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Bacterial Alkaline Laccase and Its Environmental Applications

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Abstract: *Alkaline laccase play an important role in bioremediation such as degradation of phenols, aromatic hydrocarbons, and different dyes used in textile industry. Also in denim washing, cosmetics and pulping industry. The isolates from soil sample near the vicinity of wood industry and were screened for alkaline laccase production. The darkness of brown colour of the colony due to oxidation of guaiacol was considered for efficient production. For potent isolate the significant enzyme activity was calculated using ABTS as substrate. The potent isolate was identified by 16s rRNA sequence analysis and found to be Bacillus subtilis. Optimization of incubation period, pH and temperature conditions for enzyme production were determined and found to be 72hrs, pH – 9 and* 30^0C *respectively. Optimization of carbon sources for enzyme production were determined and found to be 1% glucose and 1% wheat bran. Optimization of organic nitrogen sources showed maximum enzyme activity at 0.8 % peptone and tryptone. In case of inorganic nitrogen sources the activity was optimum for 2% of ammonium nitrate and ammonium dihydrogen phosphate. The partially purified preparation of enzyme was immobilized and optimized for sodium alginate concentration, bead size. The maximum activity was found at 2%, and 2mm. The enzyme activity of purified enzyme was found 18.8 U/ml. The immobilized beads were recycled up to four cycles for decolourization of textile waste and showed active decolourization up to third cycle. The enzyme was efficient in degrading phenol, model dyes like reactive black and textile effluent.*
Keywords: *Alkaline laccase, Reactive black, Immobilization, Decolourization, Phenol degradation*

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I. Introduction

Nowadays, water pollution is the important problem, the world is facing. Many factors are responsible like different industries but one of the important cause for this pollution is textile industry .The important process used in textile industry is dying .This dying can be done by both organic and inorganic synthetic dye that available in variety of colors. After dying the water is released in the streams which leads to increase in colours, toxic metals and suspended solids in that water body. These dyes are having a complex structure due to which it becomes very difficult to degrade and decolorize. These dyes are with high COD, high temperature, high pH, strong colour and also trace metals like Cr, As, Cu and Zn, which are capable of harming the environment[1]. They show recalcitrant property and are resistant to aerobic digestion and are stable to exposure of sunlight .The important class used for dyeing are Azodyes. They are important in pharmaceutical, food and textile industries. As they are carcinogenic and mutagenic, removal of these dyes becomes a challenge to waste water treatment facilities [2] .In India the major part of coloured effluent is from textile industry. The improper and indiscriminate disposal of textile effluents in natural waters and land is posing serious problems.[3]

There are different methods like physical by using reverse osmosis, ultra filtration and micro filtration, adsorption, chemical treatment involves use of coagulation or flocculation combined with filtration and floatation. Also precipitation – flocculation with $Fe_2+/Ca(OH)_2$, electrofloation ,oxidative agents like Fenton reagent (ironsulphate) , hydrogen peroxide , chlorine, U.V. irradiation and ozonation for the removal of residual colour from water[4].

The physical and chemical processes has drawbacks like low efficiencies and applicability to limited concentration ranges , formation of hazardous byproduct ,incomplete removal and the most important is high cost. As compared to physical and chemical processes biological processes are the best economical and ecofriendly alternative [5].

Before disposal of textile waste water in the natural water bodies it is important to treat it efficiently. This decolourization can be achieved by various microorganisms like bacteria, fungi, algae and actinomycete, among these bacteria and fungi are very popularly used for biodegradation of textile dyes[6]. The ability to degrade these dyes is due to the production of oxidoreductive enzymes like laccase, lignin peroxidase[7], horse radish peroxidase, lipase, and bilirubin oxidase they oxidize the aromatic compounds to form aromatic radicals, which in turn combine to form poly- meric structures that precipitate spontaneously from the solution due to

their low solubility.[8]Due to the complex structure of azo dyes the degradation of azo dyes by azo-reductase was incomplete and some of them might even be converted into toxic aromatic amines[9].
Laccase (p-benzenodiol: oxygen oxidoreductase, EC 1. 10. 3.2) are found to be more effective in decolorizing the dyes. It belongs to a group of enzymes 'blue oxidases'[8].
This enzyme is widely distributed in nature like bacteria, fungi, plants, & actinomycete.
Laccases are bound by copper in several sites like type 1, type 2, and type 3. Removal of type 1 copper causes a decrease in laccase activity[6].

Laccases are involved in degradation or polymerization reaction or cross coupling of pollutant phenol with naturally occurring phenol. By immobilized laccases or by laccase producing microorganism phenolic effluents and poly cyclic aromatic hydrocarbons can be degraded[10]. By using molecular oxygen as an electron acceptor laccasescatalyzes oxidation of various substrates. There are four catalytic copper atom present in these proteins[11].The industrial waste containing phenol is highly toxic and having health hazards. Laccase catalyzes oxidative coupling of phenol and form water soluble oligomeric and polymeric products which are further removed by filtration or sedimentation in waste water treatment plant. For degradation of phenol different physical and chemical methods are available which are costly and forms hazardous by-products, so it is always good to use the biological means that is laccase forthe degradation[12].

Laccases are found to be widespread among bacteria, however, until now, only a few bacterial laccases have been studied. Recently, the use of laccase in the textile industry is growing very fast to develop laccase based economic process for textile waste decolourization and degradation of industrial phenolic waste because most currently existing processes to treat textile wastewater and phenolic wastewater are ineffective and expensive. Recently used most of the industrial laccases for dye decolourization are from fungi, especially basidiomycetessuch as Zylite (Zytex Pvt. Ltd., Mumbai, India), which is capable of degrading Indigo in a very specific way based on a laccase-mediator system. Unfortunately, these fungal laccases which are generally acidic and most wastewaters from textile industries shows neutral to alkaline pH (around 7–11) these fungal laccases get inactivated in this alkaline pH range. Fungallaccasedo not have industrial potential for these process, since their application is expensive and increases wastewater toxicity, due to these disadvantages of it becomes important to develop a laccase bearing high activity at alkaline conditions. Laccases from bacteria may be used as good alternatives[13].

The present work aims to optimize the production of alkaline laccase by *Bacillus subtilis*. To develop eco-friendly method for decolourization of dyes from textile effluent and degradation of phenol.

II. Materials and methods

Sample

Soil sample was collected from wood industry situated at Dwarka (Nashik). The soil samples were collected from the top surface and from the depth using sterile spatula and transported to the laboratory and were used to prepare initial inoculums.
Textile waste sample was collected from local dying industry situated at Yeola (Nashik).

Isolation of alkaline laccase producer

Samples were serially diluted in saline solution preheated to a temperature of 55 °C for ten minutes. The dilutions were plated on nutrient agar plates containing guiaicol and $CuSO_4$. The plates were incubated at 30 °C for 5 days. The reddish brown color developed due to oxidation of guiaicol by laccase is used to determine enzyme production. The isolates that showed brown colour colonies on the isolation media indicating laccase production were selected and maintained on nutrient agar slants and stored at 4°C[9].

Qualitative screening of isolates

Qualitative screening was done by spreading isolated cultures on modified M162 agar containing - $CaSO_4.2H_2O$- 0.4, Glucose- 1.0 ,0.01M Ferric citrate solution-5ml,Na_2HPO_4- 0.5, NaH_2PO_4- 0.5,NH_4NO_3- 0.5,K_2HPO_4- 0.5,Yeast extract- 3.0,Tryptone – 3.0,Agar – 18.0 g/L and 10 ml micronutrient solution of (g/L)- $MnSO_4$ H_2O-2.2,$ZnSO_4.7H_2O$-0.5,H_3BO_3 -0.5,$CuSO_4.5H_2O$-0.05,$Na_2MO_4.2H_2O$- 0.05,$CoCl_26H_2O$ - 0.09,pH -9 and 0.5 ml of different concentration of guiaicol (1 to 5 mM), pH 9.0 and incubated at 30^0C for 5 days. Selection of efficient laccase producing colony was done on the basis of darkness of colony[14].

Quantitative Screening

Qualitative screening or Secondary screening of laccase positive cultures were carried out in to 100 ml Erlenmeyer flasks containing 25ml Modified M-162 medium which was inoculated with 5 ml culture of laccase producer. Flasks were incubated with shaking at 120 rpm at 30^0C. Sampling was done at regular intervals for laccase activity and all the experiments were carried out in triplicates.

For quantitation of enzyme production, enzyme assay was performed by using 500 mM ABTS (2, 20-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) (HiMedia, India) with 100mM Tris HCl buffer (pH -9). The oxidation of ABTS was indicated by intense blue colour which was read at 420 nm. The reaction mixture (3ml) contained 0.3 ml of culture filtrate and 2.7 ml ABTS were incubated for 10 min at 30^0C.One unit of enzyme activity was defined as 1 mM of ABTS oxidized per minute. Enzyme activity was calculated by following formula[15].

$A = \varepsilon C l$

Where,

A =Absorbance at 420 nm per min

ε = extinction coefficient of ABTS at 420nm 3.6×10^4 ($M^{-1} cm^{-1}$).

l =Path length

Characterization of bacterial isolates

Purified isolate was characterized morphologically and biochemically. Further the organism was genetically characterized by 16S rRNA, PCR and sequence analysis to confirm the species.[16]

Optimization of physico-chemical parameters

Production of alkaline laccase was optimized for various physico-chemical parameters. The selected bacteria was cultivated in synthetic basal medium containing (g/L)-Glucose- 10.0,NH_4NO_3 - 2.0,KH_2PO_4 - 0.8, Na_2HPO_4- 0.75 ,$MgSO_4$ – 0.5 , Yeast extract -2.0 devoid of guaiacol. Incubation period varied from 12 to 144 hrs. , temperature varied from 25to 50^0C and pH from 7.0 to 10. All the experiments were carried out in triplicates and enzyme activity was determined using ABTS as substrate.[17]

To optimize carbon and nitrogen sources, 250 ml flask containing 100 ml synthetic medium (pH-9) containing different concentrations of crude and refined carbon sources, also organic and inorganic nitrogen sources was inoculated separately and incubated at 30^0C on a rotary shaker at 120 rpm[17].Enzyme activity was calculated by laccase assay.

Purification of enzyme

The culture supernatant was obtained by centrifugation of cultured broth at 10,000 rpm for 15 min at 4^0C. Ammonium sulphate was added in to culture supernatant and the precipitate was obtained at 60-70% saturation was collected and dialyzed.[18]

Enzyme immobilization

Enzyme immobilization was done by using sterile sodium-alginate solution (2%, w/v) mixed with 10 ml of partially purified enzyme obtained from *B.subtilis.* The partially purified enzyme and sodium-alginate mixture was thoroughly mixed using a magnetic stirrer and the obtained mixture was then extruded drop wise through a syringe into 0.2M $CaCl_2$ solution from about 5 centimeters height. The beads were left in the $CaCl_2$ solution for about 30 min and then thoroughly washed with distilled water three times, kept in saline at 4 ^{0}C and used for further investigations. The activity of immobilized enzyme determined by ABTS assay[18.] Further the immobilized beads were optimized for size and recycling efficiency for decolourization of model dye reactive black and textile industry waste[19 20].

Dye decolourization using free and immobilized enzyme

Model dye Reactive black (0.02%) solution was prepared in laboratory and textile effluent was collected from local dying industry of Yeola, MS, India. In order to test ability of the enzyme to decolourize synthetic dye and industrial effluent, experiment was carried out by using purified free enzyme and immobilized enzyme, and decolourization was carried out up to 48 hrs. Stock solutions of the dye prepared in distilled water to the final concentration of 0.02 %. Reactive black and the textile effluent solution (50 ml) were adjusted to pH 9 and separately incubated with purified free enzyme (5ml) on shaker incubator at 30^0C. Control without free enzyme were run in parallel with test. After each six hours sample was removed and used to measure the dye absorbance and to calculate percent decolourization. Textile effluent without free enzyme was used as control.

Reactive black and textile effluent decolourization by immobilized beads was studied by adding 8-10beads of size 2mm in place of free enzyme as mentioned above. Control prepared by using stock solution of dye and bead without purified enzyme, incubated at the same conditions like test.After each six hours sample was removed and used to measure the dye absorbance and to calculate percent decolourization. The beads were reused for decolourization of textile waste for next three cycles.

Decolourization was measured by UV-spectrophotometer by measuring the absorbance at 600nm.All the experiments were carried out in triplicate and the findings are average of three independent experiment. The

percentage decolourization was calculated from the following equation[10, 20]. % Decolourization= (Initial A^0-Final A^0)/Initial $A^0 \times 100$

Phenol degradation using free and immobilized laccase

Phenol degradation was carried out to study potential of free enzyme and immobilized enzyme to degrade phenol. Different concentrations of phenol from 100-500 ppm were prepared. Experiment was performed in 100 ml Erlenmeyer flasks containing 50 ml phenol of different concentration added with 5 ml purified free enzyme and enzyme immobilized beads separately. The flasks were incubated on a rotary shaker 120 rpm for 72 hrs. The residual phenol was estimated by 4Aminoantipyrine method (Mohamed et al, 2003) by measuring the absorbance at 600nm. The control was designed by inserting of heat-inactivated laccase to the reaction mixture for free enzyme and immobilized beads. The percentage degradation of phenol was calculated by the equation[21]. % of Degradation= [Initial conc.-Final conc.]/Initial conc.x100.

III. Results and Discussion

Isolation of bacteria producing alkaline laccase

Alkaline laccase producing bacteria were isolated based on the brown color zone produced on nutrient agar containing guaiacol and $CuSO_4$.Altogether 15 bacterial and actinomycete strains showing positive reactions on indicator plates were isolated (Figure-1).

Qualitative and Quantitative screening of bacteria producing alkaline laccase

Fifteen isolates were screened on modified M162 medium. Out of which five isolates showing dark zone around the colony were selected for the quantitative estimation of enzyme production. For quantitative screening enzyme activity of five isolates was determined by ABTS assay. Out of five AL5 gave maximum enzyme activity i.e. 23.8 U/ml as shown in Figure-2.

Figure 1- Alkaline

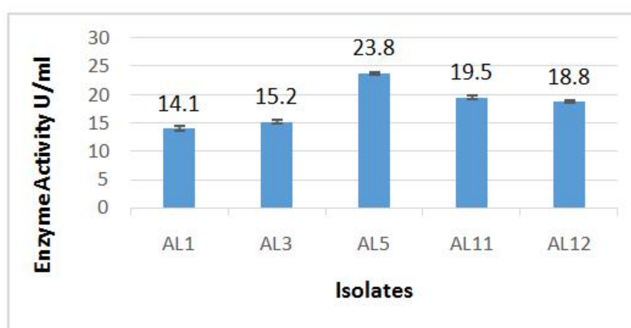

Figure 2- Enzyme activities of isolates laccase producer bacteria

Characterization and identification of selected bacteria producing alkaline laccase

Characterization and identification of alkaline laccase producing bacterial isolate was done on the basis of BLASTn search of isolate with most similar 16S rRNA.

Sequence analysis revealed that AL5 belongs to *Bacillus subtilis* as it showed maximum homology (99%) with *Bacillussubtilis* strainKY818955.1.

CGCGAAGCGGCTCTATACATGCAGTCGAGCGAACAGACAAGGAGCTTGCTCCTTTGACGT
TAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGG
GAAACCGGAGCTAATACCGGATAATATTTCGAACCGCATGGTTCGATAGTGAAAGATGGT
TTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTA
CCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACA
GGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGAC
GGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAG
AACATACGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCG
TAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAG
GGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCG
GTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAAC
TGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTACACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCCTTAGTGCTGCAGCTAACGC
ATTAAAGCACTCCGCCTTGGGGTAGTTACGACCGCAAGGATTGAAAAACCTCCAAAAGGG
AAAAT

Effect of incubation period on enzyme production
The maximum biosynthesis of alkaline laccase was recorded at incubation period of 72 hrs. The production of enzyme proportionally increased with the incubation time up to 72hrs, whereas after 72 hrs. Production was decreased as mentioned in figure-4 and biomass production reached its maximum after 96 hrs. The decreased enzyme production may be due to depletion of macro and micronutrients in production medium.

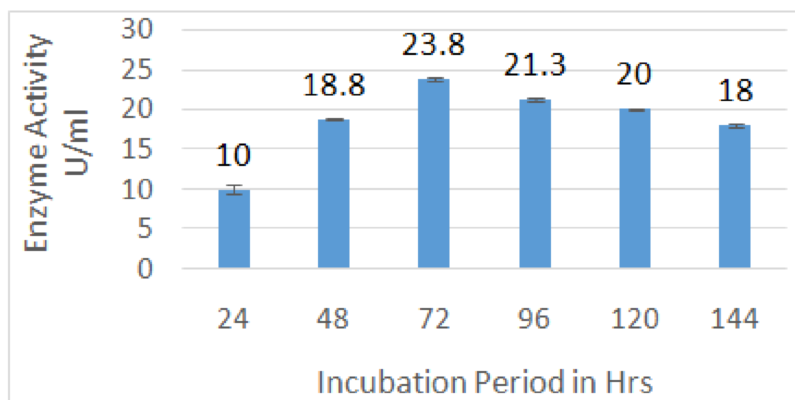

Figure- 4 Effect of incubation period on enzyme production

Effect of incubation temperature on enzyme production
The results of effect of different incubation temperatures of alkaline laccase by bacterial isolates were recorded and shown in figure 5.The maximum activity of enzyme 21.9U/ml was determined at 30^{0}C.Decreased enzyme activity may be due to thermal inactivation of enzyme.

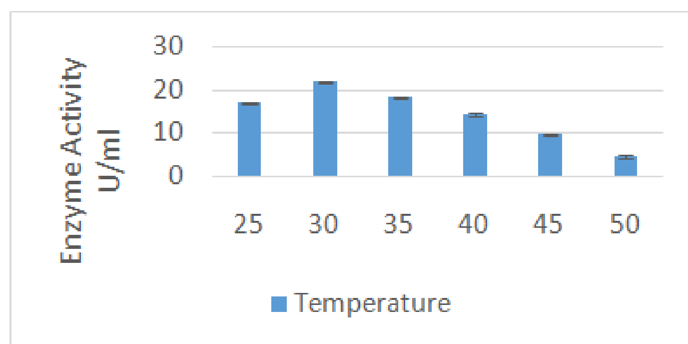

Figure-5 Effect of incubation temperature on enzyme production

The result represented in figure-6 indicated that the maximum production of laccase was recorded in medium at pH9 and the enzyme activity was dramatically decreased after pH 9. As the pH value increases, the potential difference between the phenolic substrate and the T1 copper can increase the substrate oxidation while the hydroxide anion (OH^{-}) binds to the T2/T3 copper center of laccase. This leads to inhibition of laccase.

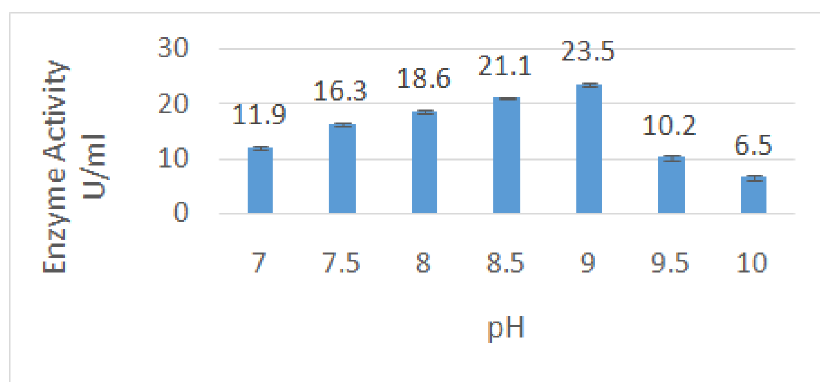

Figure-6 Effect of pH on enzyme production

Optimization for carbon sources

Effects of different refined and crude carbon compounds were evaluated in synthetic basal medium with 1% glucose as a sole source of carbon. The carbon source in the basal medium was replaced by lactose and fructose. It was found that lactose and fructose also acted as good organic carbon source and gave highest activity at concentration of 2% and 1.5 % respectively. The enzyme activity for both was 20.8 and 23.0 U/ml respectively but it was maximum for 1% of glucose i.e.24.4 U/ml as mentioned in figure-7.

Crude carbon sources also showed effective enzyme activity in case of wheat bran and waste paper it was 21.9 and 20.2 U/ml respective as shown in figure-8.

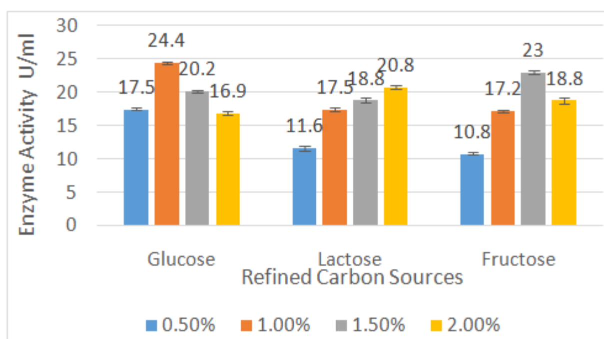

Figure -7. Effect of refined carbon sources on enzyme production.

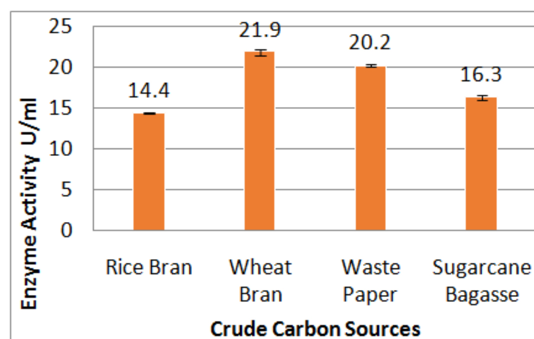

Figure-8 Effect of crude carbon sources on enzyme production

Optimization of different nitrogen sources

Effect of different nitrogen containing compounds was evaluated in basal medium with glucose (1%) as sole source of carbon. The nitrogen source in the basal medium (NH_4NO_3) was replaced by their nitrogen equivalent of different organic and inorganic nitrogen sources. In case of organic nitrogen sources peptone and tryptone were showing maximum enzyme production at 0.8% as shown in figure-9.Enzyme activity determined was 24.1 and 23.6 U/ml respectively.

Inorganic nitrogen sources such as ammonium nitrate and ammonium dihydrogen phosphate gave maximum enzyme production at 2% i.e. 23.3 and 22.5 U/ml respectively as mentioned in figure-10.

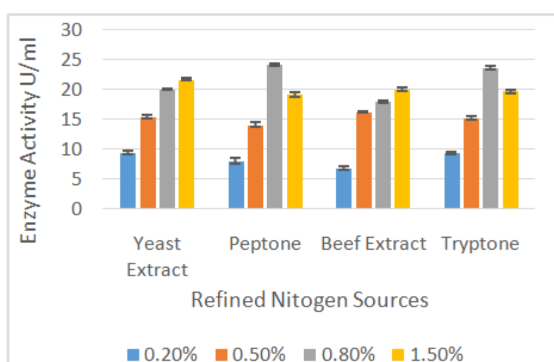

Figure -9Effect of organic nitrogen sources on sources on enzyme production

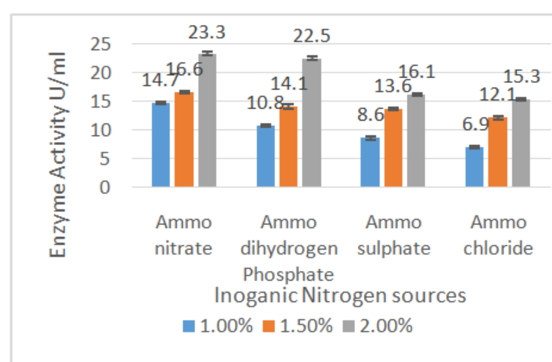

Figure-10 Effect of inorganic nitrogen enzyme production

Purification of enzyme

Crude enzyme activity was found to be 11.9 U/ml and after partial purification by ammonium sulfate precipitation and dialysis the activity of the fraction was calculated as 18.8 U/ml.The activity increased by 1.58 times or 57.98%.

Effect of alginate concentration on enzyme production

The influence of sodium alginate concentration of enzyme entrapment was studied. The alkaline laccase enzyme activity was determined by using different concentration of sodium alginate ranges from 1.5% to 3.0 % as given in table-1. The maximum activity was observed at 2.0 % of sodium alginate concentration and was found to be 20.8 U/ml after 72 hrs. incubation period. However, further increase in alginate concentration beyond 2.0% resulted in lower enzyme activity. This is due to smaller pore size of beads at higher concentrations of sodium alginate and lower sodium alginate concentration leads larger pore size of the less

tightly cross linked fragile Ca alginate beads. So enzyme activity is efficient in case of lower percentage sodium alginate concentration but due to larger pore size and consequently greater leakage they are inefficient in process.

Table 1 Effect of alginate concentration on enzyme activity

% concentration of Sodium alginate	Enzyme activity in U/ml
1.5%	23.4±0.31
2.0%	20.8±0.33
2.5%	17.2±0.32
3.0%	14.4±0.34

Optimization of bead size

For optimization of size of beads different size beads were prepared like (2mm, 3mm, and 5mm). Among these different sizes of immobilized beads 2mm bead size showed maximum enzyme activity that was 22.4 U/ml. As there is increase in bead size, the activity of enzyme has been decreased due to mass transfer resistance of bead(Table-2).

Table -2 Effect of bead size on enzyme activity

Bead size (mm)	Enzyme activity in U/ml
2	22.4± 0.21
3	18.8±0.26
5	15.2±0.36

Reactive black and textile waste decolourization by free and immobilized enzyme

The model dye Reactive black decolorized up to 70% by free enzyme and 55 % by immobilized enzyme after 48 hrs. of incubation as shown in figure-11.

As there are variations in capability of purified free and immobilized enzyme to decolorize the dye hence there existed much variation in decolourization. The study also involves possible removal of colour due to dye absorption by Ca- alginate beads by preparing a control reaction with Ca alginate beads without laccase. It was observed that alginate beads became coloured when treated with reactive black due to bio-absorption and was able to remove dye up to 8%. Thus the predominant mechanism involved in Reactive black decolourization was due to action of laccase on the nitro groups in the structure attract electrons and hence inhibit the oxidation reaction catalyzed by the laccase enzyme.

To study effect of alkaline laccase on textile effluent both free and immobilized enzyme were treated with effluent. Figure-12 shows decolourization obtained by free enzyme 50.1 % and by immobilized 59.6 % after 48hrs. Decrease in decolourization may be due to presence of toxic substances in effluent making enzyme inactive and reducing pore size. Anions such as the halides, azide, cyanide and hydroxide bind to the type 2 and type 3 copper atoms of laccase, which disrupts the electron transfer system, resulting in enzyme inhibition.

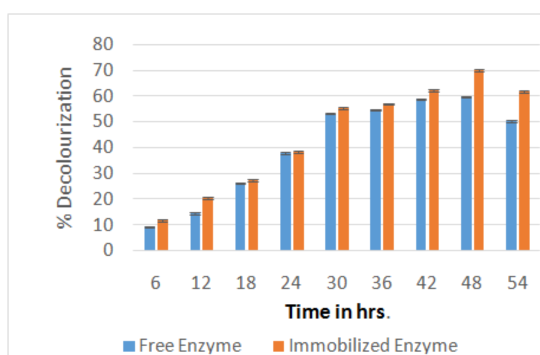

Figure-11 Decolorization of reactive black by free and immobilized enzyme

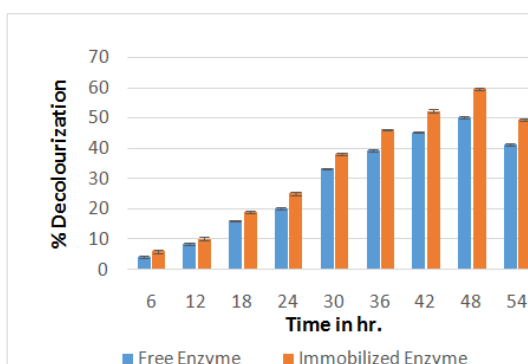

Figure-12 Decolourization of textile waste by free and immobilized enzyme

Recycling of Beads for decolourization of industrial waste

Textile effluent was again treated with immobilized beads for next three cycles to investigate whether Ca alginate immobilized enzyme beads could be successfully reused after storage at 4 ^{0}C. Reusability of immobilized enzyme exhibits important aspect for industrial application as it decreases the cost of the process. Thus the reusability of immobilized laccase in four successive batches of textile waste decolourization was investigated. From the figure-13 it was detected that enzyme was active in I, II, and IIIcycle, so efficient decolourization obtained up to 61.9%. But in fourth cycle the value gradually decreased. This decrease may be

related with enzyme inactivation, blocking of pores or leakage of enzyme from beads during repeated washing at the end of each cycle. This restriction may cause decrease inefficient activity of immobilized beads.

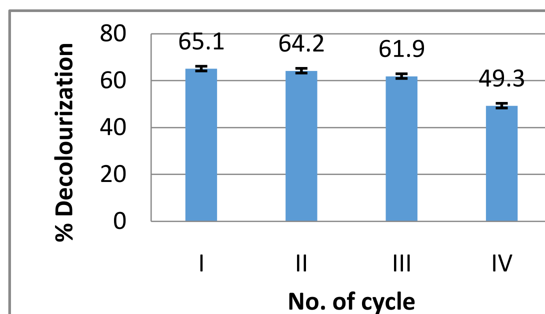

Figure-13 Recycling of Beads for decolourization of industrial waste

Phenol degradation by free and immobilized enzyme

Purified free and immobilized enzyme both were treated with different concentrations of phenol from 100 ppm to 500 ppm.Degradation of phenol by purified free enzyme was 50.7 % for 100 ppm and 31.1% for 500ppm as shown in figure-14.

In case of immobilized enzyme it was found to be 53 % for 100 ppm and 40 % for 500 ppm as mentioned in figure -15. Increase in concentration of phenol leads to decrease in degradation activity of enzyme.

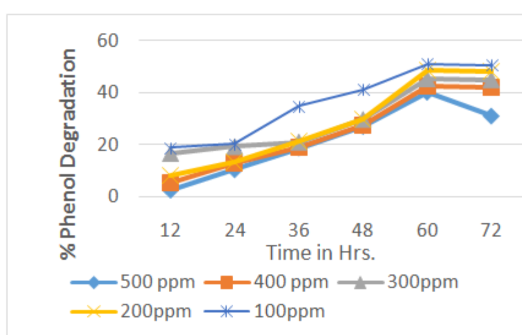

Figure 14- Degradation of phenol by free enzyme

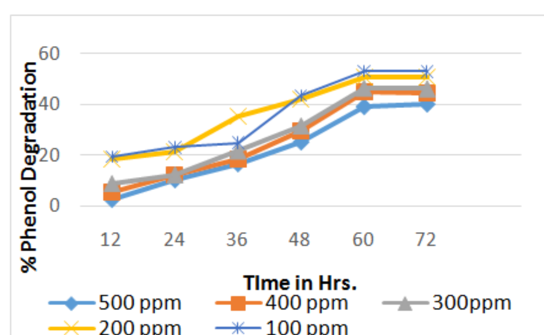

Figure 15- Degradation of phenol by immobilized enzyme

IV. Conclusions

The potent alkaline laccase producing bacteria isolated based on the brown color colony and screened for maximum enzyme production. The isolate identified as *Bacillus subtilis* by 16s rRNAsequencing. The maximum enzyme production atoptimization incubation time, pH and temperature was found to be to be 72hrs., pH-9 and 30^{0}C respectively. Optimization of carbon sources for enzyme production were determined and found to be 1% glucose and 1% wheat bran. Optimization of organic nitrogen sources showed maximum enzyme activity at 0.8 % peptone and tryptone. In case of inorganic nitrogen sources the activity was optimum for 2% of ammonium nitrate and ammonium dihydrogen phosphate.

The immobilized enzyme showed maximum activity at 2%sodium alginate concentration, and 2 mm bead size. The immobilized beads were recycled up to four cycles for decolourization of textile waste and showed active decolourization up to third cycle.

The immobilized alkaline laccase enzyme was very efficient in model dye decolourization Reactive black, also efficient in textile dye decolourization and phenol degradation than partially purified free alkaline laccase. As partially purified free enzyme can be used once, immobilized enzyme beads were used repeatedly and shown efficient textile waste dye decolourization up to three cycles.

The result of the study indicates that the *Bacillus subtilis*strain can be hopefully employed for the decolourization of dye and degradation of phenol.

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