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Biodegradation of pyridine raffinate using bacterial laccase isolated from garden soil

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ABSTRACT

Pyridine raffinate, the residual mixture after the extraction of pyridine, discharges as pollutants from pyridine manufacturing industries. Pyridine and its derivatives are of foremost concern as environmental pollutants. Removal of contaminant or pollutant can be achieved using three kinds of approaches: Physical, Chemical and Biological. Paralleled with other physical and chemical methods such as combustion, photolysis. Biodegradation is a fiscal and environment-friendly alternative. In the present study, bacterial laccase, a multi copper enzyme that uses molecular oxygen to oxidize wide variety of aromatic and non-aromatic compounds, was used for the biodeterioration of pyridine raffinate, sample taken from Jubilant Organosys, Gajraula. Laccase was isolated from the bacterium ***Pseudomonas fluorescens*** and found to be an effective method to biodeteriorate pyridine raffinate instead of using harmful chemicals. It was observed that O.D. was reduced (max. 0.2327 at 501 nm) measured by UV/VIS Spectrophotometer taking raffinate as a reference. It signifies the oxidation of compounds present in the effluent that leads to the reduction in C.O.D. and found to be an effective method for the treatment of effluent.

1. Introduction

In present era due to increased industrialization, with lots of advancement in human civilization, there is also a threat developing for our environment i.e release of harmful chemicals to the surrounding. A vast amount of aromatic compounds as pollutants are being discharged into the environment by different industries, following the wide range of implications of aromatic compounds among the top chemicals utilized. Amongst this aromatic heterocyclic compounds, pyridine and its derivatives are of foremost concern as environmental pollutants.

Pyridine raffinate is residual reaction mixture after extraction of pyridine, discharge as pollutants from pyridine manufacturing industries. This contains large quantities of pyridine, formaldehyde, phenolics, and picolines with high alkalinity (pH 12.0) and water solubility (Chandra and Singh, 2005). Due to its toxic nature and nauseous odor, discharge of pyridine raffinate causes irreversible damage to human health and environmental quality (Chandra et al., 2010).

Pyridine is used as a solvent in paint and rubber industries, as an intermediate in manufacturing insecticides and herbicides for agricultural purposes and in research for successful extraction of plant hormones. On the other hand, it is also employed in the alcohol denaturation process and to formulate diverse products for instance medicines, vitamins, food flavorings, dyes, adhesives and in waterproofing of fabrics (Mohan et al., 2005; Lataye et al., 2006). USEPA has documented pyridine as a hazardous substance in its list of priority pollutants (Lataye et al., 2006; Padoley et al., 2006). The toxicity of pyridine, picoline, formaldehyde and phenolics has been well documented (Sims et al., 1986). Pyridine is naturally produced from coal also and widely used as an industrial solvent and raw materials in the pharmaceutical, dyes, pesticides, herbicides manufacturer and agrochemical industries (Verschueren, 1977).

Laccases (EC 1.10.2.3); benzenediol: oxygen oxidoreductase are multicopper-containing enzymes, often extracellular in nature. They use molecular oxygen to oxidize a wide range of aromatic and non-aromatic compounds by a radical catalyzed mechanism (Thurston, 1994). Several industrial applications of laccases are pulp delignification, textile dye bleaching, effluent detoxification, biopolymer modification and bioremediation as cited in many literatures (Xu, 1999; Lu et al., 2010).

Very important environmental application for laccases is the bioremediation of contaminated soil,water as laccases are able to oxidize toxic organic pollutants, such as polycyclic aromatic hydrocarbons (Manzanares et al., 1995; Cañas et al., 2010; Kunamneni et al., 2008) and chlorophenols (Gianfreda et al., 1999; Michizoe et al., 2005; Ford

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et al., 2007).

This time, many of the industries are following chemical methods to treat such wastes

Biodegradation is the use of living organisms to remove pollutants. It may involve the use of enzyme or cell to degrade pollutants. The biological degradation of pollutants is considered as an environment friendly, feasible technique requiring low cost and minimum maintenance. It has been reported that pyridine could be used as the sole source of carbon and nitrogen during the degradation process by soil microorganisms (Shukla and Kaul, 1974; Watson and Cain, 1975; Korosteleva et al., 1981).

However the inhibitory effect of phenol for pyridine degradation is reported (Kim et al., 2006) but the bacterial degradation of pyridine raffinate is not reported so far due to high content of formaldehyde, phenol, pyridine, and picoline in mixed condition. The microbial degradation of pyridine raffinate could be a better approach for its safe disposal into the environment (Chandra et al., 2010). The objective of the present study was to isolate bacterial strain capable of degrading pyridine raffinate by using enzyme bacterial laccase.

2. Material and methods

2.1. Isolation and screening of laccase producing bacterium

Samples from the garden soil were collected and taken to laboratory for the isolation of bacteria producing laccase. The sample was serially diluted and aliquot was streaked on Media plate using sterilized wire loop for the isolation of bacteria. Media used for isolation purpose was Modified M162 along with guaiacol (1 mM) as substrate. Media was composed of(gm/l): 0.4 $CaSO_4{\cdot}2H_2O$, 2.0 $MgCl_2{\cdot}6H_2O$, 1.0 Glucose, 5.0 Ferric Citrate Solution (0.01 M), 0.5 Na_2HPO_4, 0.5 NaH_2PO_4, 0.5 NH_4NO_3, 0.5 K_2HPO_4, 3.0 Yeast extract,10 ml micronutrients containing (gm/l): 2.28 MnSO4. H2O, 0.5 ZnSO4.7H2O, 0.5H_3BO_4, 0.05 $Na_2MO_4{\cdot}2H_2O$, 0.09 $CoCl_2{\cdot}6H_2O$, 0.5(ml) H_2SO_4. Laccase producing bacteria showed brown colonies on M162 media plate.

2.2. Morphological characterization of laccase producing bacterium

To find the morphology and nature of bacteria producing laccase, Gram's staining was performed. Thin smear was prepared on clean glass slide and then heat fixed. The smear flooded with crystal violet for 1 min and rinsed with water. Few drops of the mordent (Gram's iodine solution) added to the smear and left for 1 min. After rinsing with distilled

Water, decolourizer (ethanol) was added drop by drop for 10 s. The smear then, flooded with saffranin for 30 s, rinsed with water, air-dried and observed under the microscope.

2.3. Biochemical characterization of laccase producing bacterium

The bacterial isolate was characterized based on biochemical tests using KB002 HiAssorted™ Biochemical test kit (HIMedia Laboratories Pvt. Ltd, Mumbai, India). Each kit has a standardized colorimetric identification system and consists of seven conventional tests (Citrate utilization, lysine decarboxylase, ornithine decarboxylase, urease, phenylalanine deamination, nitrate reduction H_2S production) and carbohydrate utilization (Glucose, adonitol, lactose, arabinose, and sorbitol) were tested.

A result was interpreted according to the specification provided by the manufacturer.

2.4. Screening of bacterial isolates for laccase production in liquid medium

Inoculum was prepared by using culture which grew in M162 (without Agar) then incubated at 30 °C shaking at 120 rpm for 24 h. Three inoculums sample (flask) were prepared for garden soil.

2.5. Laccase production

The enzyme was produced in 250 ml Erlenmeyer flask, containing 50 ml of Modified Production Media having composition (gm/l): 0.4 $CaSO_4{\cdot}2H_2O$, 2.0 $MgCl_2{\cdot}6H_2O$, 1.0 Glucose, 5.0 Ferric Citrate Solution 0.01 M, 0.5 Na_2HPO_4, 0.5 NaH_2PO_4, 0.5 NH_4NO_3, 0.5 K_2HPO_4, 3.0 Yeast extract, 3.0 Tryptone. To adjust the pH to 8.0, NaOH was used, prior to sterilization (121 °C, 15lbs, 15 min). The medium was inoculated with 1% inoculums and inoculated at 30 °C under shaking condition for 5 days. The culture broth was centrifuged at 10,000 × *g* for 10 min at 4 °C after incubation and the cell free supernatant was used as crude enzyme to measure laccase activity.

2.6. Laccase activity determination

The activity of the enzyme Laccase was determined with substrate guaiacol (Ride JP, 1980). Centrifugation of bacterial culture filtrate was performed at 10,000 × *g* for 10 min and incubated at 37 °C for 10 min. One unit of laccase activity was defined as the amount of enzyme that increases the absorbance by 0.148 by 470 nm per min per ml at under assay condition.

$$Laccaseactivity(U/ml) = \frac{A_{470}\ oftestsample - A_{470}\ ofblank\,(v)(df)}{V\,(volumeofguaiacol)}$$

$$V = volumeofguaiacol,\ v = totalvolumeofassay,\ df = dilutionfactor$$

2.7. Estimation of total soluble protein

Lowry Method was used for the estimation of total soluble protein using bovine serum albumin (BSA) as standard and reagents (in mg/ml): 2% solution of sodium carbonate in 0.1 N sodium hydroxide,0.5% solution of cupric sulphate in distilled water, 1% solution of sodium potassium tartarate in distilled water, Working reagent will be prepared by adding 100 ml of 1 and 1 ml each of 2,and 3 solution,1:1 Folin Ciocalteau's phenol reagent was diluted with distilled water.

2.8. Incubation of pyridine raffinate with an enzyme

After the enzyme production, pyridine raffinate was incubated with the crude enzyme for 24 h in test tube. The two part of the volume was crude enzyme and 8 parts was raffinate in 10 ml volume. Before incubation, O.D.(optical density) was taken using UV/VIS Spectrophotometer and after the period of 24 h., again O.D. was taken and the changes in the value of O.D. were recorded and reported by plotting a graph between Absorbance vs Incubation period.

Sample of pyridine raffinate was collected aseptically in plastic containers (Capacity 5 l) from M/S Jubilant OrganosysLtd, Gajraula (UP), India.

3. Results and discussion

3.1. Identification of bacteria

3.1.1. Gram staining

LCD microscope was used for the determination of the morphology of bacterial colonies and was found to be pink in color that showed the bacteria in the colony was gram-negative, small rods which appeared white to creamy colony, circular smooth, slightly moist, non-spreading, with center slightly raised and, margin flattened (Fig. 1).

3.1.2. Biochemical characterization of bacteria

KB002 HighAassorted™ test chemical kit (HiMedia laboratory Pvt. Ltd., Mumbai, India) was used to perform the biochemical tests. It is standardized colorimetric identification system utilizing seven different conventional biochemical tests and five different carbohydrate test. On

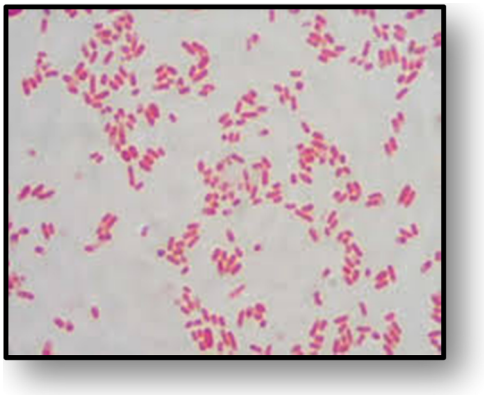

Fig. 1. Pink color shows gram's negative species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

the basis of change in pH and substrate utilization, the bacterial strain is identified. Metabolic changes undergo on incubation in organisms are identified by changes in color in the medium which was interpreted visually (Table 1).

The bacterium isolated was identified by Biochemical test kit by comparing the above data with the given Identification Index provided with the kit as ***Pseudomonas fluorescens***

3.2. Laccase activity determinations

For the quantitative screening of laccase, centrifugation of the culture broth was performed at 10,000 × *g* for 10 min and crude enzyme in cell free supernatant was used to measure laccase activity. Guaiacol was used as a substrate while determining the laccase activity. Determination of laccase activity in supernatant of bacterial culture was performed in 25 mM and tris buffer, containing 7 mM guaiacol as substrate and incubated at 37 °C for 10, 20 and 30 min with different concentrations. The results of laccase activity are shown in the table given below (Fig. 2 and Table 2).

3.3. Protein estimation

For the estimation of total soluble protein Lowry method was performed using BSA (Bovine Serum Albumin) as a standard and absorbance was measured at 660 nm (Table 3)

BSA Standard – 1 mg/ml

The calculation according to the above data gives that garden soil has concentration of 82.99 mg/ml (Fig. 3).

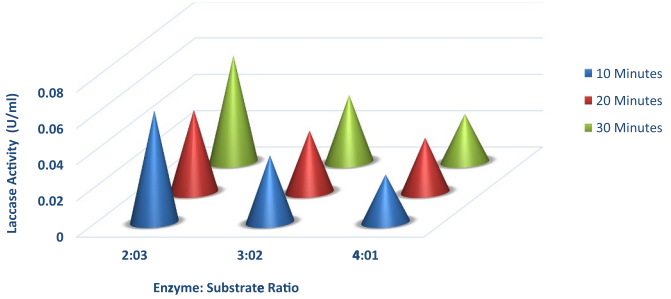

Fig. 2. Figure representing garden soil laccase activity.

Table 2
Laccase activity determination.

Sr No.	Ratio	Incubation period		
	Enzyme: substrate	10 min Garden soil (U/ml)	20 min Garden soil (U/ml)	30 min Garden soil (U/ml)
1	2:3	0.06163	0.04544	0.05875
2	3:2	0.03692	0.03397	0.037
3	4:1	0.02638	0.03005	0.02652

Table 3
Protein concentration determination.

Ratio (Water: Protein)	Concentration of protein (mg/ml)	Absorbance (nm)
10:0	0	0.8919
8:2	20	0.9032
6:4	40	1.0452
4:6	60	1.0585
2:8	80	2.0887
0:10	100	2.3876
Garden Soil Sample	82.9944	1.6408

3.4. Specific activity measurement

Specific activity is a way to measure how much of measured protein there is with all of other contaminating protein it can be calculated by dividing Laccase activity (U/ml) by Protein (mg/ml) and it is expressed as U/mg

Mathematically,

$$SpecificActivity = \frac{laccaseactivity(U/ml)}{TotalProteinConcentration(mg/ml)}$$

Specific activity of enzyme in different ratio of enzyme: substrate is shown in table given below and the maximum specific activity was observed after 10 minutes of incubation with ratio 2:3 i.e. 0.00074 (Table 4).

Table 1
Different biochemical tests are shown in the following table.

Sr. No	Test	Original color of medium	Positive test	Negative test	Test result
1	Citrate Utilization	Green	Blue	Green	Positive
2	Lysine decarboxylase	Olive Green to light purple	Purple/Dark purple	Yellow	Negative
3	Ornithine Decarboxylase	Olive Green to light purple	Purple/Dark purple	Yellow	Negative
4	Urease	Orangish Yellow	Pink	Orangish Yellow	Positive (11–89%)
5	Phenylalanine Deamination	Colorless	Green	Colorless	Negative
6	Nitrate Reduction	Colorless	Pinkish Red	Colorless	Positive (11–89%)
7	H_2S Production	Orangish Yellow	Black	Orangish Yellow	Negative
8	Glucose	Pinkish Red/Red	Yellow	Red/Pink	Positive
9	Adonitol	Pinkish Red/Red	Yellow	Red/Pink	No Data
10	Lactose	Pinkish Red/Red	Yellow	Red/Pink	Positive (11–89%)
11	Arabinose	Pinkish Red/Red	Yellow	Red/Pink	Positive (11–89%)
12	Sorbitol	Pinkish Red/Red	Yellow	Red/Pink	Negative

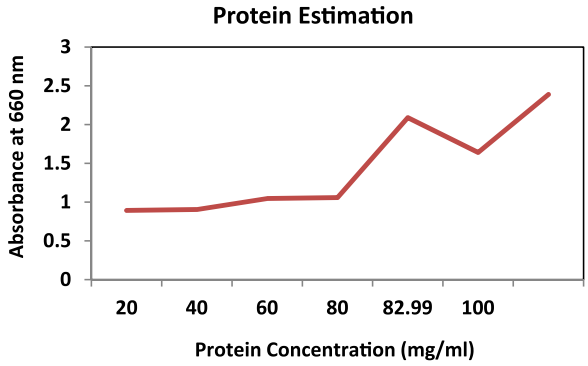

Fig. 3. Graphical representation of protein estimation by Lowry Method.

Table 4
Table showing specific activity of laccase.

Sr No.	Ratio	Incubation period		
	Enzyme: substrate	10 min Garden soil (U/mg)	20 min Garden soil (U/mg)	30 min Garden soil (U/mg)
1	2:3	0.00074	0.00055	0.00071
2	3:2	0.00044	0.00041	0.00045
3	4:1	0.00032	0.000362	0.00033

Table 5
Measurement of O.D. to analyse reduction in COD.

Wavelength (nm)	O.D of raffinate reference	O.D. of pyridine raffinate with laccase enzyme
501	1.287025	1.054315
502	1.281111	1.048929
503	1.280876	1.049645
504	1.277296	1.047034
505	1.268864	1.041887
506	1.275283	1.049079
507	1.258006	1.034760
508	1.267874	1.045081
509	1.262077	1.040959
510	1.254347	1.034775
511	1.258372	1.040399
512	1.243096	1.025991
513	1.245517	1.300235
514	1.246342	1.033092
515	1.238267	1.026950

(Absorbance of pyridine raffinate at Different Wavelenghts with Garden Soil Enzyme).

3.5. Treatment of pyridine raffinate with laccase enzyme isolated from garden soil sample

The pyridine raffinate was incubated with crude laccase enzyme to monitor the degradation of organic components present in pyridine raffinate.

O.D. was measured to check the result of COD reduction. Before incubation, the measured O.D. at 470 nm was 1.926 and after 24 h of incubation it was decreased to 0.801 (Table 5).

Conflict of interest

The authors declare that there is no conflict of interest.

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