

LACCASE PRODUCING *STREPTOMYCES BIKINIENSIS* CSC12 ISOLATED FROM COMPOST

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ABSTRACT

Lignin degradation by actinobacteria is more attractive than well known white rot fungal system by the fact of their higher survival rate, sporulating ability, economic production of useful chemicals rather than evolution of CO₂ as in white-rot fungi. One of the important enzymes in ligninolysis is laccase, which oxidize a broad range of substrates, preferably phenolic and non-phenolic compounds. Among the laccase producing microbes in nature, white rot fungi have been characterized much, very little is known about the laccase producing actinobacteria. The present investigation aims to isolate and screen active laccase producing actinobacteria from diverse environments. Fifteen isolates of actinobacteria were isolated from various organic substrates viz., coir compost, groundnut shell compost, fully and partially decomposed municipal solid waste and identified based on molecular characterization. With initial screening using p-anisidine and Poly-R 478, seven actinobacterial isolates had more efficient ligninolytic activity than others. Of which, isolate CSC₁₂ secreted highest laccase and was identified as *Streptomyces bikiniensis* CSC12. Laccase from *S.bikiniensis* CSC12 was partially purified as monomer having molecular mass ~69 kDa based on SDS-PAGE and had pH and temperature optima of 6 to 7 and 50 to 60 °C respectively.

Keywords: Actinobacteria, compost, laccase, *Streptomyces*

INTRODUCTION

Lignin is the most abundant natural aromatic polymers in the biosphere after cellulose. It comprises 20-30 per cent in woody plant cell walls by forming a matrix surrounding the cellulose and hemicelluloses, and provides strength and protection against biodegradation (Boerjan et al., 2003). Lignin exists in close association with cellulose and other polysaccharides in plant tissues that limit the efficiency and extent of utilization of those compounds to fuels and chemicals through microbial conversion (Wang et al., 2011). Low molecular weight lignins are released into the environment as a major waste product in the pulp industry and its disposal has decreased over recent years in spite of increased pulp production (Magalhaes and Milagres, 2009). Several microorganisms that include a variety of filamentous fungi are known to attack lignin to various extents through secretion of oxidoreductases, such as laccases that offer most promise because of their high redox potential. However, lignin degradation by filamentous bacteria requires detailed study because of their definite role in biosphere carbon cycle and production of value added products from lignocellulose biomass/waste. Even though the ligninolytic potential of actinobacteria has been established long before, the studies on lignin degrading enzymes from this group are still in its infancy compared to fungi. Laccase activity has been demonstrated in a few species of *Streptomyces* such as *S. cyaneus* (Berrocal et al., 1997), *S. lavendulae* (Suzuki et al., 2003) and *S. coelicolor* (Machczynski et al., 2004). Economic production of useful chemicals by actinobacteria rather than evolution of CO₂ as in white-rot fungi, sporulating property, and survival are said to be advantageous characters of actinobacteria. Hence the present study was undertaken to isolate ligninolytic actinobacteria associated with different wastes and their enzyme systems for biomass conversion more specifically for lignin bioconversion eventually this will lead to accelerated lignin bioconversion, which could be used for production of humic polymers from lignocellulosic biomass/waste.

MATERIALS AND METHODS

Isolation of actinobacteria

Compost samples like coir compost, groundnut shell compost, fully decomposed municipal solid waste and partially decomposed municipal solid waste were

collected from Coimbatore and Namakkal (Tamil Nadu, India). Actinobacteria were isolated from these compost samples by adopting serial dilution and plating technique using Kuster's agar medium (Jayashree et al., 1991). One of our earlier actinobacterial isolate, *Streptomyces violaceusniger* (MTCC 3959) lignin degrader deposited at Institute of Microbial Technology, Chandigarh, India was used as a reference culture. *S. violaceusniger* and other actinobacterial isolates were maintained in Crawford's agar slants and slopes of mineral medium containing indulin (2 %) (Crawford et al., 1982a).

Screening for ligninolytic activity

The isolated cultures and *S. violaceusniger* were tested for ligninolytic activity on Crawford's agar medium containing different indicators viz., 0.02 per cent Poly R-478 and 10 mM p-anisidine. p-anisidine was added to sterilized Crawford's medium where as Poly-R-478 was filter sterilized and added to the medium. The actinobacterial cultures were streaked over the plate and incubated for 7 days at room temperature. Cultures which show positive reaction for ligninolytic activity exhibit initial browning and later clearing zone in the p-anisidine medium and clearing zone around the colony in Poly-R medium were selected for further studies (Rittstien et al., 2002).

Decolorization assay

Filter sterilized Poly-R, the polymeric dye was added to the liquid medium as an aqueous minimal medium inoculated with all the actinobacterial cultures to a final concentration of 0.02 per cent with gentle swirling. At 1, 2, 3, 4, and 5 days of intervals, 0.1 ml of the cell free culture medium was diluted 10-fold with water and measured absorbance ratio A₅₁₈/A₃₄₆ of visible absorption spectra and routine absorbance measurements of the dye were determined on the spectrophotometer (Glen and Gold, 1983).

Enzyme production and assay for laccase

The enzyme in the cell-free culture medium obtained after 7 days of growth (450 ml) of ligninolytic culture was precipitated at 60 per cent saturation of (NH₄)₂SO₄ and the resulting precipitates were collected by centrifugation. The precipitates were dissolved in a minimal volume of buffer 0.1M sodium phosphate buffer, pH

6.8 and dialyzed against the same buffer (Arias et al., 2003). All the purification steps were carried out at 4 °C. Cell free culture supernatants obtained from 7 days old culture was used as an enzyme source. The reaction mixture consists 1.5 ml of 0.1 mM syringaldazine in 50 mM sodium phosphate buffer pH (6.0) and 0.1 ml of enzyme extract. The change in absorbance of the reaction mixture was recorded at 530 nm at 30 sec interval for 3 min at room temperature (28 ± 2 °C). The enzyme activity was expressed as change in absorbance of the reaction mixture per min⁻¹ml⁻¹ of culture extract (Chefetz et al., 1998).

Temperature and pH optima of laccase

To optimize the conditions for maximum laccase activity, the culture supernatant from actinobacteria culture grown in Crawford’s broth with 0.1 percent glucose at room temperature was used as an enzyme source and the enzyme activities were determined by measuring change in absorbance at 530nm at different temperature conditions (The reaction mixture contained 0.4 ml of the enzyme sample, 1 ml of 0.1 mM syringaldazine and 50 mM sodium phosphate buffer). To estimate the optimum pH, enzyme activity was monitored at pH values from 3 to 10 using buffers viz., 50 mM citrate buffer for pH 3 to 5; 50 mM PO₄ buffer for pH 6 to 8; 50 mM and Tris buffer for pH 8 to 10 (The reaction mixture contained 0.4 ml of enzyme sample, 1 ml of 0.1mM syringaldazine and 50 mM sodium phosphate buffer (Chefetz et al., 1998).

SDS-PAGE analysis of laccase protein profile

To determine the purity of the protein and its molecular weight, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 10% polyacrylamide gel containing 0.1% SDS. Samples (10 µg of protein) were treated before they were loaded onto the gel with 0.5% SDS and 5% β-mercaptoethanol and boiled at 100 °C for 10 min. The protein bands were visualized after staining the gel with coomassie blue and compared with molecular weight markers (Chefetz et al., 1998).

Identification of laccase producing Streptomyces CSC12

The genomic DNA from the actinobacterial isolates was carried out using Ctab method (Azadeh and Meon, 2009). The 16SrRNA target gene amplification was performed using 27F (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 1492R (5’-GGT TAC CTT GTT ACG AC TT-3’) primers. PCR amplification was performed with an initial denaturation step at 95 °C for 4 min; followed by 35 cycles consisting of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min; final extension at 72 °C for 10 min, and end at 4 °C. The PCR product was purified (Qiagen, Germany) and sequencing reaction was performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer.

RESULTS AND DISCUSSION

Isolation and screening of actinobacteria for ligninolytic activity

Until present much of the information on lignin degradation using laccase produced by basidiomycetous fungi serves as a model organism in lignin degradation and extensive research has yielded some understanding about the biochemistry and enzymology of ligninolysis (Martínez et al., 2009). Unfortunately laccases from fungal origin show lower activity and stability in alkaline pH or at high temperature (Singh and Chen, 2008), while bacterial laccases are highly active and stable at high temperature or high pH value (Jordaan, 2005). After the first report (Crawford and Sutherland, 1979) on the isolation and characterization of lignocellulose degrading actinobacteria, few attempts were made to assess the ligninolytic activity (Kalaichelvan, 1987; Sivakumar, 1991, Karthikeyan, 2001 and Sivakumar et al., 2004) of these filamentous bacteria. Streptomyces spp. are promising ligninolytic enzyme producers having great application under extreme conditions. Laccase from Streptomyces lavendulae are stable at 70 °C (Suzuki et al., 2003) and withstand a pH from 3.0 to 9.0 (Dube et al., 2008). In the present study, fifteen isolates of actinobacteria isolated from various organic substrates were identified as actinobacteria based on molecular identification by 16S rRNA gene sequences. The isolates were tested for growth, polymerization and depolymerization activity on p-anisidin and poly-R enriched agar plates. Besides one of our earlier isolate S. violaceusniger which has been deposited at IMTECH (MTCC 3959), Chandigarh, was obtained and used as a reference culture. Based on the oxidation of p-anisidin and clearing zone on poly-R in Crawford’s medium, seven isolates viz., CC₁, CC₂, CC₇, CSC₁₂, NPS₄, CFYMC₈, and CFYMC₉ were found as more efficient ligninolytic organisms (Table 1). Initial growth of seven actinobacteria on p-anisidin caused oxidation and upon further incubation showed a clearing zone around growth which might be due to the utilization of oxidized products by the organism during the later stage of growth. The initial polymerization and later depolymerization by the phenol oxidizing enzyme (laccase) indicates the positive ligninolytic activity (Sivakumar, 1991). Streptomyces spp. produce intermediate

lignin derived water soluble low molecular weight compound called APPL (Acid Precipitable Polymeric Lignin), instead of mineralization to CO₂ by P. chrysosporium. This intermediate compounds are much of interest in economic point of view to produce value added chemicals from the lignocellulosic materials rather than converting them to CO₂. Only, a few Streptomyces sp. have the ability to degrade lignocellulosis (Crawford, 1978), and the conversion efficiency is lower than P. chrysosporium.

Table 1 Screening of different actinobacteria isolates for ligninolytic activity

Designation	Source	p-anisidin	Poly R- 478
CC ₁	Coir compost	+++	++
CC ₂	"	+++	+++
CC ₄	"	-	+
CC ₇	"	++	+++
NFS ₅	Partially decomposed municipal compost	+	-
CSC ₇	Groundnut shell compost	-	-
CSC ₈	"	+	-
CSC ₂	"	-	+
CSC ₁₂	"	+++	+++
NPS ₄	Fully decomposed municipal compost	++	+
NPS ₅	"	+	-
NPS ₁₀	"	+	+
NPS ₁₂	"	+	-
CFYMC ₈	Farmyard manure compost	+++	+
CFYMC ₉	"	+++	++

+++ : Highly efficient, ++ : Moderately efficient, + : less efficient - : no activity

Decolourization of polymeric dye

Degradation of lignocellulosics, decolourization of polymeric dye (Poly-R), polymerization and depolymerization activity on p-anisidin plates and APPL production were the criteria used for selection of efficient strains of actinobacteria for ligninolytic activity. Poly-R decolourizing ability of the organism is considered as an useful indicator of ligninolytic activity in Phanerochaete chrysosporium. It is necessary to record decolourization as a ratio of two peaks because cell adsorption and degradation reduce the absorbance of the dye (Ball et al., 1989). To confirm the ligninolytic efficiency of the isolates, quantitative test viz., decolourization of polymeric dye, Poly-R by the isolates was carried out. It was measured as the ratio between the absorbance at 518 nm and 346 nm. In this study, S. violaceusniger was used as reference culture for comparing ligninolytic efficiency. Among the seven isolates CSC₁₂ performed better than the other isolates and the reference culture S. violaceusniger (Table 2). Present study also showed a decrease in the absorbance level due to inoculation of actinobacteria cultures. On further evaluation with the reference culture S. violaceusniger, the isolate, CSC₁₂ was found better than the other isolates based on the decolourization of polymeric dye, Poly-R 478. Hernandez et al. (1994) studied the decolourization of paper mill effluent by 50 actinobacteria strains isolated from lignocellulosic substances. The colour reactions with synthetic dye were more easily detectable and thereby detect more laccase positives, and thus these compounds can reliably be used for the screening of laccase activity (Kiiskinen et al., 2004).

Table 2 Decolourization of polymeric dye (Poly-R) by actinobacteria isolates (Change in OD₅₁₈/OD₃₄₆ ratio)

Actinobacteria isolates	Number of days				
	1	2	3	4	5
CSC ₁₂	0.79	0.71	0.66	0.63	0.61
CC ₂	1.11	1.09	1.07	1.02	0.99
NPS ₄	1.18	1.11	1.06	1.00	0.98
CC ₇	1.15	1.05	0.99	0.96	0.95
CFYMC ₉	1.09	0.98	0.91	0.83	0.81
CC ₁	0.99	0.94	0.89	0.80	0.78
CFYMC ₈	1.02	0.98	0.97	0.80	0.78
S.v	0.82	0.79	0.76	0.72	0.69
SEd	0.032	0.030	0.030	0.027	0.027
CD(0.05)	0.069	0.064	0.064	0.058	0.057

Laccase activity of actinobacteria isolates

In the present investigation, the activity of laccase was assayed and their activities were expressed as change in absorbance $\text{min}^{-1}\text{ml}^{-1}$ enzyme preparation as described by Chefetz et al. (1998) and Mayer et al. (1965). The results revealed that there was a gradual increase in laccase activity of cultures upto 14th day of incubation and later a slow decline was noticed. The isolate, CSC₁₂ recorded higher activity compared to other isolates. On 14th day, this culture exhibited maximum activity of 1.56 $\text{min}^{-1}\text{ml}^{-1}$ cell extract, which was significantly higher than other cultures but lower than the reference culture *S. violaceusniger* (Fig. 1).

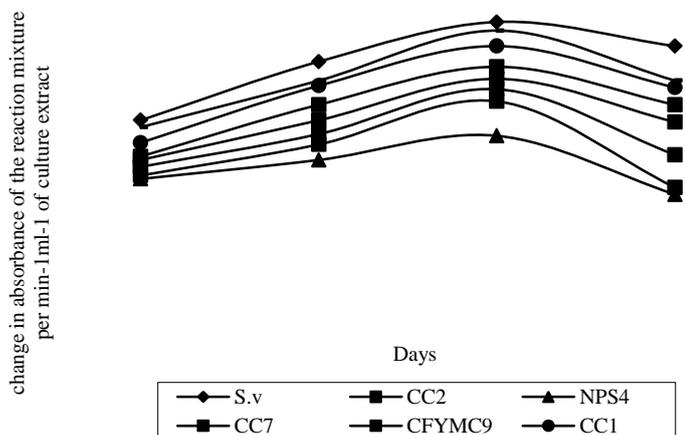


Figure 1 Production of laccase by actinobacterial isolates

One unit of enzyme activity was expressed as change in absorbance $\text{min}^{-1}\text{ml}^{-1}$ culture extract (Chefetz et al., 1998)

The phenol oxidizing enzymes such as laccase is frequently found in the culture broths of lignin degrading microbes, play a significant role in lignin degradation. It is possible that laccase may cause initial demethylation of phenol rings and the O-quinone moieties left after demethylation can be reduced to catechol, compounds which are known to be substrates of ring cleaving oxygenases (Ishiraa, 1980). This primary role of laccase may explain not only its early appearance but also the dependence of lignin degradation on laccase (Ander and Eriksson, 1976). To support this statement, our results on laccase activity of actinobacteria showed that the enzyme synthesis initiated 3rd day and extended upto 21st day, such early synthesis and extended production of laccase can be exploited for commercial production of laccase using lignocellulosic biomass/agricultural wastes. The independent action of laccase in lignin biodegradation by white-rot fungi in the absence of ligninase (Lip) and Mn-peroxidases (Mn-P) has been reported by Eggert et al., (1997). Ardon et al. (1998) reported the maximum ligninolytic enzymes of *Pleurotus ostreatus* on 8th day of incubation in cotton stalk. Castillo et al. (1997) showed that lignin and manganese peroxidase activity in extracts from straw solid substrate fermentation was maximum at 6th day of inoculation. Eggert et al. (1997) reported that the white-rot fungus, *Pycnoporus cinnabarinus*, an excellent organism to elucidate the controversial role of laccase in lignin degradation and indicated that laccase is absolutely essential for lignin degradation. Earlier, Kalaichelvan and Ramasamy (1989) reported the presence of ligninolytic enzyme viz., peroxidase, polyphenoloxidase, laccase and ligninase in *Streptomyces* sp. RK-1 and laccase hyper producing strains were also developed by Sivakumar et al., (2004). Ramasamy et al. (1989) reported that peroxidase, laccase and phenol oxidases of *Pleurotus ostreatus* were associated with the ligninolysis of coir dust. Pusti et al. (1991) reported that extracellular peroxidases of both *S. chromofuscus* and *S. viridosporus* appear extracellularly after cell cease to grow and nutrients depleted from the medium.

Temperature optima for laccase produced by Streptomyces CSC2

The optimal temperature range for the activity of laccases obtained from the actinobacteria viz., CSC₁₂ and *S. violaceusniger* reached a maximum between 50 and 60 °C (Fig. 2). The enzymes were inactive below 20 °C and above 70°C determined during 10 min reactions. Laccase from actinobacteria isolate CSC₁₂ and *S. violaceusniger* had an optimal activity at 50 to 60 °C. Chefetz et al (1998) reported that laccase of *Cheatomium thermophilum* had an optimum temperature range of 50 to 60 °C. The laccase purified from *Trametes sanguinea* and *Botrytis cinerea* showed increased activity with increasing temperature (Slomczynski et al., 1995).

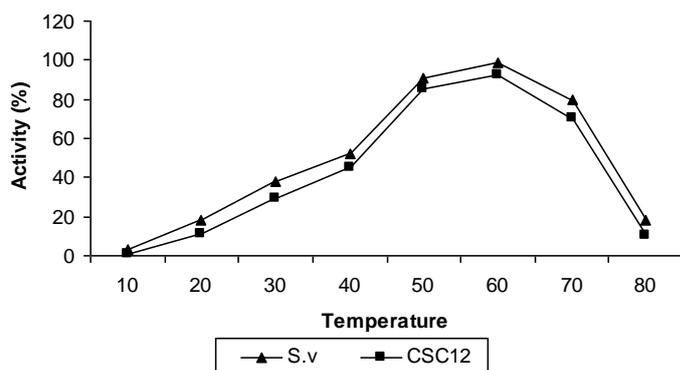


Figure 2 Influence of different temperature on the activity of laccase produced Streptomyces CSC12

pH optima for laccase produced by Streptomyces CSC12

The pH of laccase will be one of the key parameters to affect oxidation rates. Hence the influence of different pH on the activity of laccase enzyme was studied. The results obtained in the present study indicate that the pH optima of laccase (with syringaldazine oxidation) by *Streptomyces* CSC₁₂ and *S. violaceusniger* was pH 6.0-7.0 (Fig.3). Most of the fungal laccases are active at pH values 3.0-5.0 (Slomczynski et al., 1995). But studies on laccase of *C. thermophilum* showed maximum activity at pH 6.0 to 8.0 (Chefetz et al., 1998).

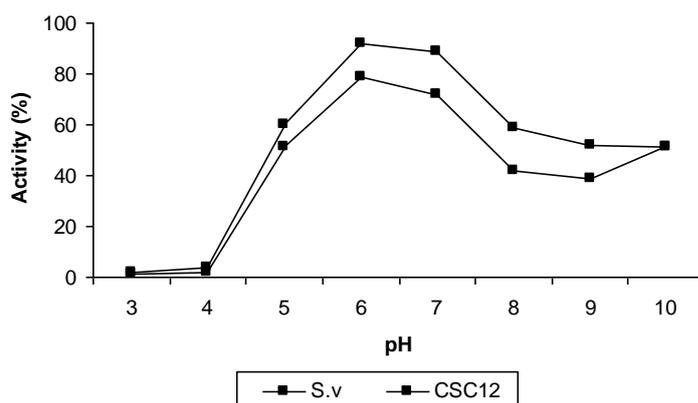


Figure 3 Influence of different pH on the activity of laccase from Streptomyces CSC12

SDS- PAGE profile of laccase from actinobacteria

Cell free culture supernatant obtained from both *S. violaceusniger* and *Streptomyces* CSC₁₂ was analyzed in 12 per cent SDS-PAGE and size of these proteins ranged from 14 to 97 kDa. The results indicated that the banding pattern in the protein profile was almost similar in these two isolates and majority of the proteins have the molecular size between 60 and 75 kDa. However, among the isolates, variations were observed in the protein profile at same molecular sizes. Molecular weight of most fungal laccase proteins fall between 43 to 110 kDa (Yoropolov et al., 1994). Majority of laccases from basidiomycetous fungi were reported to have molecular weights in the range of 55 to 72 kDa. Based on in-gel laccase activity assay, laccase from the isolate CSC₁₂ and *S. violaceusniger* was found to have a molecular weight of 69 kDa (Fig 4). The *Streptomyces* CSC₁₂ and *S. violaceusniger* laccase appears to occur in only one single, isoform, an unusual feature among laccases especially fungal laccases. The purified laccases of *Streptomyces cyaneus* produced one band on SDS-PAGE gel at a molecular mass of approximately 75 kDa. Hence the band obtained from the protein profile of cell free cultures of CSC₁₂ and *S. violaceusniger* is with molecular weight of 69 kDa, it may probably a laccase enzyme which was confirmed with zymogram obtained from native PAGE. This preliminary finding paves the way for expanding our dimensions on various biotechnological applications of laccase on energy and environment.

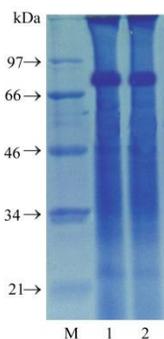


Figure 4 SDS PAGE Profile of laccase from actinobacteria (M- Molecular weight marker, 1-CSC 12, 2-S.v)

Identification of the actinobacteria

The 16S rRNA gene sequence of the actinobacteria was aligned with homologous regions from various actinobacteria, and the phylogenetic tree was constructed using neighbor-joining method (Saitou and Nei, 1987). A bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the distance tree topologies obtained (Felsenstein, 1985). The graphic representation of the resulting tree was obtained using MEGA software (version 5.05). Based on the phylogenetic data obtained from the isolate CSC₁₂ showed a maximum similarity (98 per cent) with *Streptomyces bikiniensis* (Fig.2).

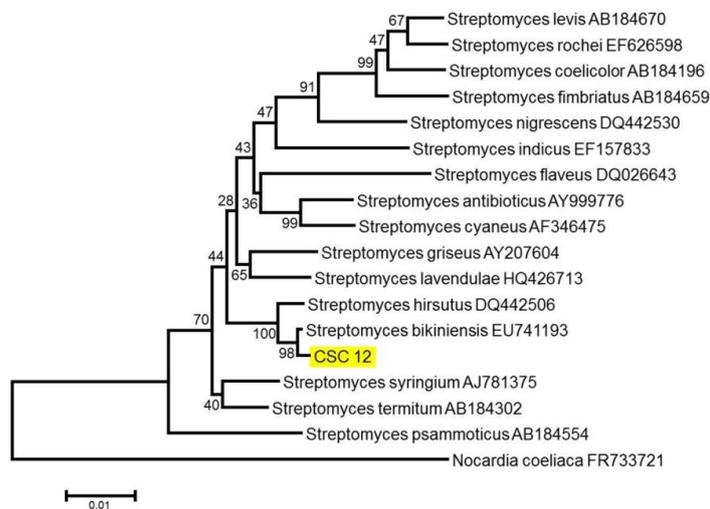


Figure 5 Phylogenetic relationship of CSC12 based on 16S rRNA gene sequences. The tree was constructed using the NJ method. Branch support is recorded at the nodes as a percentage of 1,000 bootstrap iterations

CONCLUSION

This study revealed that organic substrates viz., coir compost, groundnut shell compost, fully and partially decomposed municipal solid waste serve as a source of laccase producing actinobacteria. Initial screening using p-anisidin and Poly-R 478 yielded seven potential actinobacterial isolates that were more efficient in ligninolytic activity. Among them, one potential isolate CSC₁₂ secreted laccase at comparative levels with reference culture *S. violaceusniger* MTCC 3959 and was identified as *S.bikiniensis* CSC12. Laccase from *S.bikiniensis* CSC12 had pH and temperature optima of 6 to 7 and 50 to 60 °C, respectively and the partially purified monomer reported molecular mass of ~69 kDa based on SDS and Native PAGE.

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