Review Article

Application of Microbial Enzymes in Industrial Waste Water Treatment

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A B S T R A C T

The introduction and implementation of stringent standards for waste discharge into the environment has necessitated the need for the development of alternative waste treatment processes. A large number of enzymes from various microbes have been reported to play crucial role in wastewater treatment applications. Enzymes can specifically act on and remove recalcitrant pollutants by precipitation and transformation to other products and can also change the characteristics of a given waste making it more susceptible to treatment or aid in converting waste material to value added products. Immobilization increases the mechanical and thermal stability of the enzymes while decreasing the probability of enzyme leaching into solution. Horseradish peroxidase covalently immobilized onto magnetic beads retained a high activity and stability and performed higher phenol conversions. Recalcitrant pollutants in wastewater can also be treated by using nanotubes carrying oxidative enzymes as laccases and peroxidases. Immobilization of glucose oxidase and chloroperoxidase onto carbon nanotubes maintained their functionality and increased substrate conversion efficiency. Many novel applications for their catalytic activities are being suggested through a proper understanding of enzymes and their functional significance. The use of enzymes in place of harmful chemical reactions is extremely important to meet the demands for cleaner and greener technologies to preserve the planet.

Keywords
Enzymes, Microorganisms, Wastewater, Recalcitrant pollutants.

Introduction

Enzymes are biocatalysts produced by living cells to cause specific biochemical reactions generally forming the various metabolic processes of the cells and are indispensible to maintenance and activity of life. Enzymes are highly specific in their action on substrates and often many different enzymes are required to bring about sequence of metabolic reactions performed by living cells. Each strain of a microorganism produces a large
number of enzymes which can be hydrolyzing, oxidizing or reducing and metabolic in nature. Microbial enzymes are known to play a crucial role as metabolic catalysts, resulting in their use in various industrial applications. The end use market for industrial enzymes is extremely widespread with numerous industrial commercial applications [1]. Microbes have served and continue to serve as one of the largest and useful sources of many enzymes [2]. Many industrial processes have several disadvantages like low catalytic efficiency, lack of enantiomeric specificity for chiral synthesis, requirement of high temperature, low pH and high pressure. Also, the use of organic solvents leads to organic waste and pollutants. Enzymes are more useful for these applications as they work under mild reaction conditions (e.g., temperature, pH, atmospheric conditions), do not need protection of substrate functional groups, have a long half-life, a high stereo-selectivity yielding stereo- and regio-chemically-defined reaction products at an acceleration of 105 to 108-fold, and, in addition, they work on unnatural substrates [3]. Furthermore, enzymes can be selected genetically and chemically-modified in order to improve their key properties: stability, substrate specificity and specific activity.

In the case of ocean and river quality, pollution is primarily caused by the discharge of inadequately treated industrial and municipal wastewater.

On initial discharge, these wastewaters can contain high levels of inorganic pollutants which can be easily biodegraded, but whose impact load on the ecosystems, either in Total Suspended Solids (TSS), Biochemical Oxygen Demand (BOD), or Chemical Oxygen Demand (COD), may be in the tens of thousands mg/L [4]. To combat this increasing burden on our aquatic environment, increasingly strict regulation on pollution discharge is being implemented by various governmental bodies, with focus primarily on waste reduction. Compliance to environmental legislations should not necessary lead to the creation of additional costs, but can instead provide a secondary source of income. One possible source of increased revenue available to industries is through taking advantage of the incentives awarded by the Clean Development Mechanism (CDM) under the Kyoto Protocol 1997.

The characteristics of wastewater can differ considerably both within and among industries. In the treatment of wastewater, biological treatment appears to be a promising technology to attain revenue from Certified Emission Reduction (CER) credits, more commonly known as carbon credits, as methane gas is generated from anaerobic digestion and can be utilized as renewable energy. With appropriate analysis and environmental control, almost all wastewaters containing biodegradable constituents with a BOD/COD ratio of 0.5 or greater can be treated easily by biological means [5].

In comparison to other methods of wastewater treatment, it also has the advantages of lower treatment costs with no secondary pollution [6]. Both aerobic and anaerobic processes can be used; the former involves microorganisms (aerobes) which utilise free or dissolved oxygen and convert organic wastes to biomass and CO₂ while in the latter complex organic wastes are degraded into methane, CO₂ and H₂O through three basic steps (hydrolysis, acidogenesis including acetogenesis and methanogenesis) in the absence of oxygen.

Aerobic biological processes are commonly used in the treatment of organic wastewaters for achieving high degree of treatment
efficiency, while in anaerobic treatment, considerable progress has been achieved in anaerobic biotechnology for waste treatment based on the concept of resource recovery and utilization while still achieving the objective of pollution control [7].

Most of the waste treatment processes can be categorised as either physico-chemical or biological processes. Enzymatic treatment falls between these two conventional categories since it involves chemical processes based on the action of biological catalysts.

The potential advantages of enzymatic treatment as compared with conventional treatment include: application to biorefractory compounds; operation at high and low contaminant concentrations; operation over a wide range of pH, temperature and salinity; absence of shock loading effects; absence of delays associated with the acclimatization of biomass; reduction in sludge volume and the ease and simplicity of controlling the process [8].

**Types of microbial enzymes**

**Microbial oxidoreductases**

The detoxification of toxic organic compounds by various bacteria and fungi [9] and higher plants [10] through oxidative coupling is mediated with oxidoreductases.

**Microbial oxygenases**

Oxygenases belong to the oxidoreductase group of enzymes and participate in oxidation of reduced substrates by transferring oxygen from molecular oxygen (O₂) utilizing FAD/NADH/NADPH as a co-substrate.

These enzymes have an important role in the metabolism of organic compounds as they increase their reactivity or solubility in water or bring about cleavage of the aromatic rings. Oxygenases also mediate dehalogenation reactions of halogenated methanes, ethanes, and ethylens in association with multifunctional enzymes [11].

**Monooxygenases**

These enzymes catalyze oxidative reactions of substrates from alkanes to complex molecules as steroids and fatty acids and require only molecular oxygen for their activity.

These enzymes require only molecular oxygen for their activities and utilize the substrate as reducing agent [12].

The desulfurization, dehalogenation, denitrification, ammonification, hydroxylation, biotransformation, and biodegradation of various aromatic and aliphatic compounds are catalyzed by monooxygenases.

**Microbial dioxygenases**

These dioxygenases catalyze enantio specifically the oxygenation of wide range of substrates. Aromatic compounds are primarily oxidized by dioxygenases, reflecting the applications of dioxygenases in environmental remediation. The catechol dioxygenases are found in the soil bacteria and involved in the transformation of aromatic precursors into aliphatic products.

**Microbial laccases**

Laccases (p-diphenol: dioxygen oxidoreductase) constitute a family of multicopper oxidases produced by certain plants, fungi, insects, and bacteria, that catalyze the oxidation of a wide range of reduced phenolic and aromatic substrates with concomitant reduction of molecular oxygen to water [12]. Many microorganisms produce intra and extracellular laccases capable of
catalyzing the oxidation of ortho and paradi phenols, aminophenols, polyphenols, polyamines, lignins, and aryl diamines as well as some inorganic ions [13].

**Microbial peroxidases**

These enzymes catalyze the oxidation of lignin and other phenolic compounds at the expense of hydrogen peroxide (H₂O₂) in the presence of a mediator. Due to their high potential to degrade toxic substances in nature, lignin peroxidase (LiP) and manganese-dependant peroxidase (MnP) have been studied the most.

**Microbial lipases**

These enzymes can catalyze various reactions such as hydrolysis, interesterification, esterification, alcoholysis and aminolysis [14]. Along with its diagnostic usage in bioremediation, lipase has many potential applications in food, chemical, detergent manufacturing, cosmetic, and paper making industries, but its production cost has restricted its industrial use [15].

**Microbial cellulases**

During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol [16]. Cellulases cause removal of cellulose microfibrils, which are formed during washing and the use of cotton-based cloths. In paper and pulp industry, cellulases are used for the removal of ink during recycling of paper.

**Microbial proteases**

Proteases belong to group of enzymes that hydrolyze peptide bonds in aqueous environment and synthesize them in non-aqueous environment. Proteases have wide range of applications in food, leather, detergent, and pharmaceutical industry [17] (Table 2).

**Industries and pollutants**

Aromatic compounds, including phenols and aromatic amines, comprise one of the major classes of pollutants and are stringently regulated in many countries. They are found in the wastewaters of a wide variety of industries including coal conversion, petroleum refining, resins and plastics, wood preservation, metal coating, dyes and other chemicals, textiles, mining and dressing, and pulp and paper [18]. The Kraft process which is widely used in wood pulping leaves 5-8% (w/w) of residual modified lignin in the pulp. This residual is responsible for the characteristic brown colour of the pulp and is commercially removed by the use of bleaching agents such as chlorine and chlorine oxides [19].

Bleaching operations produce dark brown coloured effluents which contain toxic and mutagenic chlorinated products that constitute an environmental hazard [20]. There have been a number of studies including the use of peroxidases and laccases for the treatment of bleaching effluents.

Pesticides, which include herbicides, insecticides and fungicides, are widely used throughout the world today for crop protection and it is expected that this use will continue to grow [21]. The potential adverse effects that the pesticide industry can have on the environment arise from the disposal of wastes formed during production and formulation of pesticides, detoxification of pesticide containers and spray tanks, and the pollution of surface and groundwater by pesticide runoff [22]. It is estimated that 3 million tons of cyanide are used yearly throughout the world in different industrial
processes including the production of chemical intermediates, synthetic fibers, rubber and pharmaceuticals, as well as in ore leaching, coal processing and metal plating. Enzymes could be used to decrease food wastes via enzymatic processing to yield higher-value by-products and to aid in the clean-up of food waste streams [23]. For the past decade, there has been an increasing interest in the enzymatic hydrolysis of cellulose [24]. This interest stems from the advantages that such a process would offer, namely, the conversion of lignocellulosic and cellulosic wastes to a useful energy source through the production of sugars, ethanol, biogas or other energetic end products [25].

Heavy metals such as arsenic, copper, cadmium, lead and chromium among others, are dangerous contaminants found in a number of industrial and mining waste streams as well as in solid wastes, municipal sewage sludges and landfill leachate [26] (Table 1). Surfactants or surface active agents are organic substances that have rather large polar molecules and are basic ingredients of detergents [27]. Surfactants may cause significant pollution problems when high concentrations from shampoo formulation factories, for instance, enter municipal sewerage systems and generate undesirable conditions such as foaming [28]. Wastewaters from dairies [29] and slaughterhouses [30] are often rich in biodegradable organic molecules and nutrients and contain high levels of fats and proteins that have a low biodegradability coefficient. Some dyestuffs are highly structured polymers and are very difficult to decompose. Many of the dyes are carcinogenic, mutagenic and detrimental to the environment. Leather processing industries are also a huge source of waste which include wastes from untreated hides/skins (trimmings, fleshing wastes), wastes from tanned leather (shaving wastes, buffing dust), wastes from dyed and finished leather (trimmings from leather) [31], toluene and benzene.

**Table 1** Major contaminants in wastewater

| Contaminants          | Reason for Importance                                                                                                                                 |
|-----------------------|------------------------------------------------------------------------------------------------------------------------------------------------------|
| Suspended solids      | Can lead to the formation of sludge deposits and anaerobic conditions when untreated wastewater is discharged to the aquatic environment.            |
| Biodegradable organics| Are principally made up of proteins, carbohydrates and fats. They are commonly measured in terms of BOD and COD. If discharged into inland rivers, streams or lakes, their biological stabilization can deplete natural oxygen resources and cause septic conditions that are detrimental to aquatic species. |
| Pathogenic organisms  | Found in wastewater can cause infectious diseases.                                                                                                                                                             |
| Priority pollutants   | Including organic and inorganic compounds, may be highly toxic, carcinogenic, mutagenic or teratogenic.                                                                                                        |
| Refractory organics   | That tend to resist conventional wastewater treatment include surfactants, phenols and agricultural pesticides                                                                                               |
| Heavy metals          | Usually added by commercial and industrial activities must be removed for reuse of the wastewater                                                                                                            |
| Dissolved inorganic constituents | Such as calcium, sodium and sulphate are often initially added to domestic water supplies, and may have to be removed for wastewater reuse.                                                        |

Source: Adapted from Metcalf and Eddy, Inc., Wastewater Engineering, 3rd edition.
### Table 2 Microbial enzymes and applications

| ENZYMES                        | APPLICATIONS                                                                 |
|--------------------------------|-----------------------------------------------------------------------------|
| Alkylsulfatase                 | Surfactant degradation [32]                                                  |
| Amylase:                       |                                                                             |
| • a-amyrase                    | Starch hydrolysis and production of glucose [33]                           |
| • Glucoamylase                 |                                                                             |
| Cellulolytic enzymes:          | Hydrolysis of cellulosic sludges from pulp and paper to produce sugars and alcohol, hydrolysis of cellulose in municipal solid waste to sugars and other energy sources [34] |
| • Cellulase                    |                                                                             |
| • Celllobio-hydrolase          |                                                                             |
| • Celllobiase                  |                                                                             |
| • Exo-1,4-b-D-glucosidase      |                                                                             |
| Chitinase                      | Bioconversion of shellfish waste to N-acetyl glucosamine [35]              |
| Chloro-peroxidase              | Oxidation of phenolic compounds [36]                                        |
| Cyanidase                      | Cyanide decomposition [37]                                                  |
| Cyanide hydratase              | Cyanide hydrolysis [38]                                                     |
| L-Galactono-lactone oxidase    | Conversion of galactose from whey hydrolysis to L-ascorbic acid [39]        |
| Laccase                        | Removal of phenols, decolourization of Kraft bleaching effluents, binding of phenols and aromatic amines with humus [40] |
| Lactases                       | Dairy waste processing and production of value-added products [41]          |
| Lignin peroxidise              | Removal of phenols and aromatic compounds, decolourization of Kraft bleaching effluents [42] |
| Lipase                         | Improved sludge dewatering [43]                                             |
| Lysozyme                       | Improved sludge dewatering [44]                                             |
| Mn-peroxidase                  | Oxidation of monoaromatic phenols and aromatic dyes [45]                   |
| Parathon hydrolase             | Hydrolyzation of organophosphate pesticides [46]                           |
| Pectin Lyase                   | Pectin degradation [47]                                                     |
| Peroxidase                     | Removal of phenols and aromatic amines, decolourization of Kraft bleaching effluents, sludge dewatering [48] |
| Phosphatase                    | Removal of heavy metals [49]                                                |
| Proteases                      | Solubilisation of fish and meat remains                                    |
|                               | Improving sludge dewatering [50]                                            |
| Tyrosinase                     | Removal of phenols [51]                                                     |

**Technologies used for enzymatic treatment of wastewater**

The conformation of enzymes mainly decides their functionality. The conformation might change under harsh physical and chemical conditions of temperature, pH and ionic strength thus altering the function of enzymes. Such drastic conditions are often encountered in effluent streams. Immobilization method reduces the loss of enzymes increasing their reusability and also minimizes the chances of loss of enzyme activity under harsh conditions. Use of immobilized enzymes in effluent treatment, as compared to free enzymes, results in multiple advantages like increased stability, reusability, ease of handling, reduction in running cost. Horseradish peroxidase covalently immobilized onto magnetic beads retained a high activity and stability and performed higher phenol conversions. Fungal laccase...
immobilized using γ -aluminium oxide pellets, has been reported to decolorize solutions of azo dyes. Introduction of cells or tissues producing an enzyme into the effluent directly is one of the simplest method of enzyme administration to target effluent. This method is employed when suitably adapted strains of microorganisms are used to co-metabolize target pollutants.

Cultures of the *Staphylococcus arlettae* were shown to decolorize solutions of four azo dyes (CI Reactive Yellow 107, CI Reactive Red 198, CI Reactive Black 5 and CI Direct Blue 71) in a microaerophilic/aerated sequential process and the average decolorization obtained was 97% [52]. In cases where the effluent to be treated contains pollutants which cannot support growth, cell free or isolated enzymes are preferred for use over intact microorganism.

Nanotechnology is another area which is gaining importance in wastewater treatment. The use of nanoparticles in Reactive Remediation Technology is of great interest to wastewater treatment, since it involves the complete degradation of contaminants to harmless products such as carbon dioxide and water [53]. The remediation of contaminated wastewater can be achieved by using a combination of enzyme technology and nanotechnology known as the SEN, i.e., Single Enzyme Nanoparticle [54]. A SEN may be described as an enzyme covered by a protective few nanometers thick cage. Cell free crude extracts or purified forms of enzymes like peroxidases, polyphenol oxidases, dehalogenases, hydrolases can be used for SEN synthesis. A variety of recalcitrant compounds such as phenols, polyaromatic dyes, pesticides can be degraded by these enzymes. Similarly, recalcitrant pollutants in wastewater can also be treated by using nanotubes carrying oxidative enzymes as laccases and peroxidases.

Membrane bioreactors constitute an interesting possibility to be applied in wastewater treatment. The combination of membrane technology with enzymatic reactors for wastewater treatment has led to the development of three generic systems: Immobilised enzyme membrane reactor (IEMR), Extractive membrane bioreactor (EMB) and Direct contact membrane reactor (DCMR). In both systems, IEMR and EMB, the use of hollow-fibre bioreactors or capillary membrane reactors considerably increases the surface area volume ratio and, therefore, the treatment capacity of the system [55].

**Advantages of enzymatic treatment over other techniques**

The continuously expanding application of enzymes is creating a growing demand for biocatalysts that exhibit improved or new properties [56]. By specifically acting on certain recalcitrant pollutants, enzymes can remove them by precipitation or transformation to other products. Biological (enzymatic) processes have an added advantage over traditional chemical/physical methods as they are regarded as clean and green [57]. The various physicochemical treatments like chemical precipitation, coagulation, flocculation, floatation, membrane filtration offer advantages like ease of operation and control, flexibility to change in temperature and are rapid but their benefits, however, are outweighed by a number of drawbacks such as their high operational costs due to the chemicals used, high energy consumption and handling costs for sludge disposal [58]. Stringent government policies regarding permitted levels of pollutants, high costs of specialized chemical treatments for pollutant removal and the fact that some of these treatments create additional solid waste has led to the development of many effective, yet simple biological methods. Enzymes are
highly specific and extremely efficient catalysts [59]. They can selectively degrade a target pollutant without affecting the other components in the effluent. More importantly, they can operate under mild reaction conditions, especially temperature and pH. In this respect, enzymes outperform the regular catalysts (transition elements like Cu, Ni etc.).

From the environmental perspective, enzymes are more acceptable due to their biodegradability [60]. In the case of reactions wherein the target pollutant is oxidized, the enzyme receives one or more electrons from the substrate and donates these electrons to an electron acceptor. Hence, at the end of the reaction the enzyme is regenerated and is available for the next catalytic cycle. The biological origin of enzymes reduces their adverse impact on the environment thereby making enzymatic wastewater treatment an ecologically sustainable technique.

**Present scenario**

India’s share in the global market for industrial enzymes is estimated to be at about US $ 3387.30 million. The segment is largely export oriented including US (global share 40%), Europe (global share 25%), China (global share 20%) as the major export markets. Recent developments in the areas of protein engineering and directed evolution have provided tools for the development of new enzymes with improved properties and technical applications.

The use of enzymes like amylases and cellulases in place of chemical treatments in various industries has resulted in reduced waste chemical discharge in the environment. New enzymatic processes are being developed having potential to totally replace the use of chemicals in different industries. The use of enzymes like Xylanase has enhanced the bleaching efficacy thus reducing the consumption of chlorine. Immobilization increases the mechanical and thermal stability of the enzymes decreasing the probability of enzyme leaching into solution. Research and development is currently focused on imparting desirable properties, developing microbial systems and other heterologous proteins through gene cloning, over expression and enzyme engineering. Some of the ongoing programmes on enzymes include engineering a thermo-tolerant phytase [61], production of substrate specific aminopeptidases, production and application of chitinolytic enzymes [62,63].

**Future prospects**

Future trends may involve the development of more effective systems that employ lesser quantities of chemicals, less water and less energy to attain maximum performance. The use of modern biotechnology techniques will lead to enzyme systems with improved effects at diverse physiological conditions of temperature and pH resulting in increased efficiency with lesser energy consumption and lower costs.

Enzyme immobilization is an area of active research where the recent advances in the design of immobilization support have enabled more precise control of enzyme immobilization [64]. Many future investigations will use combinations of engineered and de novo designed enzymes coupled with chemistry to generate more (and most likely new) chemicals and materials from cheaper (and renewable) resources, which will consequently contribute to establishing a bio-based economy and achieving low carbon green growth [65].

Microorganisms provide a huge amount of catalysts with a wide range of applications across several industries such as food, textile, leather, pharmaceuticals and many more. In
wastewater treatment, enzymes can be utilized to develop remediation processes that are environmentally less hazardous than conventional techniques. Their versatile nature and efficiency even in mild reaction conditions gives them an advantage over the conventional physico-chemical treatment methods.

Enzymes can act on specific recalcitrant pollutants to remove them or transform them to other less harmful products. The characteristics of a given waste can also be changed by enzymes leaving the waste more susceptible to treatment or aid in bio converting waste material to value added products.

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