www.sphinxsai.com

International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.6, No.4, pp 1383-1391, Aug-Sept 2014

Production, and Characterization of Neutral Laccase from Marine *Streptomyces lydicus*

Mahmoud, M. G.[1], Rifaat, H. M.[2], El Sayed, O. H.[1], El Beih, F. M[3] and Selim*, M. S[1].

[1]Microbial Biotechnology Department, [2]Microbial Chemistry Department, National Research Centre, El-Bohos Street, Cairo, Egypt,
[3]Microbiology Department, Ein Shams University, Cairo, Egypt.

***Corres. Author : manalsleem@yahoo.com**

Abstract: Laccase is one of the ligninolytic enzymes isolated from marine *Streptomyces lydicus* in Egypt. This laccase has been purified to homogeneity through anion exchange and gel filtration chromatography steps with an overall purification fold of 42.04. The final recovery of the enzyme was 2.53%. The molecular mass of the purified laccase was about 60 kDa as determined by SDS-PAGE. The optimum pH was 6.5 and the enzyme stable up to 40ºC for 60min. The maximum activity was detected in case of EDTA followed by arbutin, while the enzyme was inhibited by metal ions like Fe^{2+}, Zn^{2+}, Mn^{2+}, and Ag^{+}. The enzyme showed highest K_m value with ABTS (0.41mM) followed by syringaldazine (0.36mM). The purified enzyme was a typical blue laccase with an absorption peak at 600nm; therefore, it may be potentially useful for industrial purposes.
Keywords: Laccase; Marine; Purification; Characterization; *Streptomyces lydicus.*

1. Introduction

Enzymes have gained great importance in industries; laccases are one among them which are widely present in the nature. Laccases are blue multicopper polyphenol oxidases (PPO) which couple the oxidation of various substrates with the reduction of O_2 to H_2O [1,2]. Laccases are widely distributed in higher plants, bacteria, fungi, and insects. Actinomycetes are believed to be potent producer of laccases, next to fungi. In *Streptomyces*, a laccase-type phenol oxidase was found to be produced during growth under solid-substrate fermentation condition [3]. Purification and characterization of laccases from actinomycetes, especially, different *Streptomyces* sp. has been accounted. The laccase-like phenol oxidase from *Streptomyces griseus* has been reported to have a highly unique homotrimer structure [4] while the small laccase from *Streptomyces coelicolor* has been described as a dimer, lacking the second domain [5]. Laccase from *Streptomyces lavendulae* has been reported as thermostable, being stable at 70ºC [6]. Arias *et al*. [7] have described a laccase from *Streptomyces cyaneus* that was capable of oxidizing non-phenolic compounds in the presence of mediators. Laccases play an important role in food, paper and pulp and textile industries also in synthetic chemistry, cosmetics, soil bioremediation, biodegradation of environmental phenolic pollutant and removal of endocrine disruptors [8-11]. Also lacasses are used in biosensor and analytical applications. Recently laccases have been efficiently applied to nanobiotechnology due to their ability to catalyze electron transfer reactions without additional cofactor. In this paper we report the purification and characterization of neutral laccase from a marine *Streptomyces lydicus* in Egypt.

2. Materials and methods

2.1. Microorganism and its maintenance

Streptomyces lydicus was isolated and identified from sediment of Red Sea, Hurghada, Egypt. The culture was grown and maintained on starch nitrate agar slants [12] and it used for inoculums preparation. *Streptomyces lydicus* colonies were grown in the laccase production medium [7] containing (per 500 ml of distilled water): 20 g peanut shell, 1 g KH_2PO_4 (anhydrous), 2 g Na_2HPO_4, 0.2 g NaCl, 0.2 g $MgSO_4$. $7H_2O$, 0.05 g $CaCl_2$. 2 H_2O and 500 ml sea water at pH 7. The cells (10^7/ml) were grown in flasks containing 50 ml medium and maintained at 28ºC under constant 150 rpm shaking for 4 days.

2.2. Laccase assay

Lacasse assay was performed with 0.5 ml of culture filtrate, 2.4 ml of 0.2 M phosphate buffer (pH 6.5) were equilibrate to 30ºC and 0.01 ml of syringaldazine 0.216 mM. Reaction was initiated by addition of syringaldazine, the change in absorbance per minute was measured at 526 nm [13]. A standard laccase enzyme unit was defined as the amount of enzyme catalysing the oxidation of 1µmol of syringaldazine to its quinine form per minute at 30ºC in 0.2 M phosphate buffer (pH 6.5) using a molar absorptivity of 65 $mM^{-1}cm^{-1}$ for the product [14].

2.3. Protein estimation

Total soluble protein was determined by Bradford [15] using bovine serum albumin as the standard. Absorbance at 280 nm was used for monitoring protein in column elutes [16].

2.4. Purification of laccase

The proteins were precipitated from the supernatant with ammonium sulfate (0-80% saturation). Laccase activity was detected in 40% saturated fractions. Laccase active fraction was pooled, centrifuged (5,000 rpm, 25min) and the precipitate was dissolved in minimal amount of distilled water and dialyzed overnight at 4ºC. The dialyzate was loaded on to a DEAE cellulose anion exchange column (1.5×40 cm), which was equilibrated and washed with 50 mM sodium phosphate buffer, pH 6.5. The enzyme was eluted with a linear gradient of NaCl (0-1.0M) at the flow rate 60 ml/h and the elute was monitored for absorbance at 280 nm, conductivity and laccase activity. The most active fractions from DEAE-cellulose column were pooled, dialysed as described in previous step and loaded on a (1.5×60 cm) of Sephadex G-100 equilibrated with 50 mM phosphate buffer (pH 6.5) and eluted with one liter of the same buffer at flow rate 35 ml/h. Elute (5 ml fractions) was collected for measurement of absorbance at 280 nm [17] and the enzyme activity was assayed. The active fractions were pooled and dialysed against distilled water. The protein content was measured and the laccase activity was assayed as described before.

2.5. Characterization and properties of the purified laccase

2.5.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight of the purified laccase was determined by SDS-PAGE according to the method of Laemmli [18]. The strength of the gel was 12% (w/v) and the protein bands were stained with Coomassie brilliant blue R 250. Medium range (29-205kDa). Molecular weight markers were used to determine the molecular mass of purified laccase. The markers were: carbonicanhydrase (29kDa), albumin (egg) (45kDa), bovine serum albumin (66kDa), phosphorylase (97kDa), β-galactosidase (116kDa) and myosin (205kDa).

2.5.2. Spectral studies

The spectral studies of purified laccase were carried out using a UV-Vis spectrophotometer (Shimadzu) [19].

2.5.3. Optimum pH and thermal stability

To estimate the optimum pH of the pure laccase from *Streptomyces lydicus*, the lacase activity was measeared with 0.01 ml of syringaldazine 0.216 mM in 0.2 M of citrate phosphate buffer pH (3.0-5.0), phosphate buffer pH (6.0-7.0) [20] and Tris-HCl pH (8.0-10.0) [21].To determine the thermal stability of

purified enzyme, samples were pre-incubated with different range of temperature from 30ºC to 100ºC at different times from 10 to 60 min.. Assays were performed as described above.

2.5.4. Inhibition studies

Different inhibitors have been used such as sodium azide, EDTA (ethylenediaminetetraacetic acid), L-cystein and arbutin with concentration of 0.08 mM on the purified laccase.

2.5.5. Effect of metal ions on laccase activity.

Metal ions such as Mn^{2+}, Zn^{2+}, Fe^{2+}, Ag^{+} and mixture of the ions with different concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1mM) were used to study their effect on purified laccase [21].

2.5.6. Substrate specificity

Two substrates specificity of purified laccase were tested syringaldazine at different concentrations of (0.05, 0.1, 0.15, 0.216, 0.25 and 0.3mM) and substrate (ABTS) 2,2´-azinobis-(3-ethylbenzthiazoline-6-sulfonate have been used with different concentrations (0.2, 0.4, 0.6, 0.8, 1 and 1.2mM). The apparent K_m values of syringaldazine and ABTS were determined through studies relating substrate concentrations to the velocities of the reaction. The apparent K_m and V_{max} values for these substrates were determined from Lineweaver–Burke plots [22].

3. Results and discussion

3.1. Purification of neutral laccase

Different concentrations of ammonium sulfate were added to the crude laccase produced by *Streptomyces ludicus* (Table1). Fractions from 40% ammonium sulfate saturation showed high laccase activity and specific activities in comparison with crude laccase and other concentrations. The obtained laccase produced by marine *Streptomyces lydicus* after partially purified using 40% ammonium sulfate was subjected on column of DEAE-cellulose. The elution diagram of the enzyme is illustrated graphically in Fig. (1). The obtained result showed a single peak co-eluting with laccase activity. The specific activity has increased to 53.83U/mg with a fold purification fold of 37.14. Subsequent purification by gel filtration using Sephadex G-100 was applied. The elution diagram of the enzyme is illustrated graphically in Fig. (2). The obtained result showed a single peak co-eluting with laccase activity of active DEAE cellulose fractions. The purification results are summarised in (Table 1) with an overall recovery of 2.53%. Laccase preparation obtained by the used producer had a specific activity of 60.93 U/mg and with purification folds 42.04. For second purification, ion exchange technique was applied for fraction obtained at concentration of 40% ammonium sulfate.

The purification of laccase is important from the perspective of developing a better understanding of the functioning of the enzyme [23-24]. Precipitation is the most commonly used method for the isolation and recovery of proteins from crude biological mixtures [25]. Arias *et al*. [7] found that laccase from *Streptomyces cyaneus* is precipitated by 50% saturation of ammonium sulfate. DEAE cellulose was used to purify laccase from *Streptomyces lydicus* and a single peak co-eluting with laccase activity of 40% ammonium sulfate fraction was achieved. This result was agreed with Suzuki *et al*. [6] and Niladevi *et al*. [19] where, they used DEAE cellulose to purify laccases from *Streptomyces lavendulae* and *Streptomyces psammoticus*. Sephadex G100 showed single peak co-eluting with laccase activity of active DEAE cellulose fractions. The same results obtained by Hess *et al*. [26] and Viswanath *et al*. [17] where they reported the use of Sephadex G100 for the purification of laccase from *Trametes versicolour* and *Stereum ostrea* respectively. In the other hand Suzuki *et al*. [6] used Sephadex G150 for the purification of laccase from *Streptomyces lavendulae*. Final purified protein obtained by Sephadex G-100 was subjected to SDS-PAGE. Gel was conducted using a 12% (w/v) polyacrylamide gel. Final purified laccase gave a single band by SDS-PAGE (Fig. 3) with Rf 0.666. Analysis performed on a polyacrylamide Fig. (3) From left to right: Lane 1, standard molecular weight markers (carbonicanhydrase (29kDa), albumin (egg) (45kDa), bovine serum albumin (66kDa), phosphorylase (97kDa), β galactosidase (116kDa) and myosin (205kDa) and Lane 2, final purified laccase obtained by Sephadex G-100. According to the calibration curve of electrophoretic mobilities of the marker proteins against the logarithms of their corresponding molecular weight, the molecular weight of laccase was 60kDa. The molecular weight of laccase produced from *Cyathus bulleri* was also 60kDa [21]. Kunamneni *et al*. [27] said that the molecular mass of the laccase ranges from about 50 to 100kDa, while Patel *et al*. [28] reported that the molecular mass of laccase produced from *Pleurotus ostreatus* HP-1 was 66 kDa.

Table 1. Summary of the purification of laccase from marine *Streptomyces lydicus*

Purification steps	Volume (ml)	Total laccase activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude	1800	3056.13	2108.1	1.449	1	100
Ammonium sulphate 40%	13	108.108	9.698	11.147	7.69	3.53
DEAE-cellulose	20	106.6	1.98	53.83	37.14	3.48
Sephadex G-100	25	77.385	1.270	60.93	42.04	2.53

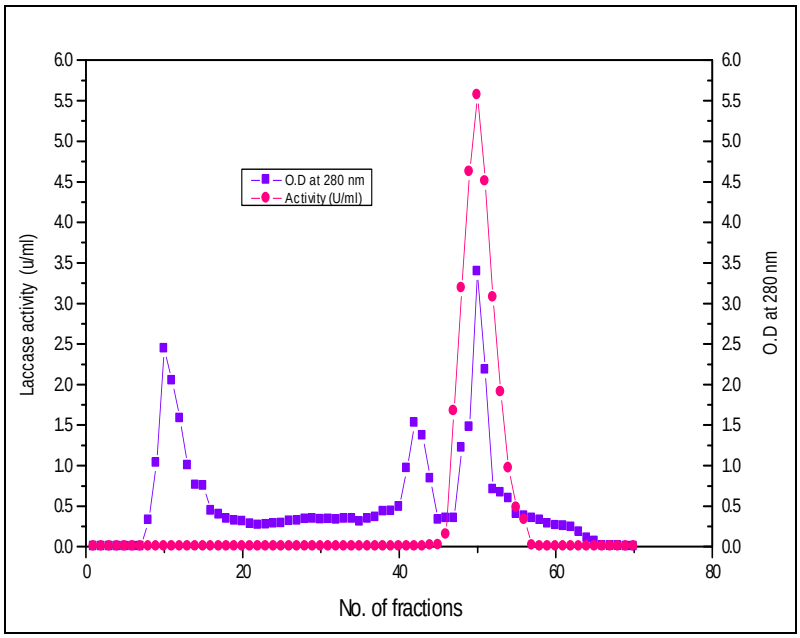

Fig. 1. Elution profile of laccase (40% ammonium sulphate saturation) on DEAE-cellulose

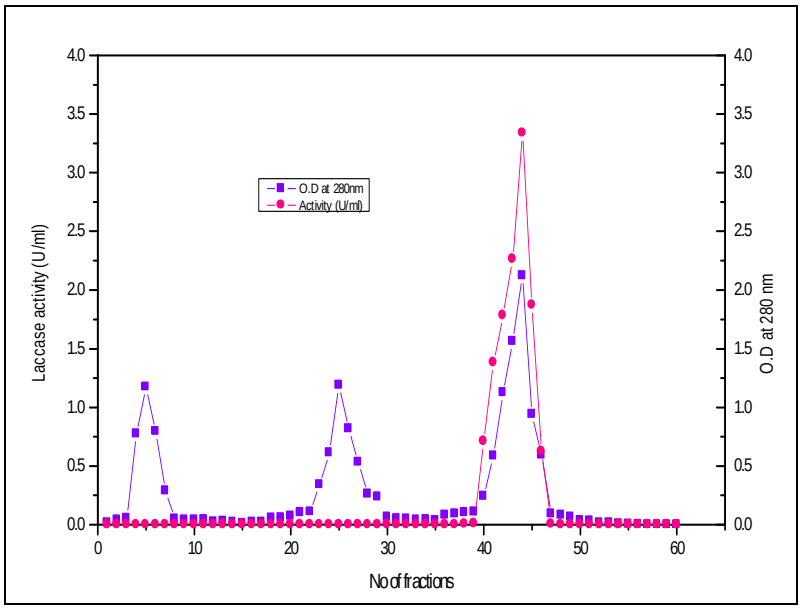

Fig. 2. Elution profile of laccase (from activated fractions of DEAE-cellulose) on sephadex G 100

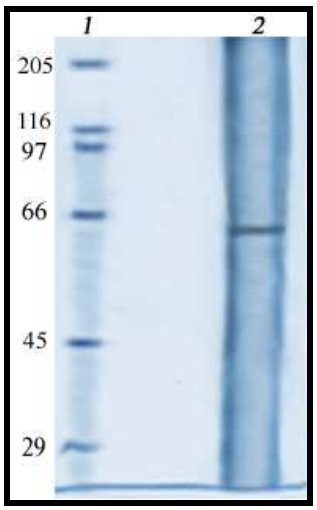

Fig. 3. SDS-PAGE of laccase enzyme produced by marine *Streptomyces lydicus* .Analysis performed on a polyacrylamide from left to right: Lane 1, standard molecular weight markers (carbonicanhydrase (29kDa), albumin (egg) (45kDa), bovine serum albumin (66kDa), phosphorylase (97kDa), β galactosidase (116kDa) and myosin (205kDa) and Lane 2, final purified laccase obtained by Sephadex G-100.

3.2 Characterization of the purified laccase

3.2.1 Spectral studies

The UV-visible spectrum of the purified laccase showed a peak around 600 nm which was characteristic of the type 1 copper of typical laccase (Fig 4). The spectral graph indicated that the purified laccase belongs to the group of well-established blue-laccase of Streptomycetes. Arias *et al*. [7] mentioned that laccase from *Streptomyces cyaneus* had a typical spectrum of the blue laccases.

3. 2.2 Optimum pH and thermal stability

The laccase activity increased by increasing the pH until the maximum laccase activity (3.095 U/ml) detected at pH 6.5 using phosphate buffer as seen in (Fig.5). The results of pH 6 and 7 was closely related to pH 6.5. Weak activities (0.639 and 0.020 U/ml) were obtained at pH 3 and 10 using citrate-phosphate and Tris-HCL buffers respectively. The optimum pH of laccase activity was found to be at 6.5, and the activity was almost stable at pH (6-6.5). A decline in activity was detected at pH 4 and 9. Niladevi *et al*. [19] mentioned that laccase from *Streptomyces psammoticus* was stable in the pH range 6.5-9.5. Laccase was stable from 30 and 40ºC at different time limits as shown in Fig. (6). At 50 ºC the residual activity showing a drastic decrease from 88.14% after 20 minutes to 12.40 % at 60 minutes. At 60ºC the residual activity decreased by increasing the time from (51.41 to 1.89%). Only 1.45% of residual activity detected after 30 min. in case of 70ºC, while at 80ºC complete inhibition of laccase activity occur. Min *et al*. [29] and Jung *et al*. [30] were reported that the stability of laccase at higher temperature increased under neutral pH conditions.

3.2.3. Effect of inhibitors on laccase activity

Laccase activity was extremely inhibited by sodium azide and L-cystein Fig.(7).The maximum activity detected in case of EDTA followed by arbutin. Inhibition of enzymatic activity was detected only for sodium azide and L-cystein (0 and 5.0%) of residual activity respectively. More *et al*. [31] stated that sodium azide was a potent inhibitor of laccase from *Pleurotus* sp. Suzuki *et al*. [6] mentioned that EDTA and arbutin had no effect on the purified laccase from *Streptomyces lavendulae.*

3.2. 4. Effect of metal ions on laccase activity

The residual laccase activity decreased by increasing the concentrations from 0.1 to 1.0 mM in case of Fe^{2+}, Mn^{2+} and Zn^{2+} (Fig 8). At concentration 0.2 mM complete inhibition occurred in case of Ag^{+} and when mixture of metal ions was used. The obtained results are in accordance with More *et al.* [31] who mentioned that laccase activity produced by *Pleurotus* sp. was decreased with Fe^{2+}, Mn^{2+} and Zn^{2+}.

3.2. 5. Substrate specificity

As seen in Fig. (9) laccase activity increased by increasing the concentration of syringaldazine till reached 0.21 mM, the activity was (3.095 U/ml) and after that the increase of the activity was very weak. Laccase activity increased by increasing the concentrations of ABTS gradually till 0.8 mM (1.155 U/ml) then the activity after that was almost stable as shown in Fig. (10). The K_m of laccase for syringaldazine and ABTS were found to be 0.365 and 0.410 mM with V_{max} 6.849 and 1.718 respectively (Figs. 9 and 10). The K_m values of laccase from marine *Streptomyces lydicus* toward the two substrates indicated that the binding affinities toward the different substrates were in the same order: syringaldazine> ABTS. The V_{max} value of syringaldazine was higher than in ABTS. Giardina *et al*. [32] regarded the same result. V_{max}/K_m ratios were expressed for the reactions of laccase towards the two substrates where the ratio in case of syringaldazine was higher than in ABTS.

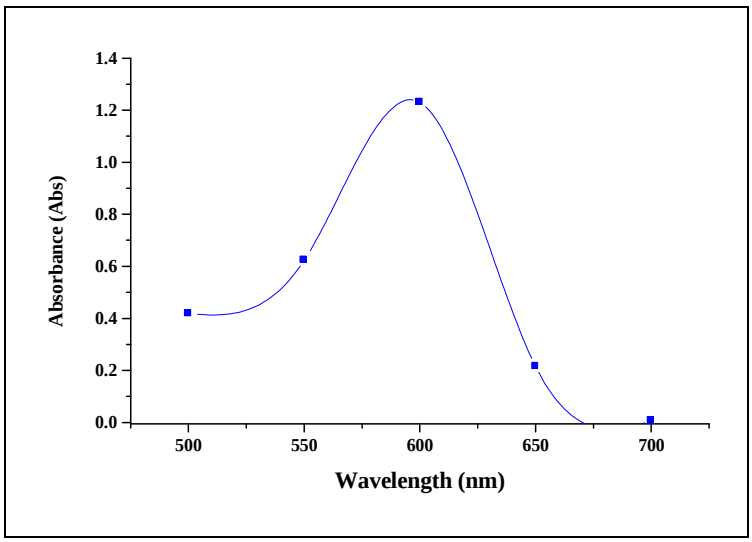

Fig. 4. Absorption spectrum of laccase from marine *Streptomyces lydicus*

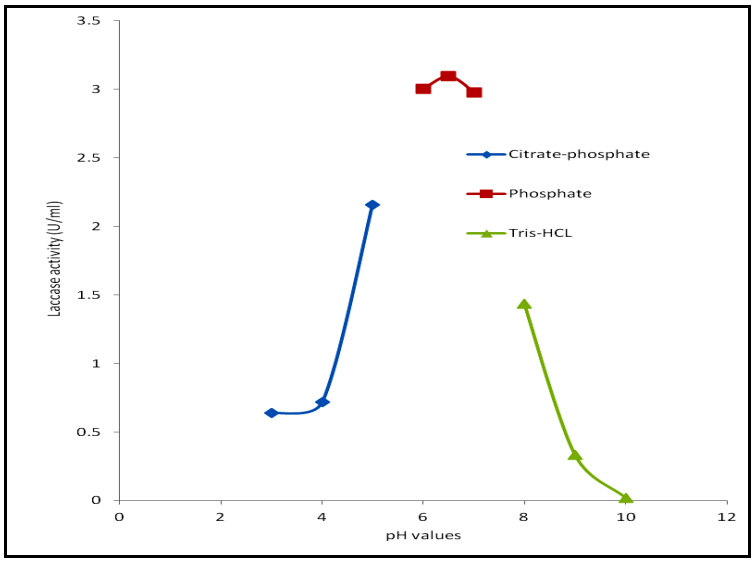

Fig. 5. Effect of pH on the purified laccase from marine *Streptomyces lydicus*

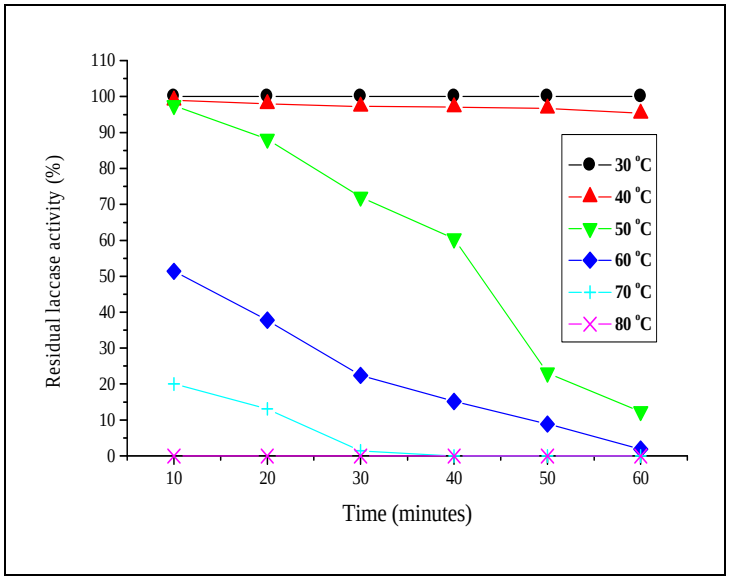

Fig. 6. Thermal stability of purified laccase from marine *Streptomyces lydicus*

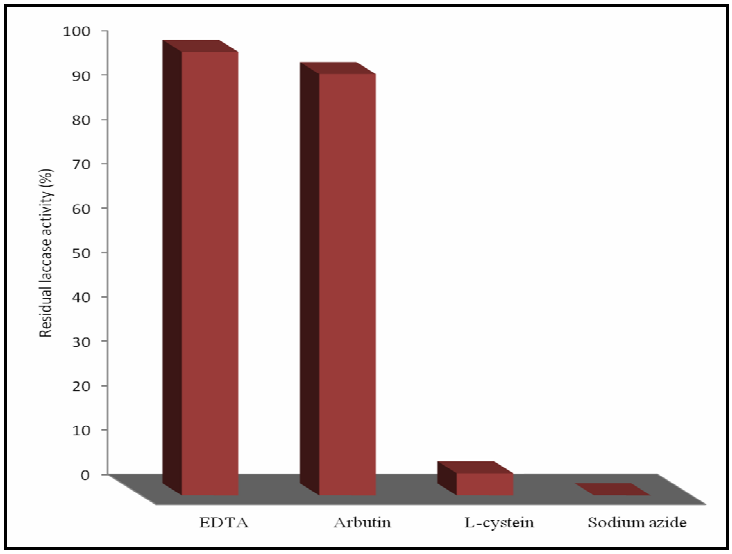

Fig. 7. Effect of inhibitors on the purified laccase from marine *Streptomyces lydicus*

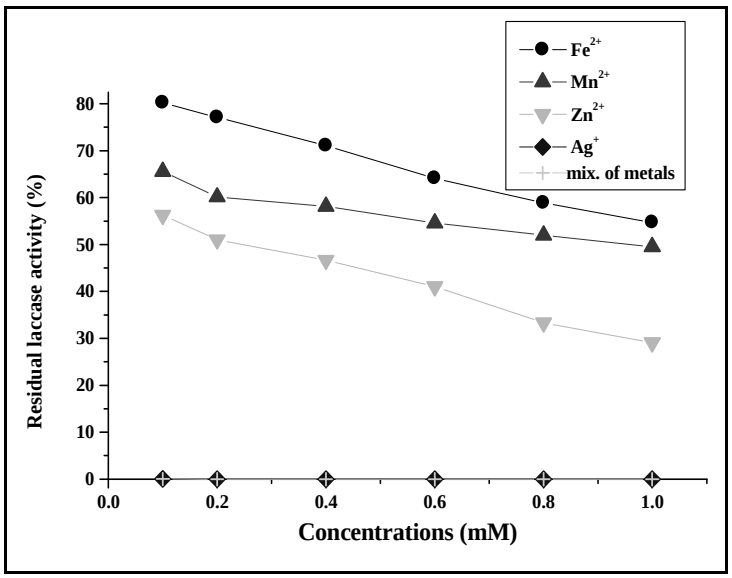

Fig. 8. Effect of metal ions on the purified laccase from marine *Streptomyces lydicus*

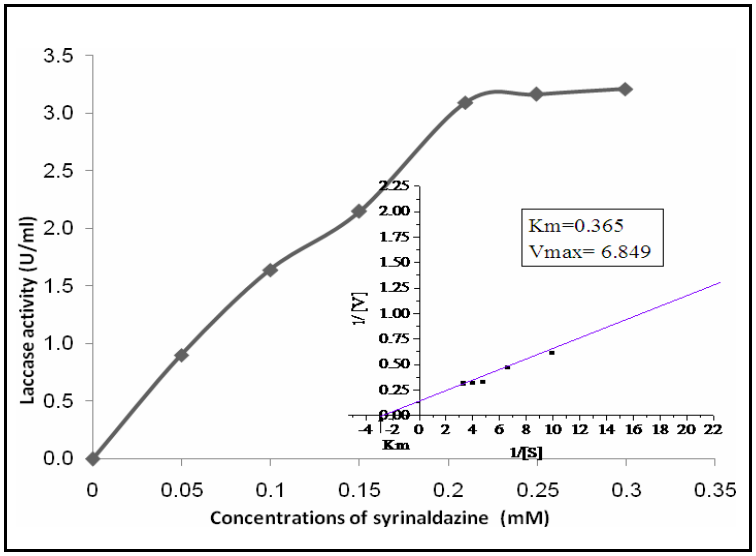

Fig. 9. Effect of syringaldazine concentrations on purified laccase from marine *Streptomyces lydicus and* Lineweaver-Burk plot of it

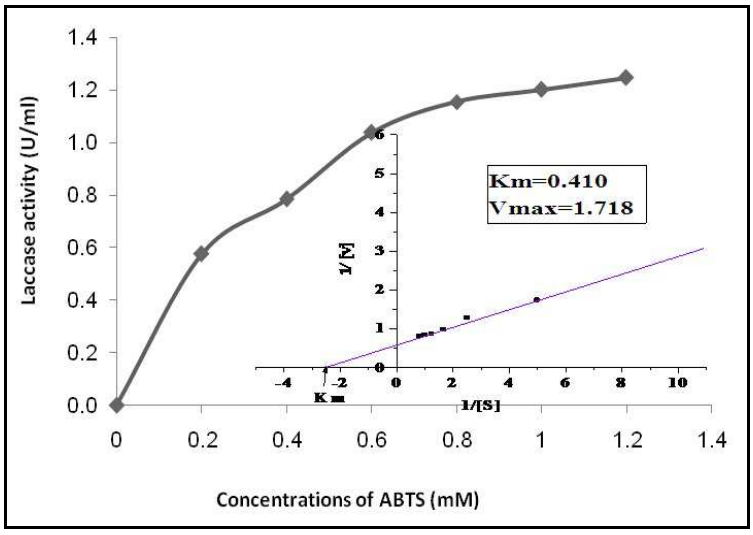

Fig. 10. Effect of ABTS concentrations on purified laccase from marine *Streptomyces lydicus and* Lineweaver-Burk plot of it.

4. References

1. Leonowicz, A., Cho, N. S., Luterek, J., Wilkolazka, A., Wojtas-Wasilewska, M., Matuszewska, A., Hoyaropolov, A. I., Skorobogat'ko, O. V., Vartanov, S. S. and Varfolomeyev, S. D. (1994). Laccase: Properties, catalytic mechanism, and applicability. Appl. Biochem. Biotechnol., 49:257-280.
2. Yaropolov, A. I., Skorobogat'ko, O. V., Vartanov, S. S., and Varfolomeyev, S. D. (1994). Laccase: properties, catalytic mechanism and applicability. Appl. Biochem. Biotechnol., 49:257-280.
3. Berrocal, M. M., Rodriguez, J., Ball, A. S., Perez-Leblic, M. I. and Arias, M. E. (1997). Solubilization and mineralization of [^{14}C] lignocellulose from wheat straw by *Streptomyces cyaneus* CECT 3335 during growth in solid state fermentation. Appl. Microbiol. Biotechnol., 48:379-384.
4. Endo, K., Hayashi, Y., Hibi, T., Hosono, K. and Ueda, K. (2003). Enzymological characterization of EpoA, a laccase like phenol oxidase produced by *Streptomyces griseus*. J. Biochem., 133:671-677.
5. Machczynski, M. C., Vijgenboom, E., Samyn, B. and Canters, G. W. (2004). Characterization of SLAC: a small laccase from *Streptomyces coelicolor* with unprecedented activity. Protein Sci., 13:2388-2397.
6. Suzuki, T., Endo, K., Ito, M., Tsujibo, H., Miyamoto, K. and Inamori, Y. A. (2003). Thermostable laccase from *Streptomyces lavendulae* REN-7:purification, characterization, nucleotide sequence and expression. Biosc. Biotechnol. Biochem., 67:2167-2175.
7. Arias, M. E., Arenas, M., Rodrıguez, J., Soliveri, J., Ball, A. S. and Hernandez, M. (2003). Kraft pulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335. Appl. Environ. Microbiol., 69:1953-1958.

8. Couto, S. R. and Toca, J. L. (2005). Industrial and biotechnological applications of laccases: a review. Biotechnol. Adv., 24:500-513.
9. Devi, V., Inbathamizh, L., Ponnu ,T., Premalatha, S. and Divya, M. (2012). Dye decolorization using fungal laccase. Bull. Environ. Pharmacol. Life Sci., 1:67-71.
10. Virk, A., Sharama, P. and Capalash, N. (2012). Use of laccase in pulp and paper industry. Biotechnol. Prog., 28:21-32.
11. Faccelo, J. and Cruz, O. (2008). Banana skin: a novel material for a low-cost production of laccase, M.S. thesis, Universitat Rovira I Virgili.
12. Waksman, S. A. (1961). The actinomycetes. Classification, identification and descriptions of genera and species. The Williams and Wilkins Co., Baltimore, 1: 1-363.
13. Petroski, J., Peczynska-Czoch, W. and Rosazza, J. (1980). Analysis, production, and isolation of an extracellular laccase from *Polypours anceps*. Appl. Environ. Microbiol., 40:1003-1006.
14. Bauer, R. and Rupe, C. (1971). Use of syringaldazine in a photometric method for estimating free chlorine in water. Anal. Chem., 43: 421-425.
15. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem., 72:248-254.
16. Warburg, O. and Christian, W. (1942). Isolation and crystallization of enolase. Biochem., Z.310: 386.
17. Viswanath, B., Subhosh Chandra, M., Pallavi, H. and Rajasekhar Reddy, B. (2008). Screening and assessment of laccase producing fungi isolated from different environmental samples. Afr. J. Biotechol., 7:1129-1133.
18. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680-685.
19. Niladevi, K., Jacob, N. and Prema, P. (2008). Evidence for a halotolerant-alkaline laccase in *Streptomyces psammoticus*: Purification and characterization. Pro. Biochem., 43:654-660.
20. Han, M. J., Choi, H. T. and Song, H. G. (2005). Purification and characterization of laccase from the white rot fungus *Trametes versicolor*. J. Microbiol., 43:555- 560.
21. Vasdev, K., Dhawan, S., Kumar Kapoor, R. and Chander Kuhad, R. (2005). Biochemical characterization and molecular evidence of a laccase from the bird's nest fungus *Cyathus bulleri*. Fung. Gene Biol., 42:684-693.
22. Lineweaver, H. and Burk, D. (1934). The determination of enzyme dissociation constants. J. Amer. Chem. Soc., 56:658-666.
23. Tsai, Y. C., Juang, R. Y., Lin, S. F., Chen, S. W., Yamasaki, M. and Tamura, G. (1988). Production and characterisation of an alkaline elastase produced by alkalophilic *Bacillus* strain Ya-B. Appl. Environ. Microbiol., 54: 3156-3161.
24. Takagi, H. (1993). Protein engineering on subtilisin. Int. J. Biochem., 25:307-312.
25. Bell, D. J., Hoare, M. and Dunnill, P. (1983). The formation of protein precipitates and their centrifugal recovery. Adv. Biochem. Eng. Biotechnol., 26:1-72.
26. Hess, J., Leitner, C., Galhaup, C., Kulbe, K. D., Hinterstoisser, B. and Steinwender, M. (2002). Enhanced formation of extracellular laccase activity by the white-rot fungus *Trametes multicolour*. Appl. Biochem. Biotechnol., 98-100:229-241.
27. Kunamneni, A., Ballesteros, A., Plou, F. J. and Alcalde, M. (2007). Fungal laccase-a versatile enzyme for biotechnological applications. Appl. Microbiol., 1:233-245.
28. Patel, H., Gupte, S., Gahlout, M. and Gupte, A. (2014). Purification and characterization of an extracellular laccase from solid-state culture of *Pleurotus ostreatus* HP-1. Biotechnology, 4: 77-84.
29. Min, K., Kim, Y., Kim, Y., Jung, H. and Hah, Y. (2001). Characterzation of a novel laccase produced by the wood-rotting fungus *Phellinus ribis*. Arch. Biochem Biophys. 392:279-286.
30. Jung, H., Xu, F. and Li, K. (2002). Purification and characterization of laccase from wood-degrading fungus *Trichophyton rubrum* LKY-7. Enz. Microb. Technol., 30:161-168.
31. More, S., Renuka, P. S., Pruthvi, K., Swetha, M., Malini, S. and Veena, S. M. (2011). Isolation, purification, and characterization of fungal laccase from *Pleurotus* sp. Enz. Res. Article ID 248735, 7 pages.
32. Giardina, P., Palmieri, G., Scaloni, A., Fontanella, B., Faraco, V., Cennamo, G. and Sannia, G. (1999). Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. J. Biochem., 341:655-663.

*****