Design of Novel Laccases for Degradation of Complex Dyes

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Abstract

Laccases also named p-diphenol: dioxide oxidoreductases are blue multicopper oxidizes (MCOs) that have the ability to catalyze the oxidation of a wide variety of organic aromatic compounds, concomitantly with the reduction of molecular oxygen to water. The present study is to isolate, extract purify and use the lacasse enzyme for degradation of complex dyes from Trichoderma viridae fungi. The fungi was isolated and maintained on PDA plates with multiple subcultures. Enzyme produced by two types of fermentation SSF and SMF. The solid state fermentation process by using wheat bran as solid support gives high production of enzyme. Screening and enzyme activity checked by using guaiacol as substrate this efficient substrate for laccase enzyme. The laccase enzyme shows the optimum activity at 35^0C and 4.00 pH. The activity was inhibited by 50% of organic solvents. Different dyes were checked for degradation by enzyme. The arylmethane group shows the high degradation percentage. The laccases are demonstrated to have a range of promising applications, they are used in bioremediation of soils, water and the development of environmentally friendly processes in the pulp and paper industry.

Keywords *: Laccases, guaiacol, SDS, ammonium sulphate precipitation, oxidoreductases, bioremediation, decolorization, effluent treatment.*

I. INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases,) are multi-copper enzymes belonging to the group of blue oxidizes. They are defined as oxidoreductases, which oxidizes diphenol and allied substances. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water [1]. The ability of laccases to oxidize phenolic compounds as well as their ability to reduce molecular oxygen to water has led to intensive studies of these enzymes [2-4].

In recent years, enzymes have gained great importance in industries; laccases are among one of them which are widely present in nature. Laccases are the oldest and the most studied enzymatic systems1. Laccase was first discovered in the sap of the Japanese lacquer tree, Rhus vernicifera and its characteristic as a metal containing oxidize was discovered by Bertrand in 19852.

Laccases are typically found in plants and fungi. Plant laccases participate in the radical- based mechanisms of lignin polymer formation [5-9], whereas in fungi laccases probably have more roles including morphogenesis, fungal plant-pathogen/host interaction and stress defense and lignin degradation [4]. Although there are also some reports about laccase activity in bacteria [10-13], it does not seem probable that laccases are common enzymes from certain prokaryotic groups. Discovery of novel laccases with different substrate specificities and improved stabilities is very important for industrial applications, besides developing an effective high yield and economic production medium enhances its utility. Several reports regarding this aspect reported thus far have been worked on both submerged and solid state fermentation. Solid state fermentation utilizing natural lignin containing substrates such as rice bran, wheat bran, coir dust, potato peel, etc. [16, 17] have received much attention because of its efficiency in production of microbial laccases.

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a) By the addition of O2 to the substrate.

b) By the removal of H from the substrate.

c) By the removal of e- from thesubstrate.

In the case of laccase it oxidizes the substrate by the removal of e- and because it belongs to the oxidize enzyme family; it requires O2 as a second substrate for enzymatic action. The copper is bound in several sites: type 1, type 2 and/or type 3. The way of assembly of types 2 and 3 is called a trinuclear cluster. Laccase contains 15- 30% carbohydrate and have molecular mass of 60-90 KDa.

A. Applications of Laccases enzyme

Laccase is important because it oxidizes both the toxic and nontoxic substrates. It is utilized in textile industry, food processing industry, wood processing industry, pharmaceutical industry, and chemical industry. This enzyme is very specific, ecologically sustainable and a proficient catalyst. Applications of laccase are as follows

a) Dye Decolourization

Textile industry utilizes large volume of water and chemicals for wet processing. These chemicals range from inorganic compounds to organic compounds. The chemical structure of dyes provides a resistance to fading when exposed to light, water, and other chemicals. Laccase degrades dye; that is why laccase-based processes have been developed which include synthetic dyes and are being used in the industry nowadays.

b) Bioremediation and Biodegradation

Due to rapid industrialization and extensive use of pesticides for better agricultural productivity, contamination of soil, water, and air take place which is a serious environmental problem of today. Polychlorinated biphenyls (PCB), benzene, toluene, ethyl benzene,xylene(BTEX) Polycyclic aromatic hydrocarbons (PAH), pentachlorophenol (PCP), 1,1,1-trichloro-2,2-bis (4- chlorophenyl) ethane (DDT), and trinitrotoluene (TNT) are the substances which are known for their carcinogenic as well as mutagenic effect and are persistent in the environment. Fungi renovate a wide variety of hazardous chemicals; that is why the researcher's interest is generated in them.

c) Paper and Pulp Industry

Chlorine and oxygen-based chemical oxidants are used for the separation and degradation of lignin which is required for the preparation of paper at industrial level. But some problems such as recycling, cost, and toxicity remain unsolved. However, in the existing bleaching process, LMS could be easily implemented because it leads to a partial replacement of ClO in pulp mills

d) Food Processing Industry

In food industry, laccase is used for the elimination of undesirable phenolic compound in baking, juice processing, wine stabilization, and bioremediation of waste water. Laccase improves not only the functionality but also the sensory properties. In beer industry, laccase not only provides stability but also increases the shelf life of beer. In beer, haze formation takes place which is stimulated by the naturally present proanthocyanidins polyphenol and is referred to as chill haze. At room temperature or above, warming of beer can redissolve the complex. After certain periods of time, phenolic rings are replaced by the sulphydryl group and permanent haze is formed which cannot be redissolved. For polyphenol oxidation, laccase has been used which is capable of removing the excess oxygen and also due to which the shelf life of beer increases. For making a fruit juice stable, laccase is commonly used. Phenol compounds and their oxidative products present naturally in the fruit juice give color and taste to the juice. Color and aroma change when polymerization and oxidation of phenolic and polyphenol takeplace.

These changes are due to the high concentration of polyphenol and referred to as enzymatic darkening. Laccase treatment removes phenol as well as substrate-enzyme complex by the help of membrane filtration, and color stability is achieved, although turbidity is present. Laccase treatment is more effective in comparison to conventional methods. For improving the texture, volume, flavor and freshness of bread, wide range of enzymes are used.

When laccase is added to the dough, strength of gluten structures in dough and baked products is improved: product volume increases, crumb structure improves, and softness of baked products takes place. Due to the laccase addition, stickiness decreases, strength and stability increase and the ability of machine are also improved which can also seen by using low-quality flour. At crushing and pressing stage, the high concentration of phenolic and polyphenolic compound plays an important role in the wine production. The high concentration of polyphenol obtained from the stems, seeds and skins which depends on the grape variety and vinification conditions contributes to of color and astringency. Due to the complex event, polyphenol oxidation occurs in musts and wines resulting in the increase in colour and flavor change which is referred to as maderization. Catalytic factors, polyphenol removal, clarification, polyvinylpolypyrrolidone (PVPP), and high doses of sulfur dioxide are utilized to prevent maderization. Minussi et al. found that polyphenol removal is selective and results in undesirable organoleptic characteristics and concluded that laccase treatment is feasible, increasing storability and reducing processing costs.

e) Other Applications

Laccase not only is used in food industry, paper and pulp industry, textile industry but also has other applications. In traditional system, PVPP is used for the removal of excess polyphenol which has low biodegradability and creates problems in wastewater treatment. Laccase has the ability to decrease odor arising from the garbage disposal sites, livestock farms and pulp mills. Since laccases catalyze the electron transfer reactions without additional cofactors, they can also be used as biosensors to detect various phenolic compounds, oxygen, and azide. As biosensor laccase can detect morphine, codeine, catecholamine, estimate phenol or other enzymes in fruit juice and plant flavonoid. Recently, laccase has been used as a biocatalyst for the synthesis of organic substance as well as in the design of biofuel cell. For the bioremediation of food industry wastewater, laccase has been utilized. In bioremediation process, contaminants are biotransformed to their original status which has no bad effects on the environment. Large amount of polyphenol is present in the beer factory wastewater which is dark brown in color and degraded by the white-rot fungus *Coriolopsis gallica.* Lactase produced from *Trametes* sp. bioremediates the distillery wastewater generated from the sugarcane molasses fermentation with high content of organic matter.

B. Mechanism of Laccas

Basically laccase reduce one oxygen molecule to water accompanied with the oxidation of one electron with wide range of aromatic compounds which include polyphones[27] and aromatic amines (Figure-1) [28and 29] .The four copper ions are classified into three categories- type 1 (T1), type 2 (T2) and type 3 (T3). These 3 types can be distinguished by using visible electronic paramagnetic resonance (EPR) spectroscopy. Type 1 copper gives blue color to protein at an absorbance of 610 nm which is EPR detectable. Type 2 copper doesn't give color which is EPR detectable. Type 3 copper gives weak absorbance in near UV region but not detected by EPR signal3.When laccase oxidizes the substrate, free radicals are generated. The lignin degradation proceeded by phenoxy radical leads to oxidation at α carbon or cleavage of bond between α and β carbon. This oxidation results in an O2 centered free radical which can be converted into second enzyme catalyst reaction to quinine. Laccase also oxidizes the non- phenolic substrates by the inclusion of organic compounds which are low molecular weight organic compounds which when oxidized by laccase can further oxidize non-phenolic compounds. Most common organic compounds that act as mediators are N- hydroxyphthalimide (NHPI), 3-hydroxy anthranilic acid [30

C. Dyes

Dyes are organic compounds which are widely used for imparting colour to textiles. They are produced either chemically or from plants. An interesting point about them is that unlike paint, they do not build up on the surface of the fibre but are absorbed into the pores of the material. This becomes possible because of two reasons. First, the size of the dye molecules is smaller than the size of the pores in the fibre. The dye molecules have a shape like narrow strips of paper, that is having length and breadth but relatively little thickness. This planar shape assists them to slip into the polymer system when the fibre, yarn or fabric is introduced into the dye bath. The second reason is the affinity of the dye to the fibre due to forces of attraction. The dye which has diffused or penetrated into the fibre is held there by the forces of attraction between the dye and the fibre.

Unlike most organic compounds, dyes possess colour because they 1) absorb light in the visible spectrum (400–700 nm), 2) have at least one chromospheres (colour-bearing group), 3) have a conjugated system, i.e. a structure with alternating double and single bonds, and 4) exhibit resonance of electrons, which is a stabilizing force in organic compounds (Abrahart, 1977). When any one of these features is lacking from the molecular structure the color is lost. In addition to chromospheres, most dyes also contain groups known as auxochromes (color helpers), examples of which is carboxylic acid, sulfuric acid, amino, and hydroxyl groups. While these are not responsible for color, their presence can shift the color of a colorant and they are most often used to influence dye solubility.

All molecules absorb electromagnetic radiation, but differ in the specific wavelengths absorbed. Some molecules have the ability to absorb light in the visible spectrum (400-800 nm) and, as a result, they are themselves colored. The dyes are molecules with delocalized electron systems with conjugated double bonds that contain two groups: the chromospheres and the auxochrome. The chromospheres are a group of atoms, which controls the color of the dye, and it is usually an electron-withdrawing group. The most important chromospheres are -C=C-, -C=N- ,-C=O, -N=N-, -NO2 and -NO groups. The auxochrome is an electron-donating substituent that can intensify the color of the chromospheres by altering the overall energy of the electron system and provides solubility and adherence of the dye to the fiber. The most important auxochromes are - NH2, -NR2, -NHR, -COOH, -SO3H, -OH and -OCH3 groups (Rocha Gomes 2001). Based on the chemical structure or chromospheres, 20-30 different dye groups can be identified . Azo (monoazo, disazo, triazo, polyazo), anthraquinone, phthalocyanine and triarylmethane dyes are quantitatively the most important chromophores.

II. MATERIALS AND METHODOLOGY

A. Isolation of Laccase Producing Microorganisms

Fungi used: *Trichoderma viridae* The Culture of T.viridae taken from RJSPM's laboratory which is exported from the NCCS, Dist. Pune, Maharashtra, was selected for this study as is already studies and recommended as good producer of laccase enzyme. The culture is transferred on PDA and incubated on 37^0C for 3 days. Further it maintained on PDA slants and transferred on new media after certain time period.

B. Screening test for enzyme producing fungi

The ability of fungal strain to secrete extracellular lactase is visualized by following assay as The assay plate contained 15 ml of 4% PDA amended with 0.01% of guaiacol and plates were incubated at 30^0C for 2-3 days. [35].

C. Laccase production in liquid media: Submerged fermentation

positive fungal strains detected in the plate-test, were subjected to qualitative determination of laccase production in submerged culture [Pant and Adholeya, 2007]. The strains were grown in 100 ml of 4% potato dextrose broth (PDB) in a 250 ml Erlenmeyer flask. The flasks were incubated at 30 C on a rotary shaker (120 rpm). Incubated for 7 days and then subjected enzyme purification

D. Laccase production in solid media: solid state fermentation

In this method the wheat bran was used as solid support for the growth of fungus. The flask containing wheat bran was sterilized by autoclaving. 100 ml of sterilized Potato dextrose broth supplemented with inoculums were added in to the sterilized bran and then incubated at 30^0C on a rotary shaker (120) for 7 days .The enzyme activity was measured from the seventh day of incubation.

E. Extraction of enzyme

The fermented broth was centrifuged at 10000 rpm for 10 min at 4^0C. Pellet was discarded and supernatant used as enzyme source.

F. Protein content estimation

The protein concentration was determined by using Lowery method using Bovine serum albumin (BSA) as standard. [37]

G. Purification of Enzyme

The laccase was purified from cell free culture medium by following methods:

H. Ammonium precipitation

The extracted crude enzyme sample subjected to ammonium precipitation method. In this method ammonium sulphate was added for 80% saturation and centrifugation of salted out proteins at 4^0C ,10000 rpm for 30 min and then the supernatant was decanted and the pellet or precipitate was dissolved in 0.2 M citric acid buffer (pH 4.0) [37]

I. Dialysis:

The precipitated sample was then subjected to dialysis. The dialysis was carried out at 4^0C for overnight against same citric acid buffer (pH 4.0) [37].

J. Guaiacol assay method for enzyme activity

Guaiacol has been reported as efficient substrate for laccase assay. The reddish brown color was developed due to oxidation of guaiacol by laccase enzyme is used to measure the enzyme activity. This activity can be measured at 450 nm at colorimeter. The blank was also prepared by using distilled water other than enzyme source. The reaction mixture was incubated for 15 min at 30^oC and then absorbance was checked at 450 nm by colorimeter. Enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1 µmol of guaiacol per min. The laccase activity in U/ml is calculated by the formula-

$E.A = A \times V / t \times e \times v$

EA=enzyme activity

A = Absorbance

V= mixture volume (ml)

v = enzyme volume (ml)

t = incubation time

e = extinction coefficient

Reaction mixture for assay given in table no.1

K. Determination of molecular mass

SDS-polyacrylamide gel electrophoresis was performed essentially to check the enzyme purity and to determine the subunit molecular weight of the recombinant enzyme. For the separation, 10% (w/v) polyacrylamide gel with 0.5% SDS was used. Volume of each sample was adjusted so that 10µg protein was applied to each well. An electric field of 100 V for 3hrs was applied to separate proteins through the gel. Then, the gel was stained with Coomassie Blue-R solution, which was followed by

distaining in Coomassie Gel Distain Solution. For the molecular weight determination standard protein markers were used. [37]

L. Characterization of purified enzyme

a) Effect of change in temperature on enzyme activity

The effect of temperature on laccase enzyme was determined carrying out the enzyme activity assay at different temperatures. In this method the reaction mixture containing 1% guaiacol (2mM) in sodium citrate buffer(10mM) and 1ml of purified enzyme. The mixture is incubated on different temperature as 15, 25,35,45,55 and 65^0C for 15 min .The enzyme activity determined by taking absorbance at 450nm by colorimetric and enzyme activity calculated by using above formula. [38]

b) Effect of change in pH on enzyme activity

The influence of change in pH on laccase activity was determined by recording the absorbance of laccase enzyme catalyzed reaction of oxidation of guaiacol as substrate at optimum temperature. In this procedure the reaction was carried out at different pH buffers as sodium citrate buffer pH-4.0,pH-5.0, phosphate buffer of pH 6.0 and pH 7.0, tri lysine buffer of pH 8 and incubated on 35^0C for 15 min and absorbance recorded at 450nm.[38]

c) Effect of organic solvents on enzyme activity

Different organic solvents (methanol, ethanol, acetone each at 5%, 20%) were mixed with the 1% Guaiacol substrate-containing reaction buffer to obtain the respective final concentrations. The effect of these additives on the laccase activity was also determined by incubating the additives at 25^0 C for 15 min. Absorbance was recorded at 450nm and enzyme activity calculated by using above formulae.

d) Enzyme immobilization

30g of sodium alginate dissolved in 1 liter to make a 3% solution. Approximately 0.015 g of enzyme mixed with 10 ml of 3% (wt.) sodium alginate solution. The concentration of sodium alginate can be varied between 6-12 % depending on the desired hardness. Then beads were formed by dripping the polymer solution from a height of approximately 20 cm into an excess (100 ml) of stirred 0.2M $CaCl_2$solution with a syringe and a needle at room temperature. The bead size can be controlled by pump pressure and the needle gauge. A typical hypodermic needle produces beads of 0.5-2 mm in diameter. Other shapes can be obtained by using a mold whose wall is permeable to calcium ions. Beads were lived in the calcium solution to cure for overnight.

M. Use of laccase enzyme for complex dye degradation

All the tested dyes were taken from laboratrotary of R.J.S.P.M. College

Detailed information was shown in Table 6. The degradation of 5 structurally different dyes by the purified laccase was determined by recording absorbance at400–700 nm at 7days and 14 days respectively. The decolonization of test dyes was calculated at 7days and 14days respectively. The reaction mixture for the standard assay contained respective dye (0.6 mg) in10 mM citric acid buffer at pH 4.0 and the enzyme solution (15 U) in a total volume of 3 ml. The decolonization rate of dye, expressed as dye decolorization (%), was calculated as the formula

Decolorization (%) = [(Ai-At)/Ai] * 100,

Where,

Ai: initial absorbance of the dye,

At: absorbance of the dye along the time

All Characters of used dyes expressed in Table no 2.

III. RESULT AND DISCUSSION

A. Isolation of Fungi

Trichoderma viridae culture subcultured on potato dextrose agar plates and maintained at 37^0C

After three days of incubation mycelium along with green color spores were observed as shown in figure. This culture was maintained at 4^0C for further use. Fig 2 shows *Trichoderma Viridae* culture on PDA mycelium along with green spores.

B. Screening test for fungi to check ability of fungi to produce the enzyme

After three days of incubation at30^0C the presence of brick red color around the mycelium was observed which was resulted from oxidation of guaiacol by laccase enzyme. This confirms the *T.Viridae* is laccase enzyme producing fungi. The results of this test are as shown in figure 3.

C. Laccase production by submerged and solid state fermentation

The amount of enzyme produced by solid state fermentation by using wheat bran is higher than the enzyme produced by submerged fermentation as the amount of crude enzyme produced by submerged fermentation was 80ml from 110 ml fermented broth and as in solid state fermentation it was 99 ml from 110 ml of fermented broth. As well as seen in enzyme

activity study the enzyme activity was greater in enzyme produced by solid state fermentation.

D. Protein content estimation

The protein content of extracted enzyme was estimated by standard Lowery method. The additions are as shown in table and concentration of protein in unknown sample was estimated by plotting Graph of absorbance against concentration of standard BSA solution For submerged fermentation [sample 1] given in table no 3. And Graph no 1 displaying the STD lowery methods to estimate protein content of unknown. Unknown protein plotted on standard curve [activity (U/ml) v/s BSA concentration ug/ml]. Protein produced by SSF. For solid state fermentation given in table no 4. And Graph no 2: Graph displaying the STD lowery methods to estimate protein content of unknown. Unknown protein plotted on standard curve [activity (U/ml) v/s BSA concentration ug/ml]. Protein produced by SMF.

The estimated protein content of sample first by graph was9.7ug/ml and of sample two was 8.5ug/ml so it seen that the amount of enzyme produced by solid state fermentation is more than the submerged fermentation process.

E. Purification of enzyme

The purification of extracted enzyme was carried out by using an ammonium precipitation and dialysis methods. The ammonium precipitation results into the 80% saturation of crude enzyme. the amount of purified enzyme obtained from this process was 64 ml from first sample (SMF) and 79.2 ml from sample two(SSF). After dialysis of the above enzyme at 4^0C for overnight obtained amount of purified enzyme were 55ml from SMF sample and 64ml from SSF sample.

F. Enzyme activity

The enzyme activity was determined before and after purification. This can be done by using Guaiacol assay as explained in materials and methods. After incubation brick red color was observed in assay test tubes it was due to oxidation of guaiacol by laccase enzyme. Fig 4 shows enzyme activity assay. The activity is determined as shown in table no 5,6,7 and 8. **As** results shows the observed enzyme activity was greater in the enzyme produced by solid state fermentation than the submerged fermentation as the mean enzyme activity by SSF is 89.27 U/ml and by SMF is 55.92 U/ml. As well as the purified enzyme activity is higher than the crude enzyme as the crude enzyme shows the activity 89.27 U/ml (SSF) and 55.92 U/ml (SMF) compare to this the purified enzyme shows higher activity as 187.76 U/ml (SSF) and 142.90 U/ml. overall enzyme activity determined is given in table no 9.

G. Determination of molecular mass of enzyme

The molecular mass and purification of isolated enzyme was checked by using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDSPAGE revealed the purity of the sample and a molecular weight of purified enzyme is 66.2kDa as shown in figure no 5.

H. Characterization of purified enzyme

a) Effect of change in temperature on enzyme activity

The influence of change in temperature was checked by performing guaiacol assay at different temperature and constant substrate concentration as well as constant incubation time. The observed results are given in table no 10 and 11. The optimum temperature for laccase enzyme activity from organism was found to be 35^0C as shown in fig. The temperature range where the enzyme is active is remarkably wide. Significant activity is detected as low as 15ºC and approximately 95% of activity is maintained at temperatures as high as 65ºC.Optimum temperatures are significantly affected by the assay used so this data should be interpreted with care. The activity of the enzyme at particular temperature; the enzyme activity reached the maximum at particular temperature that is 35^0 C and decreased thereafter.

b) Effect of change in pH on enzyme activity

The effect of pH on the enzyme activity was determined by using same process as in case of temperature but different was just temperature and substrate concentration values were kept constant and the reaction was carried out at different pH buffers as sodium citrate buffer pH-4.0, pH-5.0, phosphate buffer of pH 6.0 and pH 7.0, tri glycine buffer of pH 8 and incubated on 35^0C for 15 min and absorbance recorded at 450nm. The observed enzyme activity is given in table no 11and12.

The normal range of pH for typical laccase enzyme is 3-5. It has been also reported that optimum pH varies with type of substrate employed. Laccase from the *T. Viridae* had pH optima of 4 with guaiacol as substrate. The optimum pH value for laccases varies depending on the substrates employed, even though many reports have been reported a bell shaped profile for laccase activity. pH optimum varies considerably due to reactions caused by substrate utilized, molecular oxygen or enzyme itself. The difference in redox potential between the phenolic substrate and the T1 copper could increase oxidation of the substrate at high pH values, but the hydroxide anion OH- binding to the T2/T3 copper results in an inhibition of laccase activity due to disruption of the internal electron transfer between T1 and T2/T3 Centers. These two opposing effect can play an important role in determining the optimal pH of the biphasic laccase enzyme.

[38] The enzyme activity reached the maximum at particular pH and decreased thereafter.

c) Effect of organic solvents on enzyme activity

Effect of Different organic solvents

(methanol, ethanol, acetone each at 5%, 20%) was checked by mixing this solvents with the 1% Guaiacol substrate-containing reaction buffer to obtain the respective final concentrations. The effect of these additives on the laccase activity was also determined by incubating the additives at 35^0 C for 15 min. Absorbance was recorded at 450nm and enzyme activity calculated by using above formulae.

The addition of water miscible organic solvents caused a decrease in the enzymatic activity by altering the pH of the aqueous solution. The purified laccase retained approximately 70% of its initial activity in the presence of 5% methanol and Ethanol. These results indicated that the enzyme might be suitable for use in reactions that require a similar concentration of these solvents. However, the inhibitory effect increased with the increasing concentration of solvents. The activity was almost completely inhibited in the presence of 20% acetone... The activity was completely inhibited when the concentration of all of the tested solvents increased to 50% (data not shown). [37]

d) Immobilization of enzyme

Using enzymes instead of other molecules in reactions is useful because enzymes catalyze specific reactions and work at much lower temperatures than chemical catalysts. The molecule that an enzyme acts on is called a substrate. Enzymes can either be mixed freely with the substrate in solution or immobilized to a solid support so they do not mix freely. There are many advantages of immobilization, one of which is that the enzymes can be reused – catalyzing the same reaction many times. Binding the enzymes to a surface also makes those more stable and less likely to denature (lose their shape). In addition, there will be no enzyme left in the product at the end, so purification is not necessary. Sodium alginate solution is best prepared by adding the powder to agitated water, rather than vice versa, to avoid the formation of clumps. Prolonged stirring may be necessary to achieve the complete dissolution of sodium alginate. After sodium alginate is completely dissolved, leave the solution undisturbed for 30 minutes to eliminate the air bubbles that can later be entrapped and cause the beads to float. Although not necessary, the beads may be hardened by mixing some amines in the sodium alginate solution and cross-linking with glutaraldehyde.

IV. CONCLUSIONS

Use of laccase for dye degradation

In general, the efficiency of decolorization depends on the structure of dye and the redox-potential of the enzyme. The ability of purified laccase from *T. viridae* to decolorize various structural dyes was examined as explained in methodology. These dyes were tested at pH 5.0 where the enzyme performed a great activity to Guaiacol (acidic condition). The decolorization obtained at 7 days and 14 days showed different percentages in each case.

The efficiency of enzyme to decolorize the dye is different for different dyes as given in fig 7 to 11.The results of dye degradation are as shown in table no 15 The malachite green was shows the high percentage rate of degradation as up to 88.54% and the methylene blue dye shows less percentage of degradation than any other dyes as its percentage was 77.52%. The efficiency of enzyme to degrade the dye is dependent on the structure of dye and redox potential of enzyme as well as on concentration of enzyme. [37].

Screening for laccase producing fungi on plates containing colored indicators resulted in isolation of strain which having fast growth rate and grater reactivity towards colored indicator present in the cultivating medium that is *Trichoderma* genus. Laccase production by isolated Trichoderma strain was carried out both in submerged and solid state condition. The production level of laccase in submerged condition was quite low in medium of potato dextrose broth whereas in solid state condition employing wheat bran resulted adequate levels of laccase yields.

The crude laccase was found to have minimum activity at 35ºC and at pH 4. The addition of water miscible organic solvents caused a decrease in the enzymatic activity by altering the pH of the aqueous solution. The purified laccase retained approximately 70% of its initial activity in the presence of 5% methanol and Ethanol. The enzyme activity reached the maximum at particular pH and decreased thereafter that is the pH-4.0 and the optimum temperature for laccase activity is 35^0C. The efficiency of enzyme to degrade the dye is dependent on the structure of dye and redox potential of enzyme as well as on concentration of enzyme. This potent organism can be used for large scale laccase production and its use in treating various industrial effluents.

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