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Partial Purification And Characterization Of Laccase Enzyme Isolated From Carpet And Textile Effluent.

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I. Introduction

Enzyme production is an expanding field of biotechnology. Laccase (E.C. 1.10.3.2, *p*-benzenedial: oxygen oxidoreductase) is able to catalyze the oxidation of various aromatic compounds (particularly phenol) with the concomitant reduction of oxygen to water [1]. Although the enzyme is present in plants, insects and bacteria, the most important source are fungi and particularly basidiomycetes [1,2]. The role of laccase in lignin and phenolic compound degradations has been evaluated in a large number of biotechnological applications such as dye degradation [3,4], bioremediation of some toxic chemical wastes [3,5], wastewater and soil treatments and also biosensor developments [6,7].Due to broad substrate specificity and ability to oxidize wide range of phenols and polyphenols, laccases have received much attention from researchers in the last decade. They also play a vital role in detoxification of textile effluents and bioremediation applications [8]

The existence of laccase in bacteria was reported in *B. halodurans*, *B. subtilis* SF, *Bacillus sp*. HR03, *Azospirillum lipoferum*, *P. desmolyticum* NCIM 2112, *B. pumilus*, *B. subtilis* WP1, and *P. putida*, respectively [9]. Most bacterial laccases are highly thermotolerant and maintain their activity in neutral to alkaline conditions [10], whereas fungal laccases usually drop their activities rapidly at high temperatures and pH. It is well known fact that the majority of agroindustrial wastes are lignocellulosic in nature. The production of laccase from these agroindustrial wastes is of great significance owing to its reuse of industrial waste which reduces the problems arising during disposal and also residues can be utilized for various industrial applications [11]. The selection of suitable natural substrate is greatly influenced by high lignin contents [12]. Moreover, these substrates provide a favourable natural habitat for the secretion of lignocellulolytic enzymes in larger amounts [13, 14].

Production of laccases is affected by many factors such as medium composition, time, pH, temperature, and carbon and nitrogen ratio [15]. The optimal temperature and pH are mainly dependent on the substrate for high yield of laccase [16]. Partial purification of laccase enzyme was carried out using ammonium sulphate precipitation method followed by dialysis [17]. The stability of laccase enzymes at different pH, temperature, and metal ions effect has to be demonstrated for its betterment in industrial applications. Moreover, optimization, partial purification, and characterization of laccase were also carried out in order to study their potential towards wide applications in biotechnology.

II. Method And Material

Enzyme Purification:

Ammonium Salt Precipitation

Ammonium Salt Precipitation was done at 40%, 50%, 60%, 70%, 80% salt precipitation.For this purpose 2ml of crude extract was kept for preservation. Rest volume of crude extract was taken in a small beaker and this beaker was kept in big beaker with full of ice.The beaker was kept on the magnetic stirrer and ammonium sulphate was added pinch by pinch until the first one is completely dissolves. It is done till the ammonium sulphate which was weighed is not finished. When it is dissolved completely, it was put in the refrigerator for 24 hours. Now it was taken into the centrifuge tube and centrifuged at 1000rpm for 10mins at 4^0C.Supernatant was removed and the pellet was collected. 10ml of the Tris Buffer was added to the pellet obtained and was completely dissolved. The crude enzyme as well as the pellet was put in the refrigerator.

Dialysis

To start the process of dialysis, first dialysis bag need to be activated. Dialysis bag activation is done by boiling the bag in distilled water for 1 min twice and then transferring it to 0.1% SDS solution and boiled twice for 1mins, then again it was boiled in distilled water for 1mins, and then finally transferred to the distilled

water. Once the bag is activated, the crude enzyme was transferred to the bag and was sealed. The bag was kept in 100mM Tris buffer pH-7 for 90mins and buffer was again changed and kept overnight in fridge. After keeping it overnight, the buffer was again changed and kept for 90mins.

Ion exchange chromatography

A Biored column was extensively washed with methanol and distilled water. A bad into the column was prepared with glass wool. It was washed with methanol and distilled water. The sullry of DEAE Cellulose 1gm in 10ml, 100mM Tris HCl (prechilled) at pH-7 was added to the column and it was allowed to settle. All the chromatographic operation was conducted at 4^0C. Then dialyzed sample was added to the column. The enzyme was allowed to bind 2 min at 4^0C and was eluted with a linear gradient of NaCl (0.1- 1M) in the same buffer. A total of 50 fractions (10ml per fraction) were collected and assayed for protein and enzyme activity.

Enzyme Assay

Laccase assay was performed using syringaldazine as substrate. Reaction mixture was prepared using 3 ml of Phosphate buffer (100 mM, pH-7), 1ml of 1mM syringaldazine (in absolute ethanol) and 1ml of cultural filtrate where as in blank 1ml of distilled water was used instead of cultural filtrate.

Reagents	**Blank**	**Test**
100mM Phosphate Buffer pH- 7	3 ml	3ml
1mM Syringaldazine (in absolute ethanol)	1ml	1ml
Cultural Filtrate	1ml Distilled water	1ml

The change in absorbance due to the oxidation of syringaldazine (ε=65,000 M−1 cm−1) in the reaction mixture was monitored for 10 min of incubation at 530nm and 30^0C. Laccase activity was expressed in U/ml. U/ml was defined as production of 1uM of colored product/min/ml.

Characterization of laccase enzyme

The kinetic study of partially purified laccase was performed in terms of optimum temperature, optimum pH, effect of metal ion, effect of incubation time and effect of inhibitors.

Characterization of pH

The activity of partially purified laccase was measured at different pH. The pH was adjusted using the phosphate buffer (pH 5, 7, 9, 11). Reaction mixtures were incubated over a range of pH at 30^0C and residual enzyme activity was determined by standard assay method.

Characterization of Temperature

The effect of temperature on partially purified laccase activity was determined by performing the standard assay procedure within a temperature range from 10-100^0C.

Enzyme activity with metal ions

Partially purified laccase was pre incubated with different metal ions (2%) such as lead, zinc, iron, cooper, calcium and magnesium for 10 min at 30^0C and the residual activity was measured. Separate blank with individual metal ions were prepared.

Characterization of Inhibitors

The effect of laccase inhibitors (2%) such as EDTA, sodium azide, ethanol and SDS were investigated. After 10 min preincubation at 30^0C, residual laccase activity was measured by standard assay method.

Effect of incubation time on laccase activity

The effect of incubation time on laccase activity was investigated. Partially purified laccase was kept at 5min, 10min, 15min, and 20min to characterize optimum time for laccase activity.

Characterization of molecular mass of partially purified laccase (SDS-PAGE)

The molecular mass of partially purified laccase was determined by SDS-PAGE. SDS-PAGE of protein was performed, using a mini slab gel apparatus (Bangalore Genei Pvt. Ltd., India). The electrophoretic glass plates, spacers and comb were washed with acetone to remove any grease. Before gel casting the solution is mixed well and poured carefully without introducing any air bubble into the glass plates (10 x 10cm); and 12.5% Running and 4.5% stacking gels were used. Stacking gel is poured over the polymerized separating gel and the comb was introduced carefully without trapping any air bubbles.

After polymerization of stacking gel, comb was removed carefully and gel casted plate was introduced into electrophoresis system containing running buffer. Samples of approximately 0.3 to 10µg of protein were applied to gels in samples loading buffer. Protein samples were denatured by heating them at 100^0C with sample buffer for 3-5 min before loading them onto the gel. Variable current at 60 to 65V was applied across the gels until the tracking dye approached the end of the running gels. The protein bands were visualized with staning and destining of the gel by coomassie blue dye and destaining solution, respectively. The destaining was continued till the gel were transparent and distinct blue bands were visible. The gel was photographed and the relative molecular mass of the protein was calculated using standard protein marker run simultaneously.

III. Result And Discussion:

Purification of Crude enzyme at 40% salt concentration

In purification of enzyme at 40% salt concentration crude enzyme was showing specific activity 0.022 U/mg where as ammonium salt precipitated enzyme was showing specific activity 0.137 with 6.22 fold purification. Dialyzed sample of enzyme was showing specific activity 0.145 U/mg with 6.59 fold purification. Ion exchange fraction 1st was showing highest specific activity 0.126 U/mg with 5.72 fold purification where as fraction 8th was showing minimum specific activity 0.059 U/mg with 2.68 fold purification.

Table 4.11 Purification of Crude enzyme at 40% salt concentration

Volume 78(ml)	Protein mg/ml	Total Protein mg	Enzyme activity U/ml	Total activity Units	Specific activity U/mg	Fold Purification
Crude 82ml	6.18	506.76	0.138	11.31	0.022	1.0
Ammonium Salt Precipitation 89 ml	0.783	69.68	0.108	9.61	0.137	6.22
Dialysis 10ml	0.783	7.83	0.114	1.14	0.145	6.59
Ion Exchange Fraction -1 10 ml	0.513	5.13	0.065	0.65	0.126	5.72
Fraction -2 10 ml	1.05	10.5	0.064	0.64	0.060	2.72
Fraction -3 10 ml	1.05	10.5	0.076	0.76	0.072	3.27
Fraction -4 10 ml	1.05	10.5	0.081	0.81	0.077	3.5
Fraction -5 10 ml	1.05	10.5	0.071	0.71	0.067	3.04
Fraction -6 10 ml	1.05	10.5	0.069	0.69	0.065	2.95
Fraction -7 10 ml	1.05	10.5	0.075	0.75	0.071	3.22
Fraction -8 10 ml	1.05	10.5	0.062	0.62	0.059	2.68
Fraction -9 10 ml	1.05	10.5	0.070	0.70	0.066	3.0
Fraction -10 10 ml	0.783	7.83	0.049	0.49	0.062	2.81

4.11Purification of Crude enzyme at 50% salt concentration

In purification of enzyme at 50% salt concentration crude enzyme was showing specific activity 0.022 U/mg where as ammonium salt precipitated enzyme was showing specific activity 0.071 U/mg with 3.23 fold purification. Dialyzed sample of enzyme was showing specific activity 0.109 U/mg with 4.95 fold purification. Ion exchange fraction 9th was showing highest specific activity 0.551 U/mg with 25.04 fold purification where as fraction 2nd was showing minimum specific activity 0.126 U/mg with 5.72 fold purification.

Table4.12 Purification of Crude enzyme at 50% salt concentration

Volume (ml)	Protein mg/ml	Total Protein Mg	Enzyme activity U/ml	Total activity Units	Specific activity U/mg	Fold Purification
Crude 86ml	6.18	531.48	0.138	11.86	0.022	1.0
Ammonium Salt Precipitation 94 ml	1.32	124.08	0.094	8.83	0.071	3.23
Dialysis 10ml	1.32	13.2	0.145	1.45	0.109	4.95
Ion Exchange Fraction -1 10 ml	0 .513	5.13	.098	0.98	0.191	8.68
Fraction -2 10 ml	0.783	7.83	0.099	0.99	0.126	5.72
Fraction -3 10 ml	0.513	5.13	0.091	0.91	0.177	8.04
Fraction -4 10 ml	0.783	7.83	0.224	2.24	0.286	13.0
Fraction -5 10 ml	0.513	5.13	0.109	1.09	0.212	9.63
Fraction -6 10 ml	0.513	5.13	0.105	1.05	0.204	9.27
Fraction -7 10 ml	0.783	7.83	0.127	1.27	0.162	7.36
Fraction -8 10 ml	0.513	5.13	0.140	1.40	0.272	12.36
Fraction -9 10 ml	0.243	2.43	0.134	1.34	0.551	25.04
Fraction -10 10 ml	0.243	2.43	0.109	1.09	0.448	20.36

4.12 Purification of Crude enzyme at 60% salt concentration

In purification of enzyme at 60% salt concentration crude enzyme was showing specific activity 0.022 U/mg where as ammonium salt precipitated enzyme was showing specific activity 0.071 U/mg with 3.23 fold purification. Dialyzed sample of enzyme was showing specific activity 0.109 U/mg with 4.95 fold purification. Ion exchange fraction 10^{th} was showing highest specific activity 0.539 U/mg with 24.5 fold purification where as fraction 5^{th} was showing minimum specific activity 0.053 U/mg with 2.40 fold purification.

Table4.13 Purification of Crude enzyme at 60% salt concentration

Volume (ml)	Protein mg/ml	Total Protein Mg	Enzyme activity U/ml	Total activity Units	Specific activity U/mg	Fold Purification
Crude 80ml	6.18	494.4	0.138	11.04	0.22	1.0
Ammonium Salt Precipitation 91 ml	1.05	95.55	0.037	3.36	0.035	1.59
Dialysis 10ml	0.513	5.13	0.030	0.30	0.058	2.63
Ion Exchange Fraction -1 10 ml	1.05	10.5	0.91	9.1	0.086	3.90
Fraction -2 10 ml	1.32	13.2	0.120	1.20	0.090	4.09
Fraction -3 10 ml	0.783	7.83	0.069	0.69	0.088	4.0
Fraction -4 10 ml	1.05	10.5	0.064	0.64	0.060	2.72
Fraction -5 10 ml	1.32	13.2	0.071	0.71	0.053	2.40
Fraction -6 10 ml	1.32	13.2	0.165	1.65	0.125	5.68
Fraction -7 10 ml	1.32	13.2	0.133	1.33	0.100	4.54
Fraction -8 10 ml	1.05	10.5	0.113	1.13	0.107	4.86
Fraction -9 10 ml	0.783	7.83	0.124	1.24	0.158	7.18
Fraction -10 10 ml	0.243	2.43	0.131	1.31	0.539	24.5

4.13Purification of Crude enzyme at 70% salt concentration

In purification of enzyme at 70% salt concentration crude enzyme was showing specific activity 0.022 U/mg where as ammonium salt precipitated enzyme was showing specific activity 0.075 U/mg with 3.40 fold purification. Dialyzed sample of enzyme was showing specific activity 0.052 U/mg with 2.36 fold purification. Ion exchange fraction 9th was showing highest specific activity 0.300 U/mg with 13.36 fold purification where as fraction 2nd was showing minimum specific activity 0.002 U/mg with 0.090 fold purification.

Table 4.14 Purification of Crude enzyme at 70% salt concentration

Volume (ml)	Protein mg/ml	Total Protein Mg	Enzyme activity U/ml	Total activity Units	Specific activity U/mg	Fold Purification
Crude 87ml	6.18	537.66	0.138	12.00	0.022	1.0
Ammonium Salt Precipitation 108 ml	1.32	142.56	0.099	10.69	0.075	3.40
Dialysis 10ml	2.13	21.3	0.112	1.12	0.052	2.36
Ion Exchange Fraction -1 10 ml	1.59	15.9	0.109	1.09	0.068	3.09
Fraction -2 10 ml	1.05	10.5	0.03	0.003	0.002	0.090
Fraction -3 10 ml	1.05	1.05	.048	0.48	0.045	2.04
Fraction -4 10 ml	0.783	7.83	0.050	0.50	0.063	2.86
Fraction -5 10 ml	0.573	5.73	0.054	0.54	0.105	4.77
Fraction -6 10 ml	0.513	5.13	0.004	0.04	0.007	0.318
Fraction -7 10 ml	0.243	2.43	0.068	0.68	0.279	12.68
Fraction -8 10 ml	0.513	5.13	0.016	0.16	0.031	1.409
Fraction -9 10 ml	0.243	2.43	0.073	0.73	0.300	13.63
Fraction -10 10 ml	0.243	2.43	0.049	0.49	0.201	9.13

4.14Purification of Crude enzyme at 80% salt concentration

In purification of enzyme at 80% salt concentration crude enzyme was showing specific activity 0.022 U/mg where as ammonium salt precipitated enzyme was showing specific activity 0.077U/mg with 3.50 fold purification. Dialyzed sample of enzyme was showing specific activity 0.036 U/mg with 1.63 fold purification. Ion exchange fraction 10th was showing highest specific activity 0.144 U/mg with 6.54 fold purification where as fraction 8th was showing minimum specific activity 0.046 U/mg with 2.09 fold purification.

Table 4.15 Purification of Crude enzyme at 80% salt concentration

Volume (ml)	Protein mg/ml	Total Protein Mg	Enzyme activity U/ml	Total activity Units	Specific activity U/mg	Fold Purification
Crude 80ml	6.18	494.4	0.138	110.4/	0.022	1.0
Ammonium Salt Precipitation 97 ml	1.32	128.04	0.102	9.89	0.077	3.50
Dialysis 10ml	1.59	15.9	0.058	0.58	0.036	1.63
Ion Exchange Fraction -1 10 ml	0.783	7.83	0.069	0.69	0.088	4.0
Fraction -2 10 ml	0.783	7.83	0.071	0.71	0.090	4.09
Fraction -3 10 ml	1.05	1.05	0.082	0.82	0.050	2.72
Fraction -4 10 ml	0.783	7.83	0.082	0.82	0.104	4.72
Fraction -5 10 ml	0.783	7.83	0.096	0.96	0.122	5.54
Fraction -6 10 ml	1.05	10.5	0.091	0.91	0.086	3.90
Fraction -7 10 ml	0.783	7.83	0.058	0.58	0.074	3.36
Fraction -8	1.32	13.2	0.062	0.62	0.046	2.09

10 ml						
Fraction -9 10 ml	1.05	10.5	0.084	0.84	0.08	3.63
Fraction -10 10 ml	0.783	7.83	0.113	1.13	0.144	6.54

Section4. Characterization of Partially Purified Enzyme

4.15 Effect of temperature on laccase activity

The activity of partially purified laccase was determined at different temperatures ranging from 10^0C to 100^0C. The effect of temperature on the activity of the laccase is shown in Table 4.16. The optimum temperature for enzyme activity was 70^0C at which 39.04% activation in enzyme activity was measured which was closely followed by at 60^0C with 23.80% activation in enzyme activity. A rapid increase in enzyme activity was measured within the temperature range of 10^0C to 70^0C while at 90^0C enzyme lost its16.2% activity and 100^0C it was measured 21.91% lost in its activity. In general, laccases are stable at 30-50^0C and rapidly lose activity at temperatures above 60^0C [18, 19]. [20] reported 65^0C as optimum temperature for purified laccase from *pycnoporus sanguineus*.[21] reported 85^0C as optimum temperature for purified laccase using DMP as substrate. They also reported laccase was unstable at 85^0C but relatively stable at 75^0C whereas [22] reported purified laccase from *Pycnoporus sanguineus* showed high thermostability, retaining 40% of its original activity after 3 hours at 60^0C.[23] reported the typical half life of laccase is below 1h at 70^0C and below 10 min at 80^0C. [24] reported purified laccase has a broad temperature sensitive 35–70^0C and the optimum temperature for the laccase was observed at 65^0C.

Table4.16 Effect of temperature on laccase activity

Temperature	**Enzyme activity U/ml**	**% Inhibition/Activation**
10^0C	0.125	19.04%
30^0C	0.116	10.47%
40^0C	0.122	16.19%
60^0C	0.130	23.80%
70^0C	0.146	39.04%
80^0C	0.119	13.33%
90^0C	0.088	16.2%
100^0C	0.082	21.91%

4.16Effect of pH on laccase activity

The partially purified laccase was active over a broad pH range (pH 5.0-11.0) with maximum activity at pH 7.0. At pH 7.0 partially purified enzymes showed the maximum activity with 1% activation while below and upper this pH rapid loss in enzyme activity was considered. At pH 5 losses in enzyme activity was considered 9.53% whereas at pH 9.0 and11.0 it was 11.43 and 18.1% respectively. This also shows that the enzyme is sensitive to pH change and loses its activity at alkaline or acidic medium. The pH optima of laccases are highly dependent on substrate. The role of the T1 copper in pH optima of the enzyme was confirmed by [25], who found that the T1 copper was absent in laccase enzymes exhibiting more neutral optima.

Table4.17 Effect of pH on laccase activity

pH	**Enzyme activity U/ml**	**% Inhibition/Activation**
pH-5	0.095	9.53%
pH-7	0.106	1%
pH-9	0.093	11.43%
pH-11	0.086	18.1%

4.17Effect of incubation time on laccase activity

Partially purified laccase was kept at 5min, 10min, 15min, and 20min to characterize the optimum time for enzyme activity. It was found that at 15min it showed maximum enzyme activity 0.119U/ml with 13.33% activation in its actual activity while at 5min it showed minimum enzyme activity 0.013U/ml with 87.62% rapid loss in its activity which was closely followed by at 10 and 20 min with rapid loss in its activity 60% and 28.58% respectively.

Table 4.18Effect of incubation time on laccase activity

Time	**Enzyme activity U/ml**	**% Inhibition/Activation**
5 min	0.013	87.62%
10 min	0.042	60%
15 min	0.119	13.33%

20 min	0.075	28.58%

4.18 Effect of inhibitor on laccase activity

The effect of different inhibitors on the enzyme activity of the partially purified laccase was studied because inhibition studies primarily give an idea to the nature of active center of an enzyme. The effect of different inhibitors on the enzyme activity of the partially purified laccase was studied (Table 4.19). The laccase was incubated in 100mM phosphate buffer (pH 7.0) with various inhibitors at 37^0C for 10 min and the remaining activity was measured with syringaldazine as the substrate. SDS was the best inhibitor for laccase enzyme as its showed 35.49% inhibition which was closely followed by ethanol with 32.26% inhibition in enzyme activity. [26] reported 3.4% inhibition in purified laccase activity using 10% ethanol which was rapidly increasing with the increase in concentration of ethanol. In general, organic solvents alter the pH of aqueous solution and there by affect the enzyme activity [27] where as sodium azide and EDTA showed 1% and 2% inhibition in enzyme activity respectively. EDTA is an inhibitor of mettalo-enzymes including laccases due to its ability to form inactive complexes with inorganic cofactors of the enzyme [26,28]. However, it is not true for all laccases, *Marasmius quercophilus* and *Sinorhizobium meliloti* CE52G laccases are not significantly affected by EDTA [29,30]. [31] also reported EDTA inhibits laccase activity to a lesser extent. [32] reported EDTA and SDS slightly inhibited laccase activity at concentration of 10 mM/l.[24] reported SDS and sodium azide inhibited enzyme activity. [28] reported EDTA caused laccase inhibition. It was reported that the binding of sodium azide to the type 2 and type 3 copper sites affects internal electron transfer, thus inhibiting the activity of laccase.

Table4.19 Effect of inhibitor on laccase activity

Inhibitor (2%)	**Relative activity %**
SDS	35.49%
Sodium Azide	1%
EDTA	2%
Ethanol	32.26%

4.19Effect of metal ion on laccase activity

The interaction of metals with extracellular laccase is particularly important for the better understanding of the biotechnological processes of xenobiotic degradation. The effect of various metal ions on the activity of partially purified enzyme is shown in Table 4.20. Different metal ions viz. lead, zinc, iron, cooper, calcium and magnesium were characterized at 2% concentration. All metal ions were activator for laccase enzyme except calcium as it showed 2.42% inhibition in enzyme activity while copper showed maximum activation in enzyme activity with 208.87% which was closely followed by iron with 208.06% activation and lead with 204.83% activation where as manganese showed 194.35% and zinc showed 164.51% activation in enzyme activity.[26] reported metal ions concentration at 1mM had no significant effect over laccase activity except Hg (17.2% inhibition) whereas the metal ions concentration was increased to 5mM, Hg, Cr, Zn and Sn inhibited laccase activity by 25.4, 13.8, 11.4 and 9.7% inhibition. Copper ion at 10mM concentration activated laccase activity by 40%. The activation of laccase by Cu ion may be due to the filling of type-2 copper binding sites with copper ions [33]. The laccase activity was stimulated by 1 mM of Cu+2 and Mg+2, and essentially unaffected by 1 mM of Ca+2 reported by [32].[28] reported copper ion caused laccase activation whereas Fe and Mn ion caused laccase inhibition. Addition of Cu and Mn ion increase the activity of WRF laccases, in most cases [34,35]. The observations indicated that the effect of metal ions on laccase activity was highly dependent on its source and the type of metals used, which had a great influence on the catalytic activity of the enzyme. The activation and inhibition of proteolytic enzymes by metals could change the turnover rate of extracellular enzymes.

4.20Effect of metal ion on laccase activity

Metal Ion (2%)	**Relative activity %**
Lead	204.83%
Zinc	164.51%
Iron	208.06%
Copper	208.87%
Calcium	2.42%
Manganese	194.35%

4.20 SDS PAGE of partially purified laccase

Molecular weight of the purified enzyme was determined by SDS PAGE. Medium range's protein ladder was used for this purpose. Molecular weight of purified enzyme was found 99kDa. (Juana et al., 1996) reported molecular mass 94kDa of laccase from the *Phanerochaete flavido-alba.*

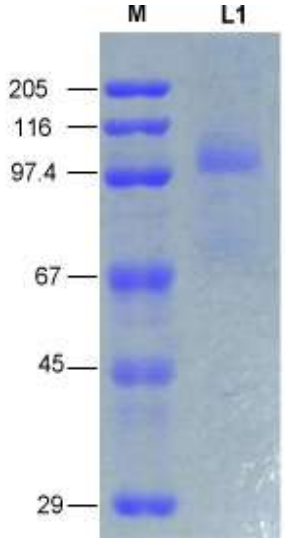

Figure 4.3 SDS Page of purified enzyme

IV. Conclusion

On behalf of result it is clear that the ammonium salt precipitated enzyme was showing highest specific activity 0.137 with 6.22 fold purification at 40% salt concentration where as ammonium salt precipitated enzyme was showing lowest specific activity 0.071 U/mg with 3.23 fold purification at 60% salt concentration. In case of dialysis, dialyzed sample of enzyme was showing highest specific activity 0.145 U/mg with 6.59 fold purification at 40%salt concentration where as lowest specific activity 0.036 U/mg with 1.63 fold purification was found at 80% salt concentration. Ion exchange fraction 9^{th} was showing highest specific activity 0.551 U/mg with 25.04 fold purification at 50% salt concentration where as fraction 2^{nd} was showing minimum specific activity 0.002 U/mg with 0.090 fold purification at 70% salt concentration. Enzyme was active over 90^0C, optimum pH was pH 7,15 min was best incubation time, SDS was best inhibitor, where as all metal ions were activator for laccase enzyme except calcium as it showed 2.42% inhibition in enzyme activity while copper showed maximum activation in enzyme activity with 208.87%. Partially purified laccase showed 99kDa its molecular weight.

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