. Appl Environ Microbiol. 63: 3164-3169.

78. Bhatia S, Sharma DK (2012) Thermophilic desulfurization of dibenzothiophene and different petroleum oils by Klebsiella sp. 13T. Environ Sci Pollut Res. 19: 3491-3497.

79. Alves L, Salgueiro Rita, Rodrigues C, Mesquita E, Matos J, Girio FM (2005) Desulfurization of dibenzothiophene, benzothiophene, and other thiophene analogs by a newly isolated bacterium, Gordonia alkanivorans strain 1B. Appl Biochem Biotechnol. 120: 199-208.

80. Tanaka Y, Matsui T, Konishi J, Maruhashi K, Kurane R (2002) Biodesulfurization of benzothiophene and dibenzothiophene by a newly isolated Rhodococcus strain. Appl Microbiol Biotechnol. 59: 325-328.

81. Tanaka Y, Onaka T, Matsui T, Maruhashi K, Kurane R (2001) Desulfurization of benzothiophene by the Gram-negative bacterium, Sinorhizobium sp. KT55. Curr Microbiol. 43: 187-191.

82. Abin-Fuentes A, Leung JC, Mohamed MES, Wang DI, Prather KL (2014) Rate-limiting step analysis of the microbial desulfurization of dibenzothiophene in a model oil system. Biotechnol Bioeng. 111: 876-884.

83. Matsui T, Onaka T, Maruhashi K, Kurane R (2001) Benzo[b]thiophene desulfurization by Gordonia rubropertinctus strain T08. Appl Microbiol Biotechnol. 57: 212-215.

84. Kirimura K, Furuya T, Sato R, Ishii Y, Kino K, Usami S (2002) Biodesulfurization of naphthothiophene and benzothiophene through selective cleavage of carbon-sulfur bonds by Rhodococcus sp. Strain WU-K2R. Appl Environ Microbiol. 68: 3867-3872.

85. Martinez I, Santos VE, Alcon A, Garcia-Ochoa F (2014) Enhancement of the biodesulfurization capacity of Pseudomonas putidaCECT5279 by co-substrate addition. Process Biochem. 1: 119–124.

86. Closmann PJ, Seba RD (1990) A correlation of viscosity and molecular weight. J Can Pet Technol. 29:115-116.

87. Ratov AN (1996) Physiochemical nature of structure formation in high-viscosity crude oils and natural bitumens and their rheological differences. Pet Chem. 36: 191-206.

88. Strausz OP, Mojelsky TW and Lown EM (1992) The molecular structure of asphaltene: an unfolding story. Fuel. 71: 1355-1363.

89. Peng P, Morales-Izquierdo A, Hogg A and Strausz OP (1997) Molecular structure of Athabasca asphaltene: sulfide, ether, and ester linkages. Energy Fuels. 11: 1171-1187.

90. Kilbane JJ, Bielaga BA (1990) Towards sulfur free fuels. Chemtech. 20: 747-751.

91. Omori T, Monna L, Saiki Y, Kodama T (1992) Desulfurization of dibenzothiophene by Corynebacterium sp. strain SY1. Appl Environ Microbiol. 58: 911-915.

92. Rhee S, Chang JH, Chang YK, Chang HN (1998) Desulfurization of dibenzothiophene and diesel oils by a newly isolated Gordona strain, CYKS1. Appl Environ Microbiol. 64: 2327-2331.

93. Visscher PT and Taylor BF (1993a) Aerobic and anaerobic degradation of a range of alkyl sulfides by a denitrifying marine bacterium. Appl Environ Microbiol. 59: 4083-4089.

94. Visscher PT and Taylor BF (1993b) A new mechanism for the aerobic catabolism of dimethyl sulfide. Appl Environ Microbiol. 59: 3784-3789.

95. Kilbane JJ, Jackowski K (1996) Biocatalytic detoxification of 2-chloroethyl ethyl sulfide. J Chem Technol Biotechnol. 65: 370-374.

96. Lee TS, Chan SH, Weigand WA, Bentley WE (2001) A metabolic model for thiodiglycol degradation: capacity constraint leads to byproduct accumulation. Bioproc Biosyst Eng. 24: 33-38.

97. Arensdorf JJ, Loomis AK, Digrazia PM, Monticello DJ, Pienkos PT (2002) Chemostat approach for the directed evolution of biodesulfurization gain of function mutants. Appl Environ Microbiol. 68: 691-698.

98. Murgich J, Abanero JA, Strausz OP (1999) Molecular recognition in aggregates formed by asphaltene and resin molecules from the Athabasca oil sand. Energy Fuels. 13: 278-286.

99. Kilbane JJ, Jackowski K (1996) Biocatalytic detoxification of 2-chloroethyl ethyl sulfide. J Chem Technol Biotechnol. 65: 370-374.

100. Omori T, Saiki Y, Kasuga K, Kodama T (1995) Desulfurization of alkyl and aromatic sulfides and sulfonates by dibenzothiophene-desulfurizing Rhodococcus sp. strain SY1. Biosci Biotechnol Biochem. 59: 1195-1198.

101. Itoh N, Yoshikda M, Miyamoto T, Ichinose H, Wariishi H, Tanaka H (1997) Fungal cleavage of thioether bond found in Yperite. FEBS Lett. 412: 281-284.

102. Omori T, Saiki Y, Kasuga K, Kodama T (1995) Desulfurization of alkyl and aromatic sulfides and sulfonates by dibenzothiophene-desulfurizing Rhodococcus sp. strain SY1. Biosci Biotechnol Biochem. 59: 1195-1198.

103. Kirkwood KM, Ebert S, Foght JM, Fedorak PM, Gray MR (2005) Bacterial biodegradation of aliphatic sulfides under aerobic carbon-or sulfur-limited growth conditions. J appl Microbiol. 99: 1444-1454.

104. Kirkwood KM, Foght JM, Gray MR (2007) Selectivity among organic sulfur compounds in one- and two-liquid-phase cultures of Rhodococcus sp. strain JVH1. Biodegradation. 18: 473-480.

105. Kayser KJ, Bielaga-Jones BA, Jackowsky K, Odusan O, Kilbane JJ (1993) Utilization of organosulfur compounds by axenic and mixed cultures of Rhodococcus rhodochrous IGTS8. J Gen Microbiol. 139: 3123-3129.

106. Kargi F (1987) Biological oxidation of Thianthrene, Thioxanthene and Dibenzothiophene by the thermophilic organism Sulfolobus Acidocaldarius. Biotechnol Lett. 9: 478-482.

107. Schreiner BP, Stevens Jr SE and Sing T (1988) Oxidation of Thianthrene by the ligninase of Phanerochaete chrysosporium. Appl and Environ Microbiol. 54: 1858-1860.

108. Squires CH, Ji W, Xi L, Ortego BC, Pogrebinsky OC, Gray KA and Childs JD (1999) Method of desulfurization of fossil fuel with flavoprotein. US Patent. 5: 985, 650.

109. Matsubara T, Ohshiro T, Nishina Y, Izumi Y (2001) Purification,characterization, and overexpression of flavin reductase involved in dibenzothiophene desulfurization by Rhodococcus erythropolis D-1. Appl Environ Microbiol. 67: 1179-1184.

110. Shavandi M, Sadeghizadeh M, Zomorodipour A, Khajeh K (2009) Biodesulfurization of dibenzothiophene by recombinant Gordonia alkanivorans RIPI90A. Bioresour Technol. 100: 475-479.

111. Shavandi M, Sadeghizadeh M, Khajeh K, Mohebali G, Zomorodipour A (2010) Genomic structure and promoter analysis of the dsz operon for dibenzothiophene biodesulfurization from Gordonia alkanivorans RIPI90A. Appl Microbiol Biotechnol. 87: 1455-1461.

112. Ma T, Li G, Li J, Liang F, Liu R (2006) Desulfurization of dibenzothiophene by Bacillus subtilis recombinants carrying dszABC and dszD genes. Biotechnol Lett. 28: 1095-1100.

113. Reichmuth DS, Blanch HW, Keasling JD (2004) Dibenzothiophene biodesulfurization pathway improvement using diagnostic GFP fusions. Biotechnol Bioengg. 88: 94-99.

114. Hirasawa K, Ishii Y, Kobayashi M, Koizumi K, Maruhashi K (2001) Improvement of desulfurization activity in Rhodococcus erythropolis KA2-5-1 by genetic engineering. Biosci Biotechnol Biochem. 65: 239-246.

115. Watanabe K, Noda K, Maruhashi K (2003) Enhanced desulfurization in a transposon mutant strain of Rhodococcus erythropolis. Biotech lett. 25: 1299-1304.

116. Reichmuth DS, Blanch HW, Keasling JD (2004) Dibenzothiophene biodesulfurization pathway improvement using diagnostic GFP fusions. Biotechnol Bioengg. 88: 94-99.

117. Takada M, Nomura N, Okada H, Nakajima-Kambe T, Nakahara T, Uchiyama H (2005) Derepression and comparison of oil-water separation activity of the dibenzothiophene desulfurizing bacterium, Mycobacterium sp. G3. Biotech Lett. 27: 871-874.

118. Li G, Li S, Zhang M, Wang J, Zhu L, Liang F, Liu R, Ma T (2008) Genetic rearrangement strategy for optimizing the dibenzothiophene biodesulfurization pathway in Rhodococcus erythropolis. Appl Environ Microbiol. 74: 971-976.

119. Arensdorf JJ, Loomis AK, Digrazia PM, Monticello DJ, Pienkos PT (2002) Chemostat approach for the directed evolution of biodesulfurization gain of function mutants. Appl Environ Microbiol. 68: 691-698.

120. Gray KA, Mrachko GT, Squires CH (2003) Biodesulfurization of fossil fuels. Curr Opin Microbiol. 6: 229-235.

121. Klein J, Catcheside DEA, Fakoussa R, Gazso L, Fritsche W, Höfer M, Laborda F, Margarit I, Rehm H-J, Reich-Walber M, Sand W, Schacht S, Schmiers H, Setti L, Steinbüchel A (1999) Biological processing of fossil fuels. Appl Microbiol Biotechnol. 52: 2-15.

122. Reichmuth DS, Hittle JL, Blanch HW, Keasling JD (2000) Biodesulfurization of dibenzothiophene in Escherichia coli is enhanced by expression of a Vibrio harveyi oxidoreductase gene. Biotechnol Bioeng. 67: 72-79.

123. Stoner DL, Wey JE, Barrett KB, Jolley JG, Wright RB, Dugan PR (1990) Modification of water-soluble coal-derived products by dibenzothiophene-degrading microorganisms. Appl Environ Microbiol. 56: 2667-2676.

124. Lee KI, Yen TF (1990) Sulfur removal from coal through multiphase media containing biocatalysts. J Chem Tech Biotechnol. 3: 71-79.

125. Luisi PL, Laane C (1986) Solubilization of enzymes in polar solvents via reverse micelles. Tibtech. 4: 153-161.

126. Singh P, Ahmad A, Faraz S, Macwan A, Kumar A and Srivastava P (2011) Desulfurization of recalcitrant organosulfur compound 4, 6-DMDBT by the isolated strain IITR100 (Abstract). Proceedings of the 80th meeting of 'Society of Biological chemists (India)' held at Lucknow, India.

127. Ahmad A, Chauhan AK, Kushwaha HN, Javed S, Kumar A (2014). Preferential desulfurization of Dibenzylsulfide by an isolated Gordonia sp. IITR100. Biotechnology DOI 10.1007/s13205-014-0221-1.

128. Ahmad A, Chauhan AK, Javed S, Kumar A (2014). Desulfurization of Thianthrene by Gordonia sp. IITR100. Biotechnology Letter 36: 2209-2214.

129. Chauhan AK, Ahmad A, Singh SP, Kumar A (2015). Nucleotide sequencing of dszABC genes from a desulfurizing strain Mycobacterium phleiSM120-1. International Journal of Applied Biochemistry 3: 1-5.

130. Chauhan AK, Ahmad A, Singh SP, Kumar A (2015). Biodesulfurization of Benzonaphthothiophene by a newly isolated Gordoniasp. IITR100. International Biodeterioration and Biodegradation 104: 105-111.

131. Adlakha J, Singh P, Saurabh, Ram K, KumarM, Singh, M.P. Singh D, Sahai V, Srivastava P (2016). Optimization of conditions for deep desulfurization of heavy crude oil and hydrodesulfurized diesel by Gordonia sp. IITR100. Fuel, 184, 761-769

132. Jaishankar J, Singh P, and Srivastava P (2017). Draft Genome Sequence of a Biodesulfurizing Bacterium, Gordonia sp. Strain IITR100. Genome Announc. 5(17): e00230-17.

Current Research in Microbiology

Chapter 4

# Solid State Fermentation: A Source of Bioactive Molecule Production

***Seema Anil Belorkar***

*Microbiology and Bioinformatics Department, Bilaspur University, Bilaspur(C.G), India.*

*Email: seema.belorkar@gmail.com*

**Abstract**

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].

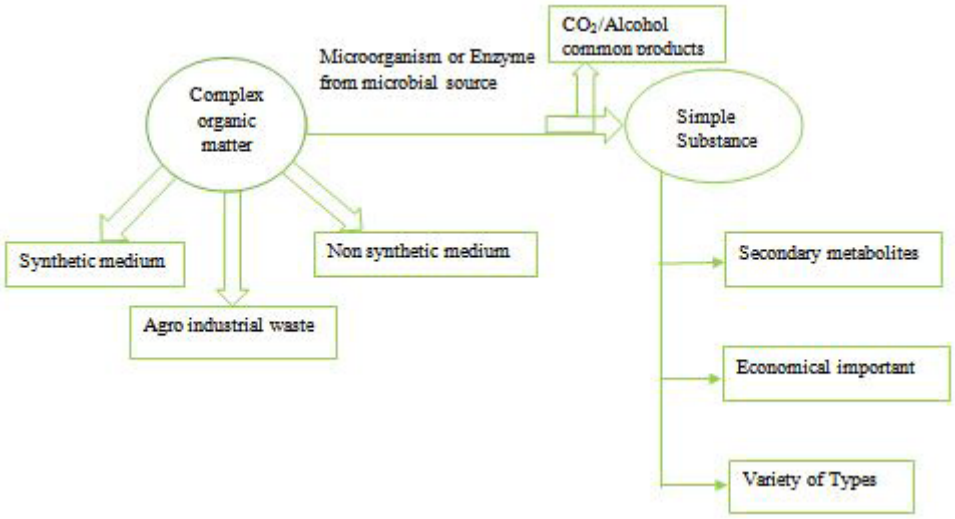



**Figure1:** Underlying principle of fermentation

## 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.**

**Table 1**

| Products | Products | Substrate used | References |
|---|---|---|---|
| Antibiotics | Iturin | soybean curd residue | Ohno et al., 1995 |
| | Griseofulvin production | Rice bran | Saykhedkar and Singhal, 2004 |
| | Anti bacterial | chicken meat | Maragkoudakis et al., 2009 |
| | Antimicrobial, antihypertensive and antioxidant properties | Cheddar cheeses | Pritchard et al., 2010 |
| Pigments | carotenoid | - | Dharmaraj et al., 2009 |
| Enzyme | Tannases, pectinases, caffeinases, mannanases, phytases, xylanases and proteases, | wheat straw or barley, sugar cane bagasse, coffee pulp, grape wastes, copra pasta | Aguilar et al., 2008 |
| | Amylase | Media | Kokilaand Mrudula, 2010 |
| Antihypertensive agents | Fermented Soybean Seasoning | Peptides | Nakahara et al., 2010 |
| Antitumor agents | Taxol | medium M1D | Ruiz-Sanchez et al., 2010 |

## 3. References

1. Demain AL (1999). Pharmaceutically active secondary metabolites of microorganisms, Applied Microbiol ogy and Biotechnology, 52: 455-463.

2. Rossi SC (2009). Improving fruity aroma production by fungi in SSF using citric pulp,Food Research International, 42: 484-486.

3. Daverey A, Pakshirajan K (2009). Production of sophrolipids by the yeast Candida bombicola using simple and low cost fermentative media. Food Research International, 42: 499-504.

4. Balakrishnan K,Pandey A (1996). Production ofBiologically Active Secondary Metabolites in Solid State Fermentation, Journal of Scientific and Industrial Research, 55: 365-372.

5. Machado CM, Oishi BO,Pandey A, Soccol CR (2004). Kinetics of Gibberella fujikori Growth and Gibberellic Acid Production by Solid State Fermentation in a Packed-Bed Column Bioreactor, Biotechnology Progress, 20: 1449-1453.

6. Robinson T, Singh D, Nigam P (2001). Solid-state fermentation: a promising microbial technology for secondary metabolite production, Applied Microbiology and Biotechnology, 55: 284-289.

7. Ohara A, Castro RJS, Nishide TG, Dias GFF, Bagagli PP, Sato HH (2015). Invertase production by Aspergillus niger under solidstate fermentation: Focus onphysical–chemical parameters, synergistic and antagonistic effects using agro-industrial wastesBiocatalysis and Agricultural Biotechnology (1): 25-06.

8. Ohno A, Ano T, Shoda M(1995) Production of a Lipopeptide Antibiotic, Surfactin, by Recombinant Bacillussubtilis in Solid State Fermentation. Biotechnology and Bioengineering, 47: 209-214.

9. Saykhedkar SS, Singhal RS (2004). Solid-State Fermentation for Production of Griseofulvin on Rice Bran Using Penicillium griseofulvum, Biotechnology Progress, 20: 1280-1284.

10. Dharmaraj S, Askokkumar B, Dhevendran K (2009). Food-grade pigments from Streptomyces sp. isolated from the marine sponge Callyspongia diffusa, Food Research International, 42: 487-492.

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

(2009) Functional properties of novel protective lactic acid bacteria and application in raw chicken meat against Listeria monocytogenes and Salmonella enteritidis . International Journal of Food Microbiology, 130: 219-226.

12. Aguilar CN, Gutierrez-Sánchez G, Rrado-BarragánA, Rodríguez-HerreraR,Martínez-Hernandez JL and Contrer as-Esquivel JC(2008). Perspectives of Solid State Fermentation for Production of Food Enzymes. American Journal of Biochemistry and Biotechnology, 4(4): 354-366.

13. Kokila R, Mrudula S (2010). Optimization of cultureconditions for amylase production by Thermohilic Bacillussp. in submerged fermentation, Asian Journal ofMicrobiology, Biotechnology and Environmental Sciences, 12(3): 653-658.

14. Xie X, Tang Y (2007). Efficient Synthesis of Simvastatinby Use of Whole-Cell Biocatalysis, Applied andEnvironmental Microbiology, 73(7): 2054-2060.

15. Pansuriya RC Singhal RS (2010). Response surface methodology for optimization of production of lovastatin by solid state fermentation, Brazilian Journal of Microbiology, 41: 164-172.

16. Nakahara T, Sano A, Yamaguchi H, Sugimoto K,Chilkata H, Kinoshita E, and Uchida R (2010). Antihypertensive Effect of Peptide-Enriched Soy Sauce-Like Seasoning and Identification of its Angiotensin I-Converting Enzyme Inhibitory Activity, Journal of Agricultural and Food Chemistry, 58: 821-827.

17. Ruiz-SanchezJ, Flores-Bustamante ZR, Dendooven L, Favela-Torres E, Soca-Chafre G, Galindez-Mayer J and Flores-Cotera LB (2010). A comparative study of Taxol production in liquid and solid state fermentation with Nigrospora sp.a fungus isolated from Taxus globose, Journal of Applied Microbiology, 109: 2144-2150.

18. Pritchard SR, Phillips M, Kailasapathy K (2010). Identification of bioactive peptides in commercial Cheddarcheese. Food Research Journal, 43: 1545-1548.

Current Research in Microbiology

Chapter 5

# Enzymatic Degradation of Lignocellulosic Biomass: A Brief Update

***Mohd Jahir Khan[1*]; Abrar Ahmad[2]; Mahmood Ahmad Khan[3]; Mohammd Kashif [4]; Anjum Bee[5] Mohamad Yusof Maskat[6]***

*[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 in1980s 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, cellobiohydrolases 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.

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

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 cellulolytic 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 $CO_2$ [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 (*Fomitopsispalustris, Fomitopsispalustris*), 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 *Cellulomonasfimi* and *Thermomonosporafusca* 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 endomannases, α-galactosidases, acetylglucomannan esterases and β-mannisidases. Degradation opens with rupturing of a polymer by endomannases; 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, multidomain 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-coumaricesterases, α-l-arabinofuranosidases and α-4-O-methyl glucuronosidases for the efficient hydrolysis of xylans and mannans [80].

Endomannases usually found in white-rot fungi like *Irpexlacteus, Haematostereum-sanguinolentum and Coriolusversicolor* as well as gram-positive and gram-negative bacteria. They are extensively studies in several nonwood decaying ascomycetes such as *Sporotrichumcellulophilum, Trichodermareesei,* and *Sclerotiumrolfsii*. Additionally, α-galactosidases, acetylglucomananeesterases and β-mannonidases are explored in *Aspergillusniger* and *Polyporussulfureus* [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 *Coriolusversicolor, Phanerochaetechrysosporium and Trametesversicolor* 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 $H_2O_2$ 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 phenoxyl 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*, Trametesversicolor, Trametes sp.* strain AH28-2*, Trametespubescens* 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 pIs 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

1. Wyman CE. Twenty years of trials, tribulations, and research progress in bioethanol technology selected key events along the way. Appl Biochem Biotechnol. 2001; 91-93: 5-21.

2. Pérez J, Muñoz-Dorado J, de la Rubia T, Martínez J. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int Microbiol. 2002; 5: 53-63.

3. Lynd LR, Laser MS, Bransby D, Dale BE, Davison B, Hamilton R, Himmel M, Keller M, McMillan JD, Sheehan J, Wyman CE. How biotech can transform biofuels. Nat Biotechnol 2008; 26: 169-172.

4. Wackett LP. Biomass to fuels via microbial transformations. CurrOpinChem Biol. 2008; 12: 187-193.

5. Sánchez C. Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv. 2009; 27: 185-194.

6. Palacios-Orueta A, Chuvieco E, Parra A, Carmona-Moreno C. Biomass burning emissions: a review of models using remote-sensing data. Environ Monit Assess. 2005; 104: 189-209.

7. Asgher M, Bashir F, Iqbal HMN. A comprehensive ligninolyticpre-treatment approach from lignocellulose green biotechnology to producebio-ethanol. Chem Eng Res Des. 2014; 92: 1571-1578.

8. Ragauskas AJ, Beckham GT, Biddy MJ, Chandra R, Chen F, Davis MF, Davison BH, Dixon RA, Gilna P, Keller M, Langan P, Naskar AK, Saddler JN, Tschaplinski TJ, Tuskan GA, Wyman CE. Lignin valorization: improving lignin processing in the biorefinery. Science. 2014; 344: 1246843.

9. Kawaguchi H, Hasunuma T, Ogino C, Kondo A. Bioprocessing of bio-based chemicals produced from lignocellulosicfeedstocks. CurrOpinBiotechnol. 2016; 42: 30-39.

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

Biotechnol. 2007; 77: 819-823.

11. Wen Z, Liao W, Chen S. Hydrolysis of animal manure lignocellulosics for reducing sugar production. Bioresour Technol. 2004; 91: 31-39.

12. vanDyk JS, Pletschke BI, A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-factors affecting enzymes, conversion and synergy. Biotechnol Adv. 2012; 30: 1458-1480.

13. Zhang Z, Liu B, Zhao Z. Efficient acid-catalyzed hydrolysis of cellulose in organic electrolyte solutions. PolymDegrad Stab. 2012; 97: 573-577.

14. Sanchez, C. 2009. Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv. 27: 185-194.

15. Zhao X, Zhang L, Liu D. Biomass recalcitrance. Part II: Fundamentals of different pretreatments to increase the enzymatic digestibility of lignocellulose. Biofuels Bioprod Bioref. 2012; 6: 561-79.

16. Ani FN, Energy and value-added products from oil palm solid wastes. In: Proceedings of the regional symposium chemical engineering. Bandung, Indonesia, pBM3. 2001: 1-6.

17. Martin C, Puls J, Saake B, Schreiber A. Effect of glycerol pretreatment on component recovery and enzymatic hydrolysis of sugarcane bagasse. Cellulose Chem Technol. 2011; 45: 487-494.

18. Howard RL, Abotsi E, Jansen van Rensburg EL, Howard S. Lignocellulose biotechnology: issues of bioconversion and enzyme production. Afr J Biotechnol. 2003; 2: 602-619.

19. Malherbe S, Cloete TE. Lignocellulose biodegradation: fundamentals and applications. Reviews Environ SciBiotechnol. 2002; 1: 105-114.

20. Bridgeman TG, Jones JM, Shield I, Williams PT. Torrefaction of reed canary grass, wheat straw and willow to enhance solid fuel qualities and combustion properties. Fuel. 2008; 87: 844-856, 2008.

21. Prassad S, Singh A, Joshi HC. Ethanol as an alternative fuel from agricultural, industrial and urban residues. Resour Conserv Recycl. 2007; 50: 1-39.

22. Rowell MR. Opportunities for lignocellulosic materials and composites. Emerging technologies for material and chemicals from biomass: Proceedings of symposium. Washington, DC: American Chemical Society. 1992; 2: 26-31.

23. Ruiz E, Cara C, Manzanares P, Ballesteros M, Castro E. Evaluation of steam explosion pre-treatment for enzymatic hydrolysis of sunflower stalks. EnzMicrob Technol. 2008; 42: 160-166.

24. Wiselogel A, Tyson S, Johnson D. Biomass feedstock resources and composition. In: Wyman CE (Ed.) In: Handbook on Bioethanol: Production and Utilization. (Wyman CE, ed.), Taylor and Francis, Washington, DC. 1996; 105-118.

25. Cheng K-K, Zhang J-A, Ling HZ et al. Optimization of pH and acetic acid concentration for bioconversion of hemicellulose from corncobs to xylitol by Candida tropicalis. BiochemEng J. 2009; 43: 203-207.

26. Stewart D, Azzini A, Hall A, Morrison I. Sisal fibers and their constituent non-cellulosic polymers. Ind Crops Prod. 1997; 6: 17-26.

27. Dijkerman R, Bhansing DC, Op den Camp HJ, van der Drift C, Vogels GD. Degradation of structural polysaccharides by the plant cell-wall degrading enzyme system from anaerobic fungi: and application study. EnzMicrob Technol. 1997; 21: 130-136.

28. Téllez-Luis SJ, Ramírez JA, Vázquez M, Mathematical modelling of hemicellulosic sugar production from Sorghum straw. J Food Eng. 2002; 52: 285-291.

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

ic enzymes production in submerged fermentation of mandarin peels and tree leaves. EnzMicrob Technol. 2006; 38: 998e1004.

30. Moldes AB, Bustos G, Torrado A, Domínguez JM. Comparison between different hydrolysis processes of vine-trimming waste to obtain hemicellulosic sugars for further lactic acid conversion. Appl Biochem Biotechnol. 2007; 143: 244-256.

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

32. Antai SP, Crawford DL. Degradation of softwood, hardwood, and grass lignocelluloses by two streptomyces strains. Appl Environ Microbiol. 1981; 42: 378-380.

33. McCarthy AJ. Lignocellulose-degrading actinomycetes. FEMS Microbiol Rev. 1987; 46: 145-163.

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.

35. Vicuna R, Gonzalez B, Seelenfreund D, Ruttimann C, Salas L. Ability of natural bacterial isolates to metabolize high and low-molecular-weight lignin-derived molecules. J Biotechnol. 1993; 30: 9-13.

36. Vicuña R. Ligninolysis. A very peculiar microbial process. MolBiotechnol. 2000; 14: 173-176.

37. White GF, Russell NJ, Tidswell EC. Bacterial scission of ether bonds. Microbiol Rev. 1996; 60: 216-232.

38. Kirk TK, Farrell RL. Enzymatic "combustion": the microbial degradation of lignin. Annu Rev Microbiol. 1987; 41: 465-505.

39. Hatakka, A. Biodegradation of lignin. In: Hofrichter, M. and Steinbuchel, A., Eds., Biopolymers, Vol. 1: Lignin, Humic Substances and Coal, Wiley-VCH, Weinheim, 2001; 129-180.

40. Nieves RA, Ehrman CI, Adney WS, Elander RT, Himmel ME. Technical communication: survey and commercial cellulase preparations suitable for biomass conversion to ethanol. World J MicrobBiotechnol. 1998; 14: 301-304.

41. Jørgensen H, Errikson T, Børjesson J, Tjerneld F, Olsson L. Purification and characterization of five cellulases and one xylanases from Penicilliumbrasilianum IBT 20888. Enzyme MicrobTechnol. 2003; 32: 851-861.

42. Esterbauer H, Steined W, Labudova I, Herman A, Hayn M. Production of Thrichodermacellulase in laboratory and pilot scale. BioresourTechnol. 1991; 36: 51-65.

43. Akin DE, Rigsby LL, Sethuraman A, Morrison WH 3rd, Gamble GR, Eriksson KE.Alterations in structure, chemistry, and biodegradability of grass lignocellulose treated with the white rot fungi Ceriporiopsissubvermispora and Cyathusstercoreus. Appl Environ Microbiol. 1995; 61: 1591-1598.

44. Gold MH, AlicM.Molecular biology of the lignin-degrading basidiomycetePhanerochaetechrysosporium. Microbiol Rev. 1993; 57: 605-622.

45. Arora DS, Chander M, Gill PK. Involvement of lignin peroxidase, manganese peroxidase and laccase in the degradation and selective ligninolysis of wheat straw. Int. Bioterior. Biodegrad. 2002; 50: 115-120.

46. Bennett JW, Wunch KG, Faison BD. Use of fungi in biodegradation. In: Hurst CJ, editor. Manual of Environmental Microbiology.Washington DC: AMS press; 2002; 960-971.

47. Limayema A, Ricke SC, Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. Progress in Energy and Combustion Science. 2012; 38: 449-467.

48. Chandel A K, Chan E, Rudravaram R, Narasu M L, Rao LV, Ravindra P. Economics and environmental impact of bioethanol production technologies: an appraisal. Biotechnol MolBiol Rev. 2007; 2: 14-32.

49. Ohgren K, Bura R, Lesnicki G, Saddler J, Zacchi G. A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. Process Biochem. 2007; 42(5): 834-839.

50. Kumar S, Singh SP. Mishra IM, Adhikari DK. Recent advances in production of bioethanol from lignocellulosic biomass. ChemEng Technol. 2009; 32(4): 517-526.

51. Ting CL, Makarov DE, Wang ZG. A kinetic model for the enzymatic action of cellulase. J PhysChem B. 2009; 113(14): 4970-4077.

52. Gilkes NR, Henrissat B, Kilburn DG, Miller RC Jr, Warren RA. Domains in microbial beta-1, 4-glycanases: sequence conservation, function, and enzyme families. Microbiol Rev. 1991; 55: 303-315.

53. Cao NJ, Krishnan MS, Du JX, Gong CS, Ho NW, Chen, ZD, Tsao GT. Ethanol production from corncob pretreated by the ammonia steeping process using genetically engineered yeast. Biotechnol Lett. 1996; 18: 1013-1018.

54. Wyman CE. What is (and is not) vital to advancing cellulosic ethanol. Trends Biotechnol. 2007; 25: 153-157.

55. Hendriks AT, Zeeman G. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour Technol. 2009; 100: 10-18.

56. Wilson DB. Microbial diversity of cellulose hydrolysis. CurrOpinMicrobiol. 2011; 14: 259-263.

57. Dashtban M, Schraft H, Qin W. Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. Int J Biol Sci. 2009; 5: 578-595.

58. Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. The Carbohydrate-Active Enzymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Res. 2009; 37: D233-D238.

59. Kumar R, Singh S, Singh OV. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol. 2008; 35: 377-391.

60. Kirk TK, Jeffries TW. Roles for microbial enzymes in pulp and paper processing. In: T. W. Jeffries and L. Viikari, (ed.) Enzymes for pulp and paper processing, ACS, Washington DC. 1996; 2-14.

61. Teeri TT. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. Trends Biotechnol. 1997; 15: 160-167.

62. Linder M, Teeri TT. The roles and function of cellulose-binding domains. Journal of Biotechnology. 1997; 57(1-3): 15-28.

63. Stahlberg J, Johansson G, Pettersson G. A new model for enzymatic-hydrolysis of cellulose based on the 2-domain structure of cellobiohydrolase-I. Biotechnology. 1991; 9: 286-290.

64. Kirk K, Cullen D. Enzymology and molecular genetics of wood degradation by white rot fungi. In: Young RA, Akhtar M. (Eds.) Environmental friendly technologies for pulp and paper industry. Wiley, New York, 1998; 273-307.

65. Rabinovich ML, Melnik MS, Boloboba AV. Microbial cellulases (review). Appl Biochem Microbiol. 2002; 38: 305-322.

66. Valaskova V, Baldrian P. Degradation of cellulose and ´ hemicelluloses by the brown rot fungus Piptoporus betulinusproduction of extracellular enzymes and characterization of the major cellulases. Microbiology. 2006; 152: 3613-3622.

67. Szijártó N, Szengyel Z, Lidén G, Réczey K. Dynamics of cellulase production by glucose grown cultures of Trichoderma reesei Rut-C30 as a response to addition of cellulose. Appl Biochem Biotechnol. 2004; 113-116: 115-24.

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

Environ Microbiol. 2007; 73: 756-767.

69. Yu H, Zeng G, Huang H, Xi X, Wang R, Huang D, Huang G, Li J. Microbial community succession and lignocellulose degradation during agricultural waste composting. Biodegradation. 2007; 18: 793-802.

70. Taylor LE, Henrissat B, Coutinho PM, Ekborg NA, Hutcheson SW, Weiner RM. Complete cellulase system in the marine bacterium Saccharophagusdegradans strain 2-40T. J Bacteriol. 2006; 188: 3849-3861.

71. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. MicrobiolMolBiol Rev. 2002; 66: 506-577,

72. Percival Zhang YH, Himmel ME, Mielenz JR.Outlook for cellulase improvement: screening and selection strategies. Biotechnol Adv. 2006; 24: 452-481.

73. Biely P, Vrsanska M, Tenkanen M, Kluepfel D. Endo-β-1,4-xylanase families: differences in catalytic properties. J Biotechnol. 1997; 57: 151-166.

74. Kirk TK, Jeffries TW. Roles for microbial enzymes in pulp and paper processing. In: Jeffries TW, Viikari L. (ed.) Enzymes for pulp and paper processing. ACS, Washington DC, 1996; 2-14.

75. Tenkanen M, Siika-aho M, Hausalo T, Puls J, Viikari L. Biotechnology in the pulp and paper industry. In: Serbotnik E, Messner K. (ed.) Facultas-universitatsverlag. Vienna, 1996; 503.

76. Tenkanen M, Thorntonb J, Viikaria L. An acetylglucomannan esterase of Aspergillusoryzae; purification, characterization and role in the hydrolysis of O-acetyl-galactoglucomannan. J Biotechnol. 1995; 42: 197-206.

77. Kanda T, Amano Y, Nisizawa K. Purification and properties of two endo-1, 4-β-xylanases from Irpexlacteus (Polyporustulipiferae). J Biochem. 1985; 98: 1545-1554.

78. Dobozi MS, Szakacs G, Bruschi CV. Xylanase activity of Phanerochaete chrysosporium. Appl Environ Microbiol. 1992; 58: 3466-3471.

79. Blanco A, Dıiaz P, Zueco J, Parascandola P, Pastor JF. A multidomain xylanase from a Bacillus sp. with a region homologous to thermostabilizing domains of thermophilic enzymes. Microbiology. 1999; 145: 2163-2170.

80. Stalbrand H, Saloheimo A, Vehmaanpera J, Henrissat B, Penttila M. Cloning and expression in Saccharomyces cerevisiae of a Trichoderma reesei beta-mannanase gene containing a cellulose-binding domain. Appl Environ Microbiol. 1995; 61: 1090-1097.

81. Johnson KG, Ross NW. Enzymic properties of β-mannanase from Polyporusversicolor.Enzyme Microb Technol. 1990; 12: 960-964.

82. Hammel KE, Jensen KA Jr, Mozuch MD, Landucci LL, Tien M, Pease EA. Ligninolysis by a purified lignin peroxidase. J Biol Chem. 1993; 268: 12274-12281.

83. Araujo A, Ward OP. Hemicellulases of Bacillus species: preliminary comparative studies on production and properties of mannanases and galactanases. J Appl Bacteriol. 1990; 68: 253-261.

84. Kirk TK, Farrel RL. Enzymatic combustion: the microbial degradation of lignin. Annu Rev Microbiol. 1987; 41: 465-505.

85. Martinez AT, Speranza M, Ruiz-Duenas FJ, Ferreira P, Camarero S, Guillén F, Martínez MJ, Gutiérrez A, del Río JC. Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int Microbiol. 2005; 8: 195-204.

86. Hatakka A.Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. FEMS Microbiology Reviews. 1994; 13: 125-135.

87. Singh D, Chen S. The white-rot fungus Phanerochaete chrysosporium: conditions for the production of lignin-degrading enzymes. Appl Microbiol Biotechnol. 2008; 81: 399-417.

88. Martinez D, Larrondo LF, Putnam N, Gelpke MD, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F, Coutinho PM, Henrissat B, Berka R, Cullen D, Rokhsar D. Genome sequence of the lignocellulose degrading fungus Phanerochaete chrysosporium strain RP78. Nat Biotechnol. 2004; 22: 695-700.

89. Wang L, Yan W, Chen J, Huang F, Gao P. Function of the iron-binding chelator produced by Coriolusversicolor in lignin biodegradation. Sci China C Life Sci. 2008; 51: 214-221.

90. Moredo N, Lorenzo M, Domínguez A, Moldes D, Cameselle C, Sanroman A. Enhanced ligninolytic enzyme production and degrading capability of Phanerochaetechrysosporium and Trametesversicolor. World JMicrobBiotechnol. 2003; 19: 665-669.

91. Orth AB, Royse DJ, Tien M.Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. Appl Environ Microbiol. 1993; 59: 4017-4023.

92. Kersten PJ.Glyoxal oxidase of Phanerochaete chrysosporium: its characterization and activation by lignin peroxidase. ProcNatlAcadSci U S A. 1990; 87: 2936-2940.

93. Piontek K, Smith AT, Blodig W.Lignin peroxidase structure and function. BiochemSoc Trans. 2001; 29: 111-116.

94. Perez J, Jeffries TW. Roles of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by Phanerochaete chrysosporium. Appl Environ Microbiol. 1992; 58: 2402-2409.

95. Asgher M, Bhatti HN, Ashraf M, Legge RL.Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. Biodegradation. 2008; 19: 771-783.

96. Wong DW. Structure and action mechanism of ligninolytic enzymes. Appl Biochem Biotechnol. 2009; 157: 174-209

97. Jensen KA, Bao W, Kawai S, Srebotnik E, Hammel KE. Manganese-dependent cleavage of nonphenolic lignin structures by Ceriporiopsissubvermispora in the absence of lignin peroxidase. Appl Environ Microbiol. 1996; 62: 3679-3686.

98. Wesenberg D, Kyriakides I, Agathos SN.White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol Adv. 2003; 22: 161-187.

99. Camarero S, Sarkar S, Ruiz-Dueñas FJ, Martínez MJ, Martínez AT. Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. J Biol Chem. 1999; 274: 10324-10330.

100. Thurston CF. The structure and function of fungal laccases. Microbiology. 1994; 140: 19-26.

101. Bourbonnais R, Paice MG. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett. 1990; 267: 99-102.

102. Selinheimo E, Autio K, Kruus K, Buchert J. Elucidating the mechanism of laccase and tyrosinase in wheat bread making. J Agric Food Chem. 2007; 55: 6357-6365.

103. Couto SR, Sanroman MA, Hofer D, Gübitz GM. Production of laccase by Trameteshirsuta grown in an immersion bioreactor and its application in the decolorization of dyes from a leather factory. Eng Life Sci. 2004; 4: 233-238.

Current Research in Microbiology

Chapter 6

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

***Mohammad Kashif[1*]; Abrar Ahmad[2]; Mohd Jahir Khan[3]; Somnath Rahangdale[1]; Bhupendra Kumar[1]; Anjum Bee[4] ; Mahmood Ahmad Khan[5] and Md. Salman Akhtar[6]***

*[1]Plant Molecular Biology and Genetic Engineering Division, CSIR-NBRI, Lucknow, India.*

*[2]Environmental Biotechnology Division, CSIR-IITR, Lucknow, India.*

*[3]School of Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India.*

*[4]Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow.*

*[5]Department of Biochemistry, University College of Medical Sciences & GTB Hospital, Dilshad Garden, Delhi 110095, India.*

*[6]Department 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 microganism 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 necked 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 resistant 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**).

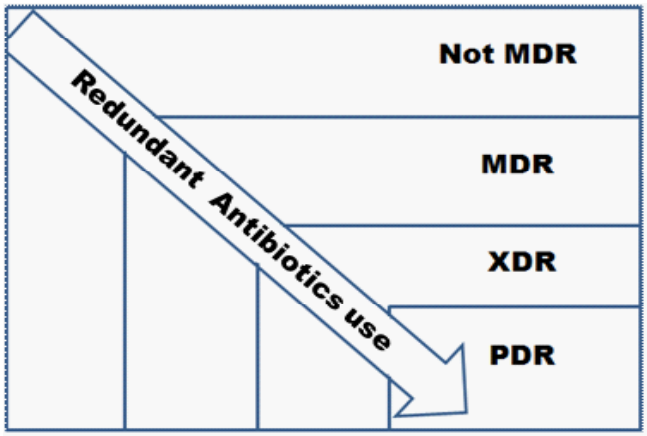



**Figure 1**: An epidemiological correlation between MDR, XDR and PDR

## 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 with azole 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 amantadenes, 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.

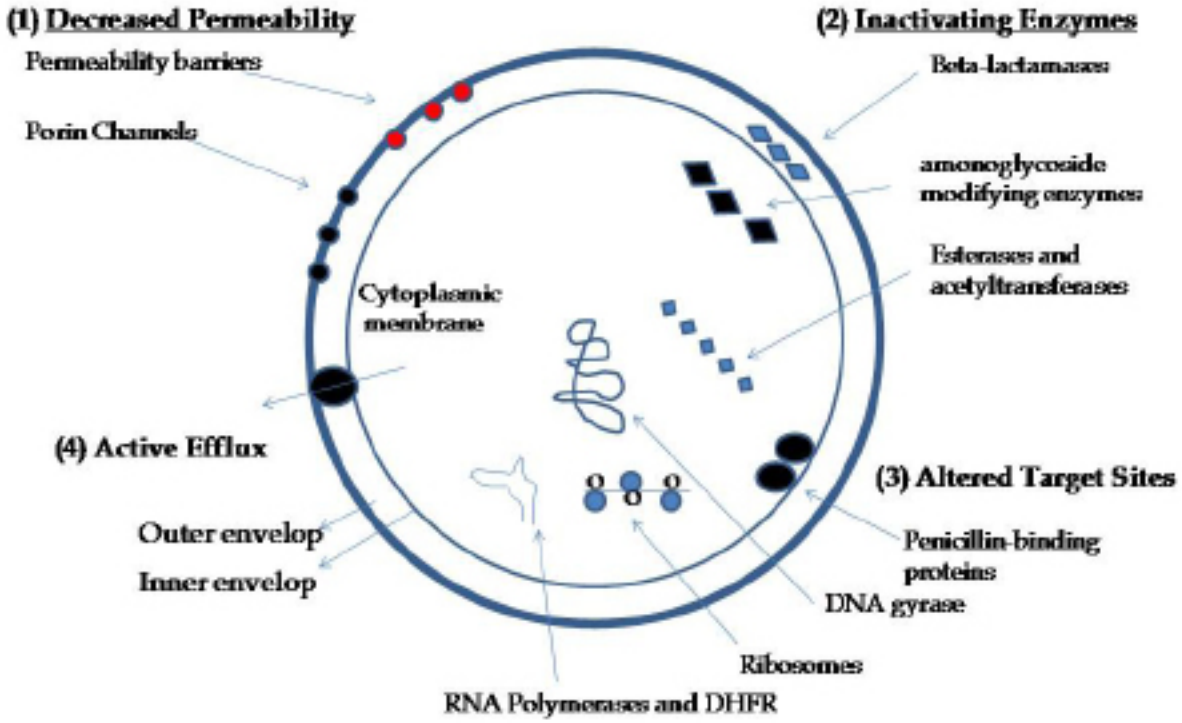



**Figure 2**: Schematic representation of various aspects of action mechanism of multidrug resistance.

## 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**

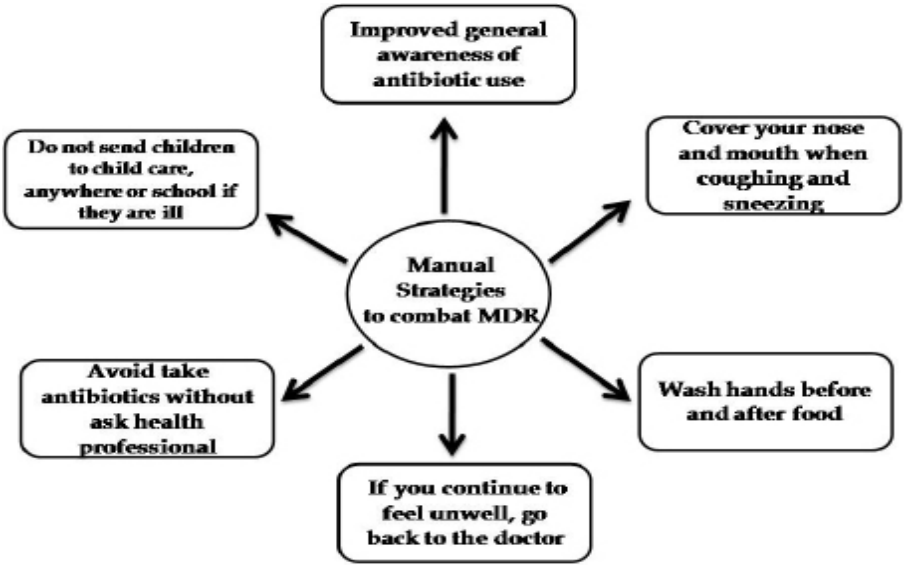



**Figure 3**: Diagrammatic representation of manual stratgies to combat multidrug resistance.

## 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 stratgies should be taken to combat the emergence and spread of antibiotic resistance worldwide described in **Figure-4** [51].

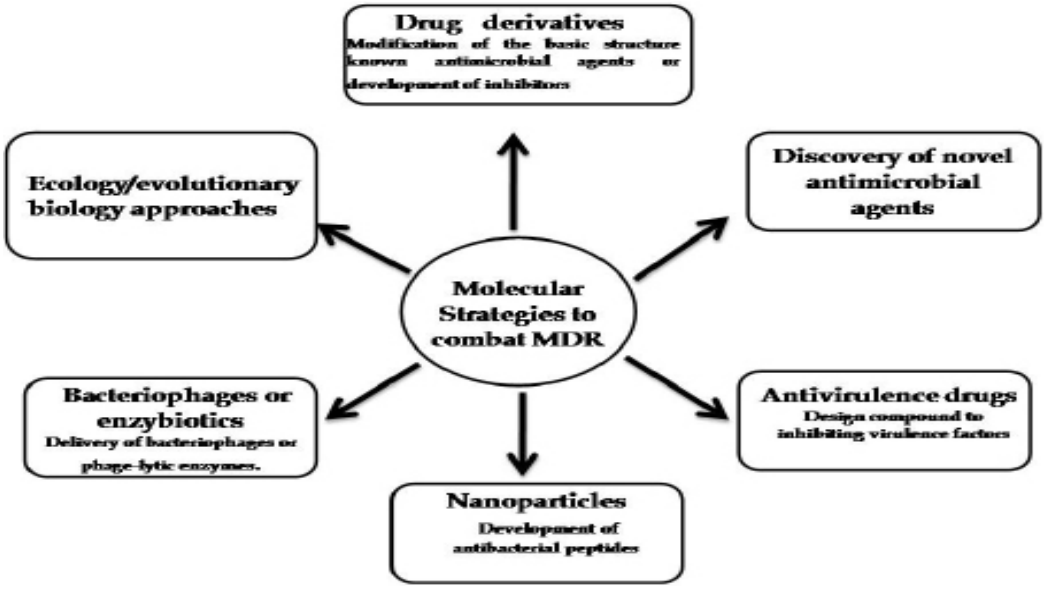



**Figure 4**: Diagrammatic representation of molecular stratgies to combat multidrug resistance.

## 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

1. Vernet, G., Mary, C., Altmann, D.M., Doumbo, O., Morpeth, S., Bhutta, Z.A. and Klugman, K.P., 2014. Surveillance for antimicrobial drug resistance in under-resourced countries. Emerging infectious diseases, 20(3), p.434.

2. Liebana, E., Carattoli, A., Coque, T.M., Hasman, H., Magiorakos, A.P., Mevius, D., Peixe, L., Poirel, L., Schuepbach-Regula, G., Torneke, K. and Torren-Edo, J., 2012. Public health risks of enterobacterial isolates producing extended-spectrum β-lactamases or AmpC β-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options. Clinical infectious diseases, 56(7), pp.1030-1037.

3. ECDC, E. and EMEA, S., 2009. Joint opinion on antimicrobial resistance (AMR) focused on zoonotic infections. EFSA Journal, 7(11), p.1372.

4. Ventola, C.L., 2015. The antibiotic resistance crisis: part 1: causes and threats. Pharmacy and Therapeutics, 40(4), p.277.

5. Kumar, S.G., Adithan, C., Harish, B.N., Sujatha, S., Roy, G. and Malini, A., 2013. Antimicrobial resistance in India: A review. Journal of natural science, biology, and medicine, 4(2), p.286.

6. Landis, S.J., 2008. Chronic wound infection and antimicrobial use. Advances in skin & wound care, 21(11), pp.531-540.

7. Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B. and Paterson, D.L., 2012. Multidrug-resistant, extensively drug-resistant and pan-drug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical microbiology and infection, 18(3), pp.268-281.

8. Seaworth, B.J. and Longfield, R.N., 2011. Therapy of Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis. In Tuberculosis and Nontuberculous Mycobacterial Infections, Sixth Edition (pp. 120-140). American Society of Microbiology.

9. Ding, P., Li, X., Jia, Z. and Lu, Z., 2017. Multidrug-resistant tuberculosis (MDR-TB) disease burden in China: a systematic review and spatio-temporal analysis. BMC infectious diseases, 17(1), p.57.

10. Prasad, R., Singh, A., Balasubramanian, V. and Gupta, N., 2017. Extensively drug-resistant tuberculosis in India: Current evidence on diagnosis & management. The Indian journal of medical research, 145(3), p.271.

11. Rahman, M.A. and Sarkar, A., 2017. Extensively Drug-resistant Tuberculosis (XDR-TB): A daunting challenge to the current End TB Strategy and policy recommendations. Indian Journal of Tuberculosis.

12. Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B. and Paterson, D.L., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical microbiology and infection, 18(3), pp.268-281.

13. Gandhi, N.R., Nunn, P., Dheda, K., Schaaf, H.S., Zignol, M., Van Soolingen, D., Jensen, P. and Bayona, J., 2010. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. The Lancet, 375(9728), pp.1830-1843.

14. Fisher, J.F., Meroueh, S.O. and Mobashery, S., 2005. Bacterial resistance to β-lactam antibiotics: compelling opportunism, compelling opportunity. Chemical reviews, 105(2), pp.395-424.

15. Mitnick, C.D., Shin, S.S., Seung, K.J., Rich, M.L., Atwood, S.S., Furin, J.J., Fitzmaurice, G.M., Alcantara Viru, F.A., Appleton, S.C., Bayona, J.N. and Bonilla, C.A., 2008. Comprehensive treatment of extensively drug-resistant tuberculosis. N Engl J Med, 2008(359), pp.563-574.

16. Goyal, N., Miller, A., Tripathi, M. and Parvizi, J., 2013. Methicillin-resistant Staphylococcus aureus (MRSA). Bone Joint J, 95(1), pp.4-9.

17. Kliiman, K. and Altraja, A., 2009. Predictors of poor treatment outcome in multi-and extensively drug-resistant pulmonary TB. European Respiratory Journal, 33(5), pp.1085-1094.

18. Spellberg, B., Powers, J.H., Brass, E.P., Miller, L.G. and Edwards Jr, J.E., 2004. Trends in antimicrobial drug development: implications for the future. Clinical Infectious Diseases, 38(9), pp.1279-1286.

19. Khan, M.S.A., Malik, A. and Ahmad, I., 2012. Anti-candidal activity of essential oils alone and in combination with amphotericin B or fluconazole against multi-drug resistant isolates of Candida albicans. Medical Mycology, 50(1), pp.33-42.

20. Herzog, T., Chromik, A.M. and Uhl, W., 2010. Treatment of complicated intra-abdominal infections in the era of multi-drug resistant bacteria. European journal of medical research, 15(12), p.525.

21. King, N.M., Melnick, L., Prabu-Jeyabalan, M., Nalivaika, E.A., Yang, S.S., Gao, Y., Nie, X., Zepp, C., Heefner, D.L. and Schiffer, C.A., 2002. Lack of synergy for inhibitors targeting a multi-drug-resistant HIV-1 protease. Protein Science, 11(2), pp.418-429.

22. Larder, B.A., 1994. Interactions between drug resistance mutations in human immunodeficiency virus type 1 reverse transcriptase. Journal of General Virology, 75(5), pp.951-957.

23. Memoli, M.J., Hrabal, R.J., Hassantoufighi, A., Eichelberger, M.C. and Taubenberger, J.K., 2010. Rapid selection of oseltamivirand peramivir-resistant pandemic H1N1 virus during therapy in 2 immunocompromised hosts. Clinical infectious diseases, 50(9), pp.1252-1255.

24. Pearson, M.L., Jereb, J.A., Frieden, T.R., Crawford, J.T., Davis, B.J., Dooley, S.W. and Jarvis, W.R., 1992. Nosoco-

mial transmission of multidrug-resistant mycobacterium tuberculosisa risk to patients and health care workers. Annals of internal medicine, 117(3), pp.191-196.

25. Frange, P., Boutolleau, D., Leruez-Ville, M., Touzot, F., Cros, G., Heritier, S., Moshous, D., Neven, B., Fischer, A. and Blanche, S., 2013. Temporal and spatial compartmentalization of drug-resistant cytomegalovirus (CMV) in a child with CMV meningoencephalitis: implications for sampling in molecular diagnosis. Journal of clinical microbiology, 51(12), pp.4266-4269.

26 Ejaz, M., Siddiqui, A.R., Rafiq, Y., Malik, F., Channa, A., Mangi, R., Habib, F. and Hasan, R., 2010. Prevalence of multi-drug resistant tuberculosis in Karachi, Pakistan: identification of at risk groups. Transactions of the Royal Society of Tropical Medicine and Hygiene, 104(8), pp.511-517.

27. Guan, J., Kyle, D.E., Gerena, L., Zhang, Q., Milhous, W.K. and Lin, A.J., 2002. Design, Synthesis, and Evaluation of New Chemosensitizers in Multi-Drug-Resistant Plasmodium f alciparum. Journal of medicinal chemistry, 45(13), pp.2741-2748.

28. Garretson, P.D., Hammond, E.E., Craig, T.M. and Holman, P.J., 2009. Anthelmintic resistant Haemonchus contortus in a giraffe (Giraffa camelopardalis) in Florida. Journal of Zoo and Wildlife Medicine, 40(1), pp.131-139.

29. Nikaido, H. and Pagès, J.M., 2012. Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. FEMS microbiology reviews, 36(2), pp.340-363.

30. Mammeri, H., Van De Loo, M., Poirel, L., Martinez-Martinez, L. and Nordmann, P., 2005. Emergence of plasmid-mediated quinolone resistance in Escherichia coli in Europe. Antimicrobial Agents and Chemotherapy, 49(1), pp.71-76.

31. Sikorski, R.S. and Hieter, P., 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics, 122(1), pp.19-27.

31. Arthur, A. and Sherratt, D., 1979. Dissection of the transposition process: a transposon-encoded site-specific recombination system. Molecular and General Genetics MGG, 175(3), pp.267-274.

32. Kapitonov, V.V. and Jurka, J., 2005. RAG1 core and V (D) J recombination signal sequences were derived from Transib transposons. PLoS biology, 3(6), p.e181.

33. Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., Iijima, Y., Najima, M., Nakano, M., Yamashita, A. and Kubota, Y., 2003. Genome sequence of Vibrio parahaemolyticus: a pathogenic mechanism distinct from that of V cholerae. The Lancet, 361(9359), pp.743-749.

34. Hall, R.M. and Stokes, H.W., 2004. Integrons or super integrons?. Microbiology, 150(1), pp.3-4.

35. Davies, J., 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science-AAAS-Weekly Paper Edition-including Guide to Scientific Information, 264(5157), pp.375-381.

36. D'costa, V.M., McGrann, K.M., Hughes, D.W. and Wright, G.D., 2006. Sampling the antibiotic resistome. Science, 311(5759), pp.374-377.

37. Khotimchenko, Y., Khozhaenko, E., Kovalev, V. and Khotimchenko, M., 2012. Cerium binding activity of pectins isolated from the seagrasses Zostera marina and Phyllospadix iwatensis. Marine drugs, 10(4), pp.834-848.

38. Fokina, D.A. and Belyakova, G., 2014. The adaptation of measures to stimulate an export engineering to the WTO rules. International Journal of Applied and Fundamental Research, (2), pp.181-181.

39. Brown, J.S., Aufauvre-Brown, A., Brown, J., Jennings, J.M., Arst, H. and Holden, D.W., 2000. Signature-tagged and directed mutagenesis identify PABA synthetase as essential for Aspergillus fumigatus pathogenicity. Molecular microbiology, 36(6), pp.1371-1380.

40. Hernández-Allés, S., Benedí, V.J., Martínez-Martínez, L., Pascual, Á., Aguilar, A., Tomás, J.M. and Albertí, S., 1999. Development of resistance during antimicrobial therapy caused by insertion sequence interruption of porin genes. Antimicrobial agents and chemotherapy, 43(4), pp.937-939.

41. Martinez, J.L. and Baquero, F., 2000. Mutation frequencies and antibiotic resistance. Antimicrobial agents and chemotherapy, 44(7), pp.1771-1777.

42. Gilbert, D.N., Moellering, R.C. and Sande, M.A., 2003. The Sanford guide to antimicrobial therapy (Vol. 48). Antimicrobial Therapy Incorporated.

43. Niederman, M.S., Mandell, L.A., Anzueto, A., Bass, J.B., Broughton, W.A., Campbell, G.D., Dean, N., File, T., Fine, M.J., Gross, P.A. and Martinez, F., 2001. Guidelines for the management of adults with community-acquired pneumonia: diagnosis, assessment of severity, antimicrobial therapy, and prevention. American journal of respiratory and critical care medicine, 163(7), pp.1730-1754.

44. Harbarth, S., Garbino, J., Pugin, J., Romand, J.A., Lew, D. and Pittet, D., 2003. Inappropriate initial antimicrobial therapy and its effect on survival in a clinical trial of immunomodulating therapy for severe sepsis. The American journal of medicine, 115(7), pp.529-535.

45. Rello, J., Ausino, V., Ricart, M., Castella, J. and Prats, G., 1993. Impact of previous antimicrobial therapy on the etiology and outcome of ventilator-associated pneumonia. Chest, 104(4), pp.1230-1235.

46. Southern, P.J. and Berg, P., 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. Journal of molecular and applied genetics, 1(4), pp.327-341.

48. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. Identification of acquired antimicrobial resistance genes. Journal of antimicrobial chemotherapy. 2012 Jul 10;67(11):2640-4.

49. Zhang, T., Zhang, X.X. and Ye, L., 2011. Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. PloS one, 6(10), p.e26041.

50. Exner, M., Bhattacharya, S., Christiansen, B., Gebel, J., Goroncy-Bermes, P., Hartemann, P., Heeg, P., Ilschner, C., Kramer, A., Larson, E. and Merkens, W., 2017. Antibiotic resistance: What is so special about multidrug-resistant Gram-negative bacteria?. GMS hygiene and infection control, 12.

51. Alekshun, M.N. and Levy, S.B., 2007. Molecular mechanisms of antibacterial multidrug resistance. Cell, 128(6), pp.1037-1050.