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Partial purification and characterisation of dye degrading enzyme laccase from *Enterobacter cloacae strain*

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Abstract
Textile industries are one of the most complicated industries among manufacturing industry. Increasing population and modernised civilisation trend gave rise to blooming textile sector in India especially in Gujarat. Moreover, because of industrialisation in textile industry tones of textile effluent are been released into nearby pond and rivers that are harmful to the terrestrial as well as aquatic life. A Recent study is based on the dye degrading enzyme i.e. Laccase which is quite useful for the degradation of the textile dyes that are released by the textile plants. Purification and characterisation of the enzyme are carried out. It gave the best optimum condition growth of laccase enzyme using isolated strain from textile effluent GIDC P1. The activity found to be 478 U/ml.

Keywords: Textile effluent, Laccase, partial purification, Characterisation

Introduction
From last decade, the world is set to witness major environmental revolution that could be potentially game-changing in efforts aimed to achieve sustainable development. Numerous strategies are being studied to achieve sustainable development to lessen the various industrial wastes. For, these biocatalysts are a most vital tool for the sustainable development and to reduce and reuse the effluent of different industries. Various enzymes are being used or studied for various applications. Broadly, they are used in bioremediation. Industrial units involved in the manufacture of textile are using various dyes products for processing of textile. These dyes are the major cause of pollution resulting in serious health and environmental hazards. Synthetic dyes in which Azo dyes are widely used in several industries such as textile, paper, printing, cosmetics, pharmaceuticals, and as an additive in the petroleum industry. Many potentially hazardous organic compounds are continuously introduced with an exponential increase into various components of the environment.

Dyes absorb and reflect sunlight entering the water and so they can interfere with the growth of bacteria and hinder photosynthesis in aquatic plants (A.K.Tripathi et al 2011). Also it is well known that dye effluents from dyestuff manufacturing and textile industries, may exhibit toxic effects on microbial populations and can be toxic and/or

carcinogenic to mammalian animals. Therefore, removal of dyes from industrial effluents before the discharge is an important aspect of wastewater treatment; it is an important environmental problem (N. Manikandan et al 2012). Conventional methods for the removal of dyes in effluents include physical, chemical, and biological processes. However, because of the some or the other disadvantages of using physical and chemical methods for removal of dye from the textile effluent leads to the high cost and it becomes the biggest drawback. So to overcome it the alternative prospect for dye removal from the textile effluent biological methods are used. Moreover, these biological methods proved to be the best methods for the dye removal from the textile effluent (Patrick Huber et al 2012).

Azo dyes are widely used in the textile processes because of their ease and cost effectiveness of synthesis compared to other dyes; they are toxic, carcinogenic, and mutagenic characterized by the presence of one or more azo group (-N=N-) It has become inevitable to develop novel bioremediation technologies in order to dilute the impact of pollution (G.MILIKLI et al 2012).

Among the enzymes used for degradation process, Laccase has acquired good attention as they are favourable for the development of biodegradation systems and catalyse reductive cleavage of azo groups (-N=N-) under mild conditions (Savitha S. Desai et al 2011). Identification, purification and characterization of Laccase constitute a straightforward approach to the development of azo dye biodegradation systems. Present work focused on the characterization of this enzyme. Various parameters were studied which are effect of carbon source, effect of pH, effect of temperature, effect of heavy metal, effect of nitrogen source and further partial purification of the enzyme was carried out using Ammonium sulphate precipitation.

In the current study microbe isolated from the textile effluent, GIDC P1, was investigated for the bioremediation of azo dye using enzyme laccase.

Laccases (benzenediol: oxygen oxydoreductases, EC 1.10.3.2) are multi-copper enzymes belonging to the group of blue oxidases. They are defined as oxidoreductases, which oxidises diphenol and allied substances. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water. The ability of laccases to oxidise phenolic compounds as well as their capacity to reduce molecular oxygen to water has led to intensive studies of these enzymes. Although there are also some reports about laccase activity in bacteria, it does not seem probable that laccases are common enzymes from certain prokaryotic groups.

From the survey of literature it was found that the organism has not been studied previously.

Materials and methods

Isolation of Bacteria:

Textile effluents were collected from effluents site as a source of bacteria from Sachin G.I.D.C., Surat, and Gujarat, India. The sample was serially diluted on BHM containing Methyl Red 200mg/l to screen for the potential bacteria.

Identification of the bacteria:

GIDC P1 was identified as ***Enterobacter cloacae*** using molecular biology approach i.e. 16s rRNA sequencing.

Syringaldazine Assay for Laccase Assay:

Laccase activity was determined by Syringaldazine assay in which oxidation of syringaldazine at 530 nm. The reaction mixture (3 ml) which contained 0.1 ml of the enzyme sample and 2.9 ml of 20M syringaldazine in 50 mM sodium phosphate buffer, pH 7.0 was incubated at 30°C for 10 min. Enzyme activity was expressed in enzyme units. 1 U being defined as the

amount of enzyme causing the formation of 1mol of product per minute under the assay conditions used. Protein assay was determined by Folin Lowry Estimation.

Characterisation of Laccase Enzyme:
The fermentation medium was used for the study of various parameters for 7 days. For each experiment inoculums medium were taken in 250ml Erlenmeyer flask.

Effect of Carbon Source:
The effect of different carbon sources like lactose, glucose, and sucrose on the production of laccase was studied. The carbon sources were amended to the different concentration % in the production medium. 24-hour old culture were transferred to Erlenmeyer flasks (250 ml) containing 100 ml of production medium. Moreover, the enzyme is harvested in 7 days.

Effect of Nitrogen Source:
To find the suitable nitrogen source for the maximum production of laccase following organic and inorganic nitrogen sources namely peptone, beef extract, were amended at the different concentrations. The 24-hour old culture were transferred to Erlenmeyer flasks (250 ml) containing 100 ml of production medium. Flasks were incubated at 27°C and enzyme is harvested in 7 days.

Effect of pH on laccase production:
The effect of pH on laccase production was carried out by incubating the culture flasks containing 100 ml of production medium inoculated with 24 hour old culture of *Enterobacter cloacae* at different pH such as 5.0, 6.0, and 6.5 and 7.0. These experiments were conducted for 7 days (R.Sahay et al 2008, Madhavi and Lele et al 2009, Irshad et al 2011).

Effect of Temperature:
Different temperature ranges were studied for the impact on the laccase enzyme. The temperature was 37.c and 50.c. and the flask were incubated and studied for the 7 days for the effect on laccase activity (R.Sahay et al 2008, Madhavi and Lele et al 2009, Irshad et al 2011).

Effect of Heavy Metal Concentration (Copper and Cadmium):
To find out the suitable concentration of copper sulphate and cadmium for the maximum production of laccase the following concentrations of copper sulphate 1 and 0.5 were used. 24hours old activated culture were transferred to Erlenmeyer flasks (250 ml) containing 100 ml of production medium amended with different concentration of Copper sulphate and Cadmium. Flasks were incubated at 27°C and enzymes were harvested in 7 days (Petr Baldrian 2002, Verneker Madhavi et al 2009).

Partial Purification of Laccase:
Loop full of the culture was inoculated into the fermentation medium. Moreover, the crude enzyme was obtained by centrifugation of the fermentation broth at 10,000 rpm for 15 minutes. Moreover, this supernatant was ruined for the study of the enzyme activity and protein concentration. The precipitation of laccase was carried out from the crude first at 40% and from the supernatant and filtrate the protein estimation and enzyme estimation was conducted. Moreover, the saturation was achieved at 80% and then obtained precipitates were dissolved in phosphate buffer and from that protein estimation was been done using Folin Lowry Estimated and enzyme assay was performed using Syringaldazine assay method (Daphne Vivienne et al 2013) (Savitha S. Desai et al 2011).

Results and discussion
The textile effluent was collected from the Sachin GIDC surat. Moreover, were serially diluted on the BHM medium.30 isolates

were isolated and further studied the potentiality of dye degradation. Among them around 6 isolates were found to be potent and were characterising further for the study of enzyme Laccase.

Among them the strain GIDC P1 which was identified using the molecular approach as *Enterobacter cloacae* was found to be laccase producing strain. *E.cloacae* was one of the isolates that were been isolated from the textile effluent and the isolate was screened for the bioremediation and enzymatic studies. During these enzymatic studies it was found that it gave high productivity for the enzyme laccase that is the main prospect for the recent research study.

Characterisation of laccase enzyme was been done by studying the different factors and its effect on the enzyme activity.

Different carbon sources were used for its effect on its activity. Carbon sources that were undertaken for the studies are Glucose, Lactose and Sucrose. BHM medium was supplemented with 1% of these carbon sources in 250 ml Erlenmeyer flask containing 100 ml of the BHM broth and the enzyme activity was observed for the 7 days. Moreover, it was observed that glucose was the optimum carbon source for the production of a higher amount of enzyme activity.

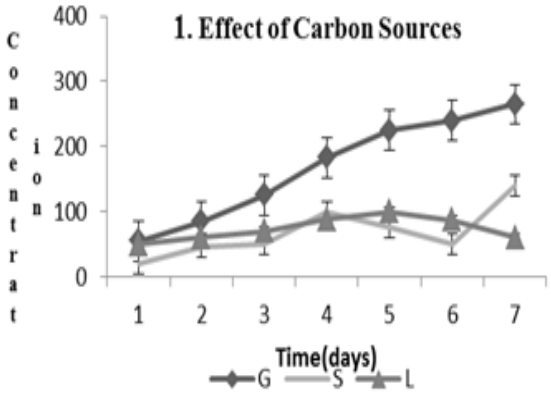

Fig. 1: Effect Carbon sources on the enzyme activity.

In the literature study, the contradictory evidence on the effect of nitrogen source on the laccase activity. High N media has given the maximum production of laccase activity in *L.edodes, Rigodosporus lignosus,* and some gave better laccase production in the nitrogen deficient medium i.e. found in the *P.cinnabarinus*, *P.sanguineus* and *Phlebia*. In the current study different nitrogen source such as peptone and beef extract were used. Moreover, it was observed that the *Enterobacter cloacae* GIDC P1gave the better enzyme activity with peptone so the optimum nitrogen source for the given strain was found to be the 1% peptone. However from the study it can be said that the specific laccase activity of laccase proves that the positive effect of additional N source on laccase enzyme activity.

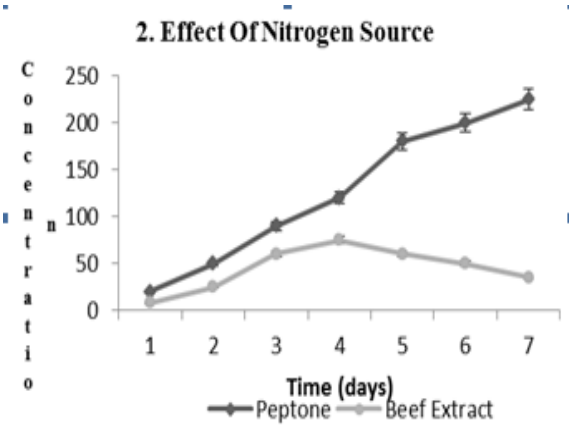

Fig. 2: Effect of Nitrogen source on enzyme activity.

Optimum pH of the laccase is mainly depended on the type of the substrate used. For e,g, when phenol is used as a substrate optimum pH was found to be in the range between 3 to 7 for fungal laccase and Ph-9 for the plant laccases. When ABTS was used as a substrate the optimum pH was found to be acidic that is the range between 3 to 5. In case the use of syringaldazine the optimum pH for *Enterobacter cloacae* was found to be 8. Moreover, the variations may result due to change in the reactions that occur because of the substrate or the enzyme itself.

We have not studied but the optimum pH reported by Soden and Dobson for laccase using 2,6-dimethoxyphenol of *Pleurotus sajor caju* strain is 6 compared to 4.5 found in the case of laccase.

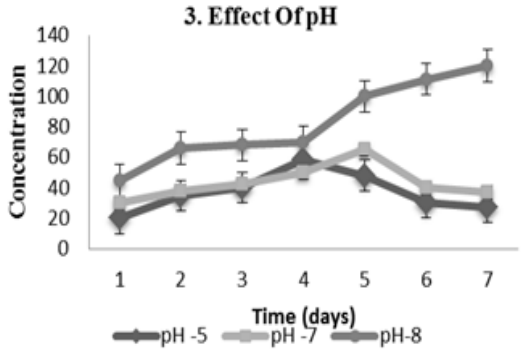

Fig. 3: Effect of pH on enzyme activity.

The optimum temperature of laccase differs greatly from one strain to another strain. Based on the literature study the laccase that was isolated from *Ganoderma lucidium* has the optimum temperature between 20-25.c and laccase which was isolated from *Marasmus querocorpium* was 60.c. for the fungal laccases the optimum temperature range is 25-80.c. However, most of the enzymes have optimum temperature 50-70.c. The optimum temperature range was been studied by us was 50.c .Enzyme activity affect was been studied on two different temperature range it was at 37.c and 50.c. Moreover, the maximum activity obtained at the temperature range of 50.c with *E.cloacae* GIDC P1 strain. Moreover, the optimum temperature ranges for enzyme production are rarely found in the literature. The data obtained under lab conditions makes conclusions about the situation under natural conditions.

The culture of *E.cloacae* growing on BHM medium was supplemented with cadmium chloride to a final concentration of 0.5 and 1%. During the initial days the laccase activity was found to increase negligible. The activity was found to be increase rapidly on the 10^{th} day. And the activity was found better in the flask having the cadmium concentration 1g/100ml and was found to be the optimum cadmium concentration for the *E. cloacae* GIDC P1 strain.

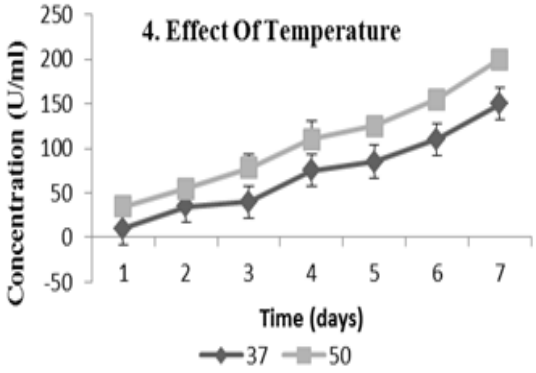

Fig. 4: Effect Of temperature on enzyme activity.

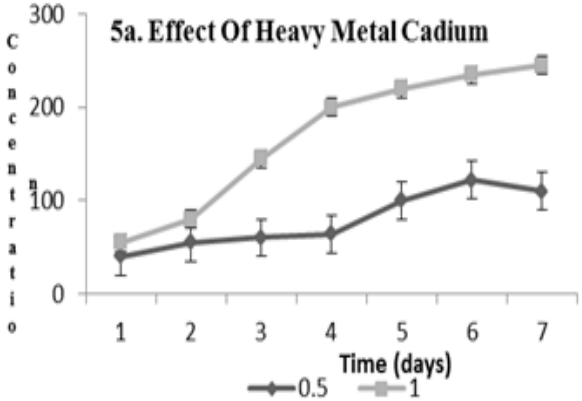

Fig. 5a: Effect of heavy metal cadmium on enzyme activity.

Based on the literature survey the *P. ostreatus* was supplemented with Cd(NO3)2 to different concentrations and it has the maximum activity obtained after day 10 and the optimum concentration was found to be 2mM where enzyme activity increased by 18.5 fold and low activity was found in the concentration of 5mM so it can be concluded that Cd helps in enhancing the laccase activity.

The addition of copper in the BHM medium lead to increasing laccase activity independent of the time of addition when the copper was added during the first day the activity was found to rise than the control flask. Two different concentrations were

studied for the experimental purpose. The concentration was 0.5 and 1% respectively. Moreover, the better results with the *Enterobacter cloacae* were found to be 1% CuSO4 flask the activity was found to be increased from the first day the control flask. Literature study also showed that the addition of copper in the minimal medium use for the laccase production increases the activity of laccase and thus these metals can be supplemented in the medium that increases the laccase activity rate rapidly.

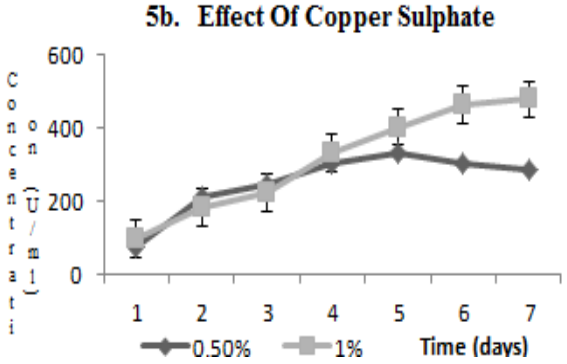

Fig. 5b: Effect of heavy metal copper sulphate on enzyme activity.

Partial purification of the enzyme was carried out using Ammonium salt precipitation procedure. 500 ml of the flask was been used for the production of the enzyme. And the initially the protein estimation was carried out and the protein concentration was found to be 0.782 mg and the specific activity of enzyme was found to be 58000 units/mg. Initially 40% precipitation was done and the fold purification was found to be 0.43 and during the 80% precipitation it was found to be 0.31.and the enzyme activity was found to be 478 U/ml.

Conclusion

Laccase is found to be present everywhere in nature found to be produced by different plants, fungi and bacteria. The functions of enzyme differ from organisms to organisms. It plays an important role in degrading xenobiotic compounds. It is an industrially important enzyme which helps in bioremediation applications such as waste detoxification, textile dye degradation etc. so these enzymes are studied because of its importance and is centre of attraction for recent research development. Current research helps in making us the increase in the laccase production by using different factors. Thus it can be concluded that the increase in the use of laccase can help us to overcome the many problems.

Competing interests

The authors declare that they have no competing interests.

Table 1: Partial Purification of Laccase enzyme.

Fraction	Volume (ml)	Total Protein (mg)	Activity (Units/ml)	Total activity (Units*ml)	Specific activity (Units/mg)	Fold purification	% Yield
Crude	**500**	**0.782**	**116**	**92800**	**58000**	**1**	**100**
Partial purification process							
40%	**100**	**0.447**	**112**	**11200**	**25055**	**0.43**	**43**
80%	**100**	**0.258**	**478**	**47800**	**18527**	**0.31**	**31**

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