Global J. Environ. Sci. Manage. 5(2): 203-212, Spring 2019

Global Journal of Environmental Science and Management
(GJESM)

Homepage: https://www.gjesm.net/

ORIGINAL RESEARCH PAPER

Bioprospecting and molecular characterization of laccase producing bacteriafrom industrial contaminated sites

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ARTICLE INFO

Article History:
Received 05 November 2018
Revised 14 January 2019
Accepted 15 February 2019

Keywords:
2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS)
Bushnell Haans medium (BHM)
Guaiacol
Laccase
Ribosomal Deoxy ribonucleic acid (rDNA)

ABSTRACT

Laccases have vast prospective for biotechnological applications due to their outstanding bioremediation potential. These include abundant applications in effluent detoxification, enzymatic conversion of chemical intermediates, wine clarification degradation of textile dyes etc. In the present study, two potential microbes were isolated on solid medium containing guaiacol and ABTS for laccase activity out of 10 microbes. Two cultures PHP7 and PKD5 were selected for molecular characterization was carried out using 16S rRNA gene technology of PHP7 revealed as *Bacillus cereus* (KU878970.1).Partial amplification of laccase gene contain conserved domain of multicopper oxidase family. The biomass produced by PHP7 was 0.053 mg/5 mL, while PKD5 was 0.058 mg/5 mL. While dye degradation of PHP7dye of 64.28% after incubation of 6 days at pH7 whereas PKD5 shows highest degradation of dye i.e. 61.90% after incubation of 8 days at pH8. PHP7 showed highest Laccase activity of 0.489 U/L at pH 7 while PKD5 showed 0.404 U/L Laccase activity at pH 8 at 8th day of incubation. Using laccase from PHP7 and PKD5 isolates, explored at industrial level for decolorization of coloured effluents that significance in environmentally friendly and play critical role as bioremediation at commercial scale.

DOI: 10.22034/gjesm.2019.02.06

INTRODUCTION

Microorganisms are the chief source of metabolic and genetic diversity on the Planet. Studying on microbial diversity, one can better know how the diversity in ecosystem regulate (Grant, *et al.,* 1990). Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2), are multicopper and lignin modifying enzyme (LME) which is leading source from microbes, fungi, plants and insects (Bourbonnais *et al.,* 1995; Palmieri *et al.,* 2000, Claus, 2004). Literature review reported that laccases are extensively dispersed among the bacteria. For example, laccases from *Azospirillum lipoferum* (Diamantidis, *et al.,* 2000), *Marinomonas mediterranea* (Solano, *et al.,* 1997), *Streptomyces griseus*(Endo, *et al.,* 2003), *E. coli* (Kim, *et al.,* 2001), *Bacillus subtilis* (Muthukumarasamy, *et al.,* 2015) and many more bacteria have been identified and characterizedby Shrestha, *et al.*(2016). In laccases, molecular oxygen work as an electron acceptor to catalyze the oxidation

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Note: Discussion period for this manuscript open until July 1, 2019 on GJESM website at the "Show Article.

of aarray of substrates (Revanker and Lele, 2006). Due to laccase activity on mixture of substrates, numerous diverse compounds used as indicators for conformation of laccase production. Some indicators used are Guaiacol, Tannic acid, 2, 2-azino-bis (3- ethylbenzthiazoline-6-sulphonic acid (ABTS), Syringaldazine and polymeric dyes like remazol brilliant blue-R (RBB-R) (Coll *et al*., 1993; Anna, *et al*., 2002; Kiiskinen *et al*., 2004; Cristina *et al*., 2004; Jiejie *et al.,* 2007; Buddolla *et al*., 2008; Teck *et al*., 2010; Chun *et al.,* 2010).Screening numerous microbial diversity isessential to select potent strains that are able to produce laccase enzyme with narrative characteristics (Monteiro and Carvalho, 1998). Laccases producing bacteria have been screened by visualizing laccase activity on a solid state fermentation of especially fungi (Nishida *et al*., 1988; De Jong *et al*., 1992) or with liquid cultivation examined with enzyme activity measurements. Laccases are recognized for their wide spread applications including ethanol production, food processing, dye bleaching, paper and pulp processing, and production of value added chemicals from lignin (Giardina *et al.* 2010; Shrestha *et al.,* 2016**)**. Literature review reported numerous industrial applications of laccases as textile dye bleaching, bioremediation, effluent detoxification and biopolymer modification (Xu, 1999; Lu *et al.,* 2010; Aravind *et al.,* 2016). The enzyme concerned in linking monomers, polymer degradation and also break down of aromatic compounds.It has received a l9ot of scientific awareness over last couple of decade for their proficiency to oxidize phenolic and non-phenolic lignin linked compounds as well as highly recalcitrant environmental pollutants treatment (Brenna and Bianchi, 1994; Roy *et al.,* 2005). Rapid industrialization and widespread use of pesticides have become a grave environmental trouble that led to soil pollution and effect agricultural productivity to major extent. Synthetic dye containing effluents causes unresponsive impact to the surrounding environment and also poisonous and hazadous to biological life (Lee *et al*., 1999). In this regard, the objectives of the present work were to isolate and molecular characterized laccase producing bacteria that could be use as bioremediation for industrial dye effluent treatment. This study has been carried out at School of Sciences, P.P. Savani University, Surat, India in 2017.

MATERIAL AND METHODS

Collection and screening of samples

The samples was collected from four different industrial chemical dye contaminated sites (Sachin G.I.D.C.(21°09’15.57’’, 72°85’73.31’’), Pandesara Khadi (21°15’93.20’’, 72°83’11.20’’), Industrial discharge from Kadodara (21°15’73.00’’, 72°95’62.00’’) and Industrial discharge from Haripura), (21°21’62.10’’, 72°83’07.20’’) with latitude and longitude respectively in surat, Gujarat (Fig. 1). Collected samples contain the mixture of polluted water and soil with soil sediments. The samples were collected in sterile plastic bags, were

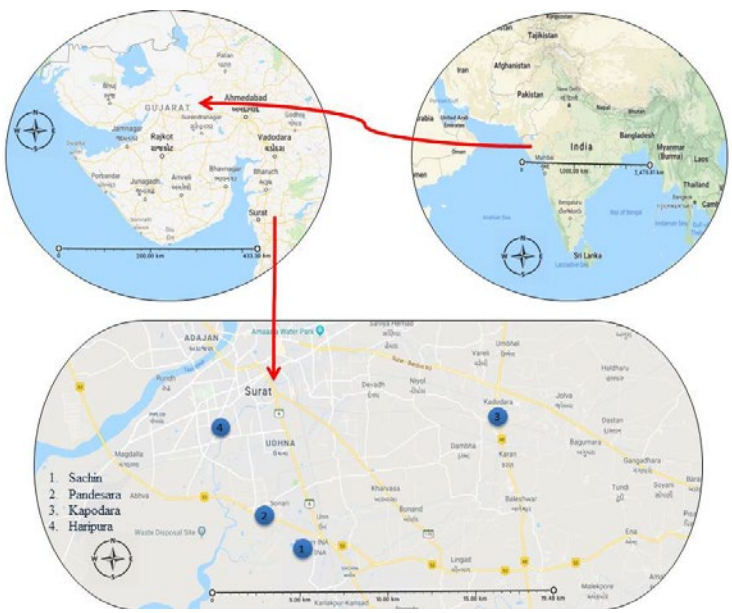


Fig. 1. Geographical location of the study area along with the sample collection sites

sealed and brought to the lab aseptically for further studies (Vantamuri and Kaliwal, 2015).

Enrichment and Isolation of bacteria

The 5 g of soil sample was inoculated individually in 500 mL Erlenmeyer flask containing 100 mL Bushnell Haans medium (BHM) (3.72 g/L) addition with wheat stalk powder (5 g/L) as the sole carbon source for enrichment purpose. The medium was incubated at 37°C at 120 rpm on rotary shaker for 7 days. Laccase producing microbes were isolated on LB agar plate incubated at 37°C for 24 h. Distinct bacterial colonies were isolated and frequently subculture until pure cultures was obtained. All the pure cultures were store on LB agar slant at 4 °C.

Qualitative screening for laccase activity

Primary screening of the isolates for laccase production was prepared on different dye containing agar plates. Toludine Blue, Methylene Blue, Congo Red, Malachite Green dye selected for screening potent microbes (Chun *et al.,* 2010). LB agar supplemented with different dye at specific concentration (25 mg/L) with substrate as a 4.0mM guaiacol and 1.0 mM ABTS (2, 2´- Azino-bis-[3-ethylbenzthiazoline-6-sulphonicacid]). All cultures were incubated on LB media containing dye. Laccase secretion was monitored by appearing clear zone surrounding the colonies. Laccase activity was specififed by the diameter of haloes on agar plate.

16s rDNA identification and laccase gene amplification of potential isolates

The isolated colonies were further taken for genomic DNA extraction for amplification of 16S rDNA gene (Ahmed *et al.,* 2007; Patel *et. al.,* 2017) by suspending in TE buffer in a micro-centrifuge tube. These cells were heated for 10 minutes at 94^{O}C and were centrifuged at 6,000 rpm for 5 minutes. The upper layerwas used as template DNA for the amplification of 16S rDNA gene and partial laccase gene amplification were carried out by ABI Veriti PCR Machine (Applied Biosystems, USA) using improved PCR Program (Sambrook *et al.*, 2009; Safary *et. al*. 2016; Patel *et. al.,* 2017).

In silico characterization of laccase gene

The obtain nucleotide sequences of gene were translated into amino acid and further check for conserved domain using EXPASY tool.

Production of laccase enzymes

Optimizationand Biomass production atpH and temperature level

The optimum pH value for Laccase production varies depending on the substrates employed, even though numerous literature have been reported a bell shaped profile as normal distribution curve for laccase activity. Four 100 mL Erlenmeyer flask containing 50 mL Laccase Production Medium were prepared. Then, some flasks were marked with different pH like 5, 6, 7, and 8 while some flask were place at different temperature RT, 30, 40 and 50°C. Different pH was set by pH meter using 1M NaOH and 1M HCl according to the marking on the flask. 25 mg/L toludinie blue dye was added, flasks were inoculated by 1 mL of microbial culture of 24 h having OD of 0.6. and then flasks were incubated for 9-10 days at 125 rpm. (Sivakumar *et al.,* 2010; Bhuvaneshwari *et al.,* 2015).

Dye degradation at different pH and temperature

The toluidine blue dye, were prepared individually with the concentration of 25 mg/L in sterilized distilled water as blank and for test the medium was prepared in LB broth along with 1 mL of culture. The culture filtrate with laccase activity was used as a source of enzymes to test its efficiency degradation of effluents and dyes. The percentage of degradation achieved was calculated with reference to the control samples that were not treated with enzyme for 8 days at an interval of 48 h at 450 nm. (Chun, *et al.,* 2010).

RESULTS AND DISCUSSION

Recently, research on pollution research has offered a simple and cost efficient ways of handling synthetic dye containing effluents. Dye decolourization using bioenzymes like laccases has been suggested as an eco-friendly and Bioremediation alternative for treatment of industrial synthetic dyes (Robinson *et al*., 2001; Gnanasalomi and Gnanadoss, 2013). However, yield of laccases produced for industrial and commercial purposes are insufficient to meet the market demand. Screening of potent laccase producing microbes that are competent and secrete elevated yields of laccase are required. In the present study, twoPotential microbes (PHP7 and PKD5) have been isolated from different coastal region of south Gujarat that could be used as bioremediation for synthetic dye degradation.

Collection and Screening of samples

Four samples from each sites were collected in sterilized plastic bag from different south Gujarat region (Fig. 1). Collected sample were immediately transferred to the laboratory for the avoiding contamination (Table 1).

Enrichment and Isolation of bacteria

Five gram (g) each of all the 4 samples collected from different collection sites were inoculatedin to Bushnell Haans medium (BHM) supplemented with wheat stalk powder separately asthe solitary carbon source for fortification of desired microbes. These enriched cultures wereused to isolate microbes having high laccase activity. After enrichment, microbes were isolated using spread plate technique (Abdulkarim,*et al.,* 2005). Colonies showing distinct morphological features were selected for further qualitative screening. Bacteria were the majorsource isolated from the different samples, followed by fungi and actinomycetes. For easy cultivation and maintenance, the bacteria were selected for further examination. Ten different colonies were selected on the basis of its morphological characteristicsie PHP7, PHP6, PHP8, PSG3, PSG9, PKD2, PKD5, PPS4, PPS1 and PPS10. The isolated bacteria were further purified and streaked on LB agar slant and preserved at 4°C in refrigerator.

Qualitative screening for laccase activity

In primary screening, Laccase producing bacteria were screened on the different dye containing agar plate (Bandounas *et al.,* 2011). Out of all the plates the microbes PHP7 and PKD5 were showing clear zone in toluidine blue, methylene blue and congo red, while the microbes PHP6, PSG3, PSG9, PPS1 and PPS10 were showing clear zone in only two, toulidine blue and methylene blue. PHP8 and PPS4 were showing clear zone in only toluidine blue and PKD9 shows clear zone in only toluidine blue.

None microbes showed clear zone in malachite green. Out of the different microbes, two potential microbes (PHP7 and PKD5) show greater zone of clearance on Toluidine blue (Table 2). It indicated that PHP7 and PKD5 may capable to produce higher laccase activity (Fig. 2).

16s rDNA identification and laccase gene amplification of potential isolates

Further sequencing of PHP7 isolates identified as *Bacillus cereus*. Original sequence is submitted to NCBI with accession no KU878970.1. KU878970.1 showed 99 % similarity with KP729612.1 (*Bacillus cereus* strain FT1 16S ribosomal RNA gene, complete sequence). A common band of ~1.5 kb laccase gene was amplified from PHP7 and PKD5 bacterial DNA (Fig. 3). Further, amplified fragment from PHP7 isolates was subjected to sequencing. A partial sequencing fragments of 484 bp was amplified and submitted to NCBI with accession no. KX215765.1 (Bacillus sp. PHPBMCST laccase-like (cotA) gene, partial sequence). KX215765.1 showed 94% similarity with CP026673.1 (*Bacillus licheniformis* strain 14ADL4 chromosome, complete genome). Nicolotti *et al.,* (2009) acknowledged decayed wood fungal samples by amplified conserved ITS region using primers pair of ITS1 and ITS4.

Table. 1 Characteristics of sample and collection site

Sr. No.	Site	Temp.	pH	Turbidity	Weather
1	Haripura	28	9	High	Cloudy
2	SachinG.I.DC	30	9	High	Cloudy
3	Kadodara	28	8.5	High	Cloudy
4	Pandesara	29	9	High	Sunny

Table 2. Zone of clearance (mm) by PHP7 and PKD5 on different dye

Sr. No.	Toluidine blue plate		Methylene blue plate		Congo red plate		Malachite green plate	
	Colony diameter	Clear zone	Colony diameter	Clear zone	Colony diameter	Clear zone	Colony diameter	Clear zone
1	14	20	13	17	14	16	14	ND
2	13	17	12	15	12	13	13	ND

*ND = Not detected

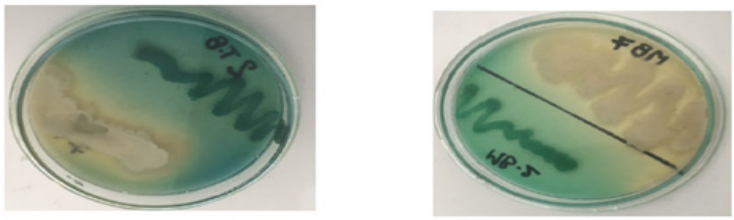


Fig. 2. PHP7 and PKD5 isolates showing dye degradation on Toulidine blue and Methylene blue on agar plate

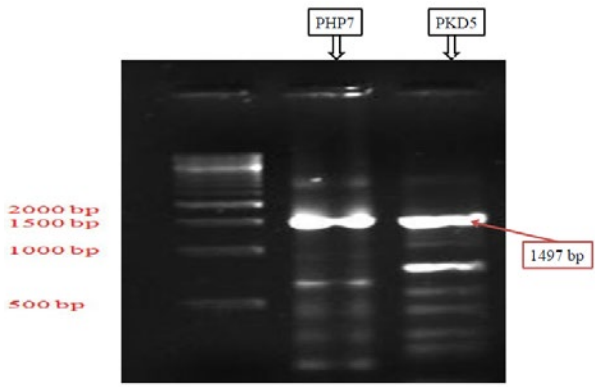


Fig. 3. Laccase gene amplification from PHP7 and PKD5 isolates DNA

Sequence analysis for conserved domain

The deduced amino acid sequence of PHP7 isolates laccase gene were analyzed for conserved domain. Different predicted ORF through NCBI ORF finder were further check for conserved domain in smart tool. The alignment results showed that the presence of PEKYRFRILNASNTRIFELYSDHDS conserved domain. It was observed that the sequence were showing conservation of histidine (H) residue at the end of the conserved domain. Similar results were also observed by (Hollo *et al.,* 2001).

The results confirmed that the sequence of laccase gene belong to the multicopperoxidase family genes. Further when the laccase amino acid sequence were analysed with ORF-BLAST, they showed the presence of Cot-A conserved domain of cupredoxin superfamilies. PHP7 showed CuRo_2 _CotA_like domain at 47-71 amino acid sequence. *In silico* conformation of laccase gene reveal the information of functioning laccase activity.

Application in dye degradation and laccase production

Laccase production could be used as dye degradation for toluidine blue dye. Toluidine blue in lignin act like indicator dye which also used fordetermination of lignolytic activity of laccase enzyme. Utilizationof this dye in the medium indicates the production of ligninolytic enzymes like, laccase. Toluidine blue complemented with LB media was used for screening method (Bandounas*etal.,* 2011).

Biomass at different pH by isolates PHP7 and PKD5

Determination of biomass indicated the growth rate of microbes. Higher the biomass,higher is the growth

rate of isolates. Efficient biomass was determined at differentpH at room temperature on shaking condition. The biomass produced by PHP7 was 0.053 mg/5 mL, while the biomass produced by PKD5 was 0.058 mg/5 mL (Table 3). PKD5 and PHP7 growth rate were higher at pH 8, pH 7 respectively at 8 days of incubation.

Dye degradation at different pH by isolates by PHP7 and PKD5

PHP7 isolate showed highest degradation of dye of 64.28% after incubation of 6 days at pH7 while from PKD5 isolate shows highest degradation of dye i.e. 61.90% after incubation of 8 days at pH8 (Table 4).

Laccase enzyme production at different pH by isolate PHP7 and PKD5

Isolate PHP7 showed highest Laccase activity of 0.489 U/L at pH 7 while PKD5 showed 0.404 U/L Laccase activity at pH 8 at 8th day of incubation, respectively in Table 5 and Fig. 4.

Biomass at different temperature by isolates PHP7 and PKD5

Growth rateof Isolate PHP7 was higher at room temperature (28-30°C approx) at 8 days of incubation. The biomass produced by PHP7 was 0.062/5 mL. While, isolate PKD5 growth rate was higher at room temperature (28-30°C approx) at 6 days of incubation. The biomass produced by PKD5 was 0.059/5 mL (Table 6).

Dye degradation at different temperature by isolates by PHP7 and PKD

PHP7 isolate showed highest decolourization of dye was 73.81% after incubation of 8 days at RT while the isolate of PKD5 isolate shows highest

Table 3. Biomass of PHP7 and PKD5 at different pH

		Biomass of PHP7 at different pH				Biomass of PKD5 at different pH			
Sr. No.	Time	pH5	pH6	pH7	pH8	pH5	pH6	pH7	pH8
1	Day2	0.006	0.012	0.023	0.016	0.004	0.014	0.023	0.024
2	Day4	0.014	0.021	0.036	0.024	0.009	0.021	0.033	0.043
3	Day6	0.019	0.029	0.049	0.032	0.016	0.027	0.049	0.056
4	Day8	0.022	0.034	0.053	0.050	0.019	0.031	0.052	0.058

Table 4.Dye degradation of PHP7 and PKD5 at different pH

		Biomass of PHP7 at different pH				Biomass of PKD5 at different pH			
Sr. No.	Time	pH5	pH6	pH7	pH8	pH5	pH6	pH7	pH8
1	Day2	2.38	7.14	21.42	2.38	2.38	7.14	11.9	23.8
2	Day 4	14.28	19.04	42.85	21.42	0.39	16.66	35.71	42.85
3	Day 6	21.42	33.33	64.28	33.33	0.37	42.85	47.61	59.52
4	Day 8	21.42	35.71	64.28	42.85	0.36	40.47	54.76	61.9

Table 5. Laccase enzyme activity of PHP7 U/L and PKD5 U/L at different pH

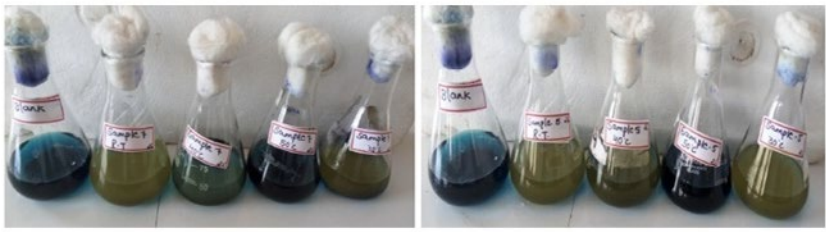
Sr. No.	pH	Laccase enzyme activity of PHP7 U/L	Laccase enzyme activity of PKD5 U/L
1	5	0.112	0.091
2	6	0.292	0.282
3	7	0.489	0.386
4	8	0.386	0.404
5	9	0.244	0.328

Table 6. Biomass of PHP7 and PKD5 at different temperature

		Biomass of PHP7 at different temperature				Biomass of PKD5 at different temperature			
Sr. No.	Time	RT	30°C	40°C	50°C	RT	30°C	40°C	50°C
1	Day2	0.026	0.023	0.015	0	0.021	0.022	0.003	0
2	Day4	0.039	0.032	0.028	0	0.04	0.039	0.01	0
3	Day6	0.052	0.048	0.028	0	0.059	0.056	0.016	0
4	Day8	0.062	0.056	0.031	0	0.059	0.031	0.021	0



(A) Dye decolourization at different pH by PHP7 (B) Dye decolourization at different pH by PKD5



(A). Dye decolourization at different temperature by PHP7 (B). Dye decolourization at different temperature by PKD5

Fig. 4. Dye decolourization at different pH and Temperature by PKD5 and PHP7 isolates

Table 7. Dye degradation of PHP7 and PKD5 at different temperature

		OD at 450nm dye degradationPHP7				OD at 450nm dye degradationPKD5			
Sr. No.	Time	RT	30°C	40°C	50°C	RT	30°C	40°C	50°C
1	Day2	26.19	7.142	7.142	0	23.8	16.66	11.9	0
2	Day 4	52.38	40.47	14.28	0	47.61	40.47	19.04	0
3	Day 6	71.42	54.76	30.95	2.38	66.66	54.76	26.19	0
4	Day 8	73.8	71.42	40.47	2.38	71.42	64.28	0.27	0

Table 8. Laccase enzyme activity of PHP7 U/L and PKD5 U/L at different temperature

Sr. No.	Temp.	Laccase enzyme activity of PHP7 U/L	Laccase enzyme activity of PKD5 U/L
1	RT	0.467	0.414
2	30°C	0.417	0.401
3	40°C	0.289	0.214
4	50°C	0	0

decolourization of dye was 71.42% after incubation of 8 days at RT (Table 7).

Laccase enzyme production at different temperature by isolate PHP7 and PKD5

Bacterial isolate PHP7 showed highest Laccase activity of 0.467 U/L and PKD5 showed0.414 U/L Laccase activity at room temperature (28-30°C approx.) at 8th day of incubation (Table. 8) and (Fig. 4). The influences of optimum temperature of laccase differ greatly from one isolates to another. The optimum temperature range for fungi is reported between 25 to 30°C (Shraddha *et al.*, 2011). Previously literature reported that optimum temperature for *Streptomyces psammoticus* was 33°C.

Results also specified that, increasing the incubation temperature, a steady decrease in laccase production and enzyme activity was observed at 45°C while no laccase production was observed at 50°C. (Lu *et al.,* 2009) found that Laccase activity was maximum when the medium was incubated at 30°C. (Niladevi *et al.,* 2009) and enzyme yield were little when the temperature was in its elevated level i.e. 37°C which concluded that the growth and physiology of this culture arecorrelated with surrounding temperature.

CONCLUSION

From the present study, Total two potential microbes capable of producing laccase enzymes. PHP7 and PKD5 showed maximum dye degradation in primary screening. Further, biochemical and molecular characterization revel that organism belong to *Bacillus cereus* (KP729612.1). Partial amplification and *in silico* characterization of laccase gene revel the presence of conserved domain of multicopper oxidase family. PHP7 and PKD5 isolates were also optimized for different pH and temperature for dye decolourization. Using laccase from PHP7 and PKD5 isolates, explored at industrial level for decolorization of coloured effluents that significance in environmentally friendly and play critical role as bioremediation at commercial scale.

ACKNOWLEDGMENT

The financial support by the Bhagwan Mahavir College of Science and technology, Veer Naramad South Gujarat University, Surat is gratefully acknowledged.Gratitude to the P. P. Savani University and Navsari Agricultural University for providing the necessary logistics and support in performing the research objectives.

CONFLICT OF INTEREST

The author declares that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been completely observed by the authors.

ABBREVIATIONS

ABTS	2, 2-azino-bis (3- ethylbenzthiazoline-6-sulphonic acid
ABI	Applied Biosystems
bp	Basepair
BHM	Bushnell Haans medium
°C	Celsius
g	grams
g/L	Gram per litre
h	Hours
ITS	internal transcribed spacer
LB	Luria Broth
nm	namometre
NCBI	National Center for Biotechnology information
M	Molar
mg/L	Milligram per litre
mL	millilitre
mM	milimolar
mm	milimitre
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
pH	potential of hydrogen
RBB-R	remazolbrilliant blue-R
rDNA	*Ribosomal Deoxyribonucleic acid*
rpm	Rotation per minute
RT	Room tempreture
TE	Tris Ethylene diamine tetraacetic acid
USA	United state of America
U/L	Unit per litre
PHP7, PHP6, PHP8, PSG3, PSG9, PKD2, PKD5, PPS4 PPS1 and *PPS10*	Abbrevation given on basis of different location and place

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HOW TO CITE THIS ARTICLE

TPatel, H.K.; Sharma, P.J.; Kalaria, R.K., (2019). Bioprospecting and molecular characterization of laccase producing bacteriafrom industrial contaminated sites. Global J. Environ. Sci. Manage., 5(2): 203-212.

DOI: 10.22034/gjesm.2019.02.06

url: https://www.gjesm.net/article_34359.html