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Demonstration of a highly thermotolerant novel Laccase from *Enterobacter cloaceae, KSB$_4$*

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Abstract

Microorganisms that produce unspecific oxidative enzymes such as laccases are a potential means to improve biodegradation of toxic compounds. Bacterial laccases are of significance because of their high stability and thereby possible extensive applications. Bacterial laccases degrade low molecular weight portions of lignin polymer and thus converts lignin to intermediate metabolites. Thermostable laccases have a wide range of biotechnological applications in paper and pulp industry, textile industries, petrochemical industries, food cosmetics, soil bioremediation and biodegration of environmental phenolic pollutants. A highly termostable laccase producing strain of *Enterobacter cloaceae* was isolated from forest soil of Kerala by plate assay technique and the stability of laccase was confirmed by different physical and chemical treatments. This is the first report of laccase from *E. cloacae* and this research work is also unique in reporting a highly thermostable laccase from bacterial origin.

Keywords: Bacterial laccase, bioremediation, guaiacol, thermostable laccase

Introduction

Laccases (EC 1.10.3.2) are polyphenol oxidases that catalyse the oxidation of various aromatic compounds, particularly those with electron-donating groups such as phenols (−OH) and anilines (−NH_2), by using molecular oxygen as an electron acceptor (Gianfreda et al. 1999). Laccase enzymes are widespread among plants, fungi and bacteria, and have various biological functions, such as degradation of complex polymers (lignin, humic acid), lignification, detoxification, pathogenicity, morphogenesis, sporulation, polymerization of melanin and spore coat resistance (Strong & Claus, 2011). Laccases have mostly been isolated and characterized from plants and fungi in contrast, little is known about bacterial laccases. In order to facilitate novel and more efficient bio-catalytic process applications, there is a need for laccases with improved biochemical properties, such as thermostability and thermo-tolerance. Thermostable laccases are important in industries, especially due to its extensive biotechnological applications.

Most bacterial laccases studied so far are located intracellularly, which is a disadvantage for micropollutant

degradation (Sharma et al. 2007). However, some strains of *Streptomyces spp.* produce extracellular laccases, such as *S. psammoticus MTCC 7334* (Niladevi et al. 2008a), *S. cyaneus CECT 3335* (Arias et al. 2003), *S. ipomoea CECT 3341* (Molina-Guijarro et al. 2009) or *S. griseus NBRC 13350* (Endo et al. 2002). Moreover, laccases from *S. psammoticus* and *S. ipomoea* showed unusually high activity at the slightly alkaline pH values (7–8) found in wastewater, as well as tolerance to high NaCl (> 1 M) concentrations (Molina-Guijarro et al. 2009; Niladevi et al. 2008a). High laccase activity was also observed in the culture supernatant of *S. psammoticus* and *S. cyaneus* (Arias et al. 2003; Niladevi et al. 2009), suggesting suitability of these strains for bioremediation applications. Due to their wide range of substrates and the sole requirement of oxygen as the co-substrate, laccases appear to be a promising biocatalyst to enhance the biodegradation of micropollutants in wastewater in a complementary treatment step (Jonas et al. 2013). Very little is known about the potential of bacterial laccases for bioremediation applications. Wastewater treatment involving bacteria is, however, considered to be more stable, as bacteria generally tolerate a broader range of habitats and grow faster than fungi (Harms et al. 2011). Moreover, in contrast to fungal laccases, some bacterial laccases can be highly active and much more stable at high temperatures, at high pH as well as at high chloride concentrations (Bugg et al. 2011; Dwivedi et al. 2011; Reiss et al. 2011; Sharma et al. 2007).

Materials and methods

Screening of Potent Laccase Producing Organisms

Soil samples were collected from Kallar forest area of Kerala and screened for laccase activity. The serially diluted soil samples were plated on Vogel's Mineral Media (VMM) agar plates (pH 5.6) containing 0.02% guaiacol (Di- methoxy phenol, DMP) (Sigma) as substrate (Vogel, 1956; Pedro et al. 1993). The positive strain which produced reddish brown zone was isolated, purified. Modified Plate Assay was conducted using an increased concentration of guaiacol (0.04%) to demonstrate the tolerance of phenolic compound by the laccase producing strain.

Laccase production was confirmed in the production media, VMM broth, pH 5.6. The *Enterobacter cloaceae KSB$_4$* bacterial culture was incubated at 37°C. The samples were collected in every 24 h and centrifuged at 10,000 rpm for 15 min. The supernatant was taken for enzyme assay. The enzyme assay was conducted using 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Biogene, USA) as substrate.

Laccase Assay

The assay mixture contained 2mM ABTS in 0.1M sodium citrate buffer, pH 3.0. Oxidation of ABTS was monitored by determining the absorbance increase at 420 nm ($\varepsilon = 3.6x10^4\ M^{-1}cm^{-1}$) (Jia Li Dong, 2004). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per min. Assay was conducted at regular intervals. The laccase production rate and the optimum day of enzyme production were noted for the *E. cloaceae KSB$_4$* strain.

Batch Production of Laccase

Batch production of laccase using *Enterobacter cloacae KSB$_4$* was carried out in VMM production media. 1% inoculum *Enterobacter cloacae KSB$_4$* was prepared in VMM broth by transferring the bacterial strain from Nutrient agar slant to

VMM broth and incubating for 24 h at 120 rpm, 37°C. 1% bacterial culture with 0.7 Abs at 600nm was used for inoculating the bacterial production media. Samples from each batch culture was collected every 24 h, centrifuged at 10,000 rpm for 15 min and ABTS assay of the supernatant was carried out.

Substrate Oxidation Studies by Laccase

The substrate tolerance was studied by incorporating 0.02% *p*-cresol, *p*-aminophenol, *p*-phenylene diamine, hydroquinone and tropolone in VMM agar plates. The organism was inoculated and incubated for 48 h at 37°C. The growth and oxidation pattern were noted.

Stability studies of Laccase from *E. cloacae KSB$_4$*

Some physical treatments were employed to study the unique characteristics of isolated laccase. The supernatant was given the following treatments: The physical treatments employed were i)The enzyme sample heated to 80°C in a water bath for 60 minutes ii) The enzyme boiled to 100°C for 5 minutes and iii) the sample heated to 121°C, at 15 lbs pressure for 15 minutes.

Some chemical treatments were also carried out to prove the unnatural characteristics of the enzyme sample. The treatments employed were i) the sample was incubated with trypsin, 10X (PAA Laboratories, Germany) in the ratio 1:1 for 1 hour at 37°C, ii) the sample was incubated with SDS in the ratio 1:1 for 1 hour at 37°C, iii) TCA was added to the sample and incubated for 1 h and 15 h at 37°C, iv) Proteinase K (20mg/ml) was added in the ratio 1:1 and incubated at 37°C for 1 h, v) 0.1N HCl was added so as to create an extreme acidic environment (pH 2.85), vi) 0.1N NaOH was added so as to create an extreme alkaline environment (pH 10), vii) The sample was treated with ethanol in the ratio 1:1.

A combination of physical and chemical treatment was carried out to study the stability of the enzyme. In this study the TCA treated sample was heated to 80ºC for 15 minutes.

Results and discussion

Screening of organisms by plate assay using guaiacol

Soil samples were plated on to guaiacol containing medium. Laccase producing organisms in the soil sample were screened by the oxidative polymerization of guaiacol to bisphenoquinone which was visualized as reddish brown zones on VMM agar plates (Figure.1)

Figure 1: Plate Assay of Laccase (0.02% guaiacol).

The tolerance of the DMP tolerant laccase producing organism was studied using the 0.04% guaiacol supplemented plates. The organism could grow in the increased concentration of guaiacol and oxidize the substrate (Figure.2).

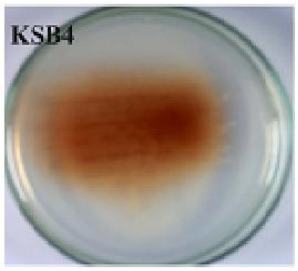

Figure 2: 0.04% DMP tolerance by *E. cloacae KSB$_4$*.

Laccase production pattern by *E. cloacae KSB$_4$*

The organism was inoculated into VMM broth for laccase production and ABTS assay was conducted every 24 h to find the optimum day of enzyme production. The quantity of enzyme produced by the strain, enzyme production pattern and the optimum day of enzyme production by the organism was studied (Figure 3). The organism produced 8.3 U/ml on 5 days of incubation.

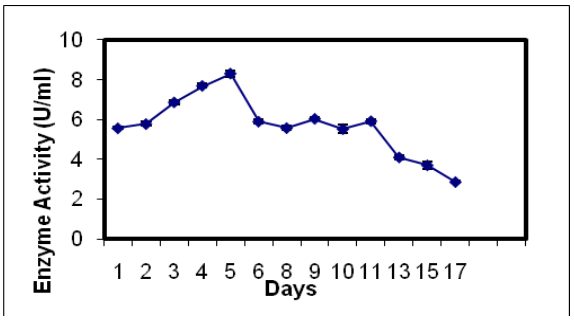

Figure 3: Laccase production by *E.cloacae KSB$_4$*.

The quantity of enzyme produced by the organism was very high compared to the laccase producing bacterial strains reported.

Substrate Oxidation Pattern of *E.cloacae KSB$_4$*

E.cloacae KSB$_4$ could grow on all substrates except tropolone and could oxidize *p*-phenylene diamine and hydroquinone. Results are tabulated in Table 1. The oxidation pattern of the two substrates is presented in Figure. 4.

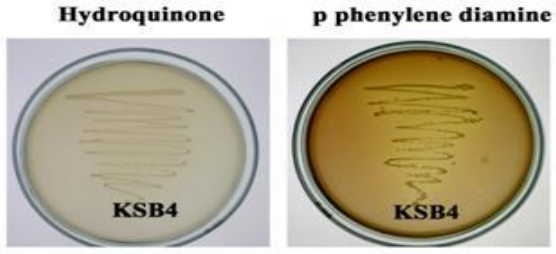

Figure 4: Oxidization of *p*-phenylene diamine and hydroquinone by *E.cloacae KSB$_4$*.

Table 1: Oxidation Pattern of Phenolic Compounds by the Screened Organisms.

Substrates	Growth/ Oxidation Pattern of *E.cloacae KSB$_4$*
guaiacol	+/+
p-cresol	+/-
p-aminophenol	+/-
p-phenylene diamine	+/+
hydroquinone	+/+
Tropolone	-/-

Stability of *E. cloacae KSB$_4$* laccase

The different treatments employed to the *E. cloacae KSB$_4$* laccase gave some unique and interesting results. The laccase activity was so stable that 56% of the total activity retained after treatment at 121°C, 15 lbs for 15 minutes. The results of heat treatments are tabulated in Table 2. This is the first report of laccase from *E. cloacae* with such a high thermostability.

Table 2: Effect of Physical Treatments.

Sl. No.	**Treatment**	**Time of treatment**	**Average Activity Retention**
i	Heated at 80°C	60min	65%
ii	Boiling	5min	58%
iii	121°C, 15 lbs	15min	56%

Different chemical compounds that denature enzymes were also tried to confirm the unnatural stability of *E. cloacae KSB$_4$* laccase. It was observed that 25% of the original activity was retained after 1hr treatment with 10X trypsin and 78% activity retention on 1hr treatment with SDS. When concentrated TCA, ethanol and Proteinase K was added to study the stability of enzyme, 40%, 44% and 46% activity retained after 1hr

respectively. The enzyme was reactive at a high pH of 10 where as at very low pH the activity was lost almost completely. Results are tabulated in Table 3.

Table 3: Effect of Chemical Treatments.

Sl. No.	Chemicals used	Time of treatment	Average Activity Retention
i	Trypsin (10X)	1 hr	25%
ii	SDS	1hr	78%
iii	TCA	1 hr	40%
		15 hrs	27%
iv	Proteinase K	1 hr	46%
		15 hrs	36%
v	HCl (0.1N) pH 2.85	5min	0.14%
vi	NaOH (0.1N) pH 10.0	5min	66%
vii	Ethanol 99%	15min	44%

Hence both physical and chemical treatments were combined to study the effect on *E. cloacae KSB4* laccase activity. It was observed that the treatment of enzyme with TCA at a temperature of 80ºC reduced the activity drastically to 13%.

Generally, thermal stability of laccases varies significantly and correlates with the temperature range of the growth of the source organism (Kristiina et al. 2009). But here the unnatural stability is surprisingly high for laccase from the mesophilic bacteria, *E. cloacae KSB4.* The molecular basis for the thermostability of proteins has been a subject of a wide variety of studies based on both theoretical and experimental investigations. The structural features of thermotolerant proteins from thermophiles and extremophiles were compared with their mesophilic counterparts, and several physicochemical factors are suggested to be the causes for protein thermostability (e.g. protein packing, hydrophobicity, increased helical fold content, density of internal hydrogen bonds and salt bridges, distribution of charged residues on the surface, proportion of certain amino acids) (Kumar and Nussinov, 2001; Sterner and Liebl, 2001). In the majority of the thermophilic proteins so far studied, a general increase in the content of internal salt bridges and hydrogen bonds, as well as an enhanced proportion of amino acid residues in a-helical conformation have been observed (Sterner and Liebl, 2001; Vogt et al. 1997).

The first prokaryotic LMCO was characterized from the soil bacterium *Azospirillum lipoferum* (Diamantidis et al. 2000). *A. lipoferum* LCMO was found to be thermostable with an estimated T1/2 value of 43 min at 70°C as determined by oxidation of syringaldazine. However, 2–3 min incubation above 80°C completely inactivated the enzyme. The most thermostable LMCO, with T1/2 of over 14 h at 80C as determined by oxidation of ABTS, has been found in the bacterium *Thermus thermophilus* (Miyazaki, 2005). Recently, a copper-activated metallo-oxidase, McoA, from the thermotolerant bacterium *Aquifex aeolicus* was reported to demonstrate heat stability even at 80°C and 90°C, with activity durable for up to 9 and 5 h, respectively (Fernandes et al. 2007).

Conclusion

Due to harsh industrial process conditions that may include high temperature and/or pressure, high salt concentrations, acidic or alkaline pH, oxidative conditions, high shear forces or short delays, resistant enzymes are required. Thermostable enzymes offer major biotechnological advantages over commonly studied mesophilic enzymes. In addition there is a

current need for enzymes with new catalytic properties for potential innovations and breakthroughs in industrial biotechnology. One of the general prerequisites for an enzyme to be applicable in industrial processes is thermotolerance (transient ability to maintain catalytic activity at elevated temperatures) or thermostability (ability to resist irreversible inactivation at high temperatures and to keep activity at least at 60C for a prolonged period of time) (Vieille et al. 1996; Vieille and Zeikus, 2001). Currently, thermostable laccases are used for various industrial applications including pulp bleaching (e.g. Lignozym-process) (Call and Mucke, 1997) and food industry (Minussi et al. 2002). Also, in textile industry thermostable laccase is already used for denim bleaching in industrial scale with products available from several suppliers (e.g. Denilite, Novozymes, Denmark; IndiStar, Genencor, US; Ecostone LCC10, AB Enzymes, Finland).

It is important to carryout biochemical studies focusing on the thermodynamics and physico-chemical properties affecting catalytic performance of metallo-enzymes like laccase. In laccases the overall 3D protein fold and packing, and minor structural modifications e.g. at the copper sites are responsible for thermotolerant response. Thermostability and thermotolerance are, in fact, results from co-action of more than one beneficial factor, such as existence of metals and cofactors, like the four Cu ions in laccases, and internal protein features, such as existence of ionic bonds (salt bridges) and dense hydrogen bonding network (Kristiina et al. 2009). In this paper we are reporting the most thermostable laccase of bacterial origin which can find very important applications in industries.

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