

Full Length Research Paper

Solid State Fermentation for the production of Laccase by *Neurospora sitophila* using agro-wastes and its partial purification

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Laccase is the enzyme that has many industrial and biotechnological. Keeping in mind the importance of this enzyme on commercial and industrial levels, the present study was designed to optimize laccase production by *Neurospora sitophila* using agro-wastes including rice straw, sugarcane bagasse and corn cobs. Among the different conditions optimized, for the enhanced production of the laccase, include fermentation period, moisture level and inoculum size and were found to be 96 hours, 70% (for corn cobs and rice straw), 60% (for sugarcane bagasse) and 5mL respectively. Peptone, yeast extract and tween-20 were used as nitrogen source, inducer and surfactant respectively, to check their effect on the production of laccase. Peptone had negative effect in all concentrations while 0.4 % yeast extract and 0.2 % tween-20 enhanced the laccase production. Ammonium sulfate was used for the partial purification of the laccase and 60% ammonium sulfate was found to be the best concentration for this purpose with a 7.5 fold increase in the laccase activity as compared to the crude extract while using corn cobs as agriculture waste. Protein concentration of crude extract and after each purification step was determined by Biuret-protein assay. Optimum temperature and pH for the laccase activity were found to be 30°C and 5 respectively. *K_m* and *V_{max}* using guaiacol were found to be 0.666mM and 20.8 µM/min respectively.

Key words: Laccase, *Neurospora sitophila*, Guaiacol, solid state fermentation, ammonium sulfate.

INTRODUCTION

Laccase (E.C. 1.10.3.2; *parabenzenediol*: oxygen: oxidoreductase) belongs to a group of Cu containing polyphenol (PP) oxidases, known as multicopper (MC) oxidases (Birhanli and Yesilada, 2006; Arora and Sharma, 2010; Elsayed *et al.*, 2012). This enzyme causes the oxidation of many phenolic compounds with the help of molecular oxygen (O₂), which act as the acceptor of electrons (Sharma *et al.*, 2007) and reduces this oxygen to water (Sathiskumar *et al.*, 2010). Laccase has low specificity towards substrate and can degrade many of xenobiotic compounds including industrial colored effluents (Riva, 2006; Dsouza *et al.*, 2006).

Laccase has been identified in different fungal species, plants (Sharm and Kuhad, 2008; Arora and Sharma, 2010), insects (Elsayed *et al.*, 2012), bacterial species (Kunamneni *et al.*, 2010). The presence of laccase is limited in higher plants than in fungi. Laccase has been reported in turnip, pears, mango, peach, prune pine, lacquer, mung bean, cabbages, potatoes, apples and other vegetable (Mohammadian *et al.*, 2010).

The utilization of laccase in various fields has been ignored in last few years because of its non-availability for commercialization (Riva, 2006). This enzyme has so many industrial and biotechnological applications due to its ability of nonspecific oxidation of many phenolic and non-phenolic compounds (Poojary and Mugeraya, 2012). Laccases are used for the cleaning of industrial effluents including paper pulp industry, textile industry and petrochemical industries. These are effectively used in cleaning of herbicide, explosive from soil, pesticide and

medical diagnostics (Davis and Perkins, 2002; Arora and Sharma, 2010).

Neurospora sitophila is a specie belonging to genus *Neurospora* and kingdom fungi and is well known model organism for experiments (Borkovich *et al.*, 2004). *Neurospora sitophila* has been exploited in the study of photobiology, molecular genetics, gene silencing, biochemistry, evolution, physiology, population studies and circadian rhythms in different projects (Patel *et al.*, 2009).

Solid state fermentation (SSF), defined as the fermentation of solids in the absence of free water, has the advantage of supporting the growth and metabolism of microorganisms under moist conditions. Production of enzymes by SSF on agro-wastes has gained much attention in biotechnology due to its higher productivity and low production cost. The use of such wastes, beside providing alternative substrates, helps to solve environmental problems, which are caused by their disposal in the open environment. Furthermore, most of them are rich in sugars, which make the whole process much more economical (Satishkumar *et al.*, 2010). Various cultural conditions can be optimized in research laboratory to increase the yield of laccase as its yield is highly dependent on these cultural conditions. Furthermore the cost of the yield can also be reduced by using agro-wastes (Arora and Sharma, 2010; Strong, 2011).

Considering the above described facts the current study was planned and carried out for enhancing laccase production by optimizing various cultural and nutritional conditions during Solid State Fermentation by *N. sitophila*.

MATERIALS AND METHODS

Substrates Preparation: Selected agro wastes (rice straw, corn cobs and sugarcane bagasse) were cut into small pieces, dried in sunlight for one week and placed in oven at 70 °C for 72 hours to remove all moisture. The dried pieces of substrate were ground with grinder from Soil Sciences Department of PMAS, Arid Agriculture University Rawalpindi and meshed with 40mm sieve, stored in small plastic jars and were used for solid state fermentation (SSF) for the production of laccase.

Fermentative Organism: The culture of *Neurospora sitophila* was grown on potato dextrose agar (PDA) slants. The composition of the medium was; agar (2.0g), glucose (2.0g), (NH₄)₂SO₄ (0.02g), Calcium chloride (0.005 gm), Magnesium sulfate.7H₂O (0.005 gm), potassium dihydrogen phosphate (0.02 gm) and distill water to make total volume of 100mL (Mahmood *et al.*, 2013). The pH of the medium was maintained at 5.5. Fungal Slants were incubated in incubator at 30 °C for 96 hours (Milner, 1997).

Inoculum: Inoculum was prepared to preserve the fungal spores for future use. Erlenmeyer flasks of 500 mL were

used for its preparation with following composition; glucose (2.0g), (NH₄)₂SO₄ (0.02g), Calcium chloride (0.005 gm), Magnesium sulfate.7H₂O (0.005 gm), potassium dihydrogen phosphate (0.02 gm) and distill water to make total volume of 100mL. Fungal culture was transferred aseptically to the flasks containing the liquid medium and the flasks were incubated in shaking incubator at 150 rpm and 30 °C for 72 hours. Number of spores was adjusted between 10⁷-10⁸ spores/mL (Krishna, 1999).

Solid State Fermentation: The grounded agro wastes were poured in Erlenmeyer flasks of 500mL capacity. These were then moistened with mineral salts solution having composition of; KH₂PO₄ (0.5%); (NH₄)₂SO₄ and MgSO₄.7H₂O (0.2%). Flasks were plugged with cotton and autoclaved at standard conditions. These were then inoculated with 5 mL of inoculum medium under aseptic conditions and were incubated at 30 °C.

Laccase Harvesting: After specified days of incubation, laccase was extracted by a simple contact method. For this purpose 100mL of tris-HCl buffer (pH 8) was added in the flasks. The flasks were placed on incubator shaker at 150 rpm for 1 hour. Mixture was then filtered with filter paper and the filtrate was centrifuged at 10,000 rpm for 10 minutes at -10 °C to remove all spores and other impurities (mahmood *et al.*, 2013). The supernatant was collected and subjected to laccase assay.

Enzyme Assay: Laccase catalyzed the hydrolysis of guaiacol which results in the reduction of its colour intensity. Enzyme activity was calculated by using method describe by Li *et al.*, 2008 with slight modification. Assay mixture containing 0.1mL of enzyme solution, 0.1mL pure H₂O₂, 1mL guaiacol reagent, 0.1mL of 0.1M sodium acetate buffer (pH 4.8) and 5mL distilled water were added into marked test tubes. Blank was also prepared containing additional 0.1mL distilled H₂O instead of enzyme solution. All of the mixtures were mixed well and were placed at 30 °C for one hour and absorbance was taken at 420nm.

Laccase activity was measured as decrease in absorbance of Guaiacol reagent (substrate) due to laccase enzyme (1mL) in 1 hour. It was calculated as follow;

$$1\text{U/mL/min} = \frac{\text{Decrease in the absorbance of guaiacol reagent} \times \text{dilution factor}}{\text{Incubation period}}$$

Experimental Design and Optimization of Different Parameters: Different parameters for the solid state fermentation were optimized by studying their effect on solid state fermentation. These parameters with their varying levels/concentrations include fermentation period (24, 48, 72, 96 and 120 hours), moisture level (40% to 80% with difference of 10) and inoculum size (3, 4, 5, 6 and 7mL), peptone as nitrogen source (0.1% to 0.5%), Yeast extract (0.1% to 0.5%) and Tween-20 as surfactant (0.1% to 0.5%). Each optimized parameter was maintained in next experiment. All of the treatments were performed in duplicates.

Table 1. Composition of Biuret reagent, use for the protein estimation in sample.

No.	Chemical	Qty in 1 Litr
1	NaOH	8.0 g
2	CuSO ₄ .5H ₂ O	3.0 g
3	KI	5.0 g
4	Sodium Potassium Tartarate	96.0 g
5	D.H ₂ O	Up to 1000 mL

Table 2. Protein estimation by Biuret assay.

Sample No.	D. H ₂ O (mL)	Protein Standard (4mg/mL) conc. (mL)	Biuret Reagent conc. (mL)	Total Conc. (mL)	Protein conc. (mg/mL)	OD at 540nm
1*	0.50	-	1.00	1.50	0.00	0.000
2	0.40	0.10	1.00	1.50	0.40	0.052
3	0.30	0.20	1.00	1.50	0.80	0.096
4	0.20	0.30	1.00	1.50	1.20	0.150
5	0.10	0.40	1.00	1.50	1.60	0.186
6	-	0.50	1.00	1.50	2.00	0.248

* Blank, which was run without the standard BSA.

Protein Estimation By Biuret Assay Method: Biuret method was used for the estimation of protein in the sample (Galhaup et al., 2002) (Table 1).

Biuret Assay: Bovine Serum Albumin (BSA) was used as a standard for protein estimation. Various concentrations of Bovine Serum Albumin (BSA) were prepared (Table 2). Standard curve was obtained by making a graph of absorbance against the different concentrations of BSA (Figure 1). Protein in the crude enzyme samples were calculated through simple linear regression equation after running samples in spectrophotometer and specific activity was also determined.

Ammonium Sulphate Precipitation: Laccase was partially purified with ammonium sulphate. Various concentrations (30%, 40%, 50%, 60%, 70% and 80%) of (NH₄)₂SO₄ were added to the 10mL of crude laccase extract. Mixture was then placed overnight for precipitation. It was then centrifuged and supernatant was subjected to laccase assay. Assay was also

performed with filtrate by dissolving it in 0.1M Na.acetate buffer (pH 4.8) and the activities were taken.

Characterization of the enzyme: Partially purified Laccase was then characterized for optimum pH, temperature, substrate affinity and kinetics parameters like Km and Vmax..

Temperature and pH Characterization: Sanyal et al method described in 1988 was used to determine optimum temperature of laccase activity. Laccase assay was performed at various temperatures ranging from 20 0C to 80 0C with the difference of 10 0C. For the optimization of pH for laccase activity, laccase assay was performed at different pH by using 0.1M Na.acetate buffer (pH 3-5.5) and phosphate buffer (pH 5.5-8).

Effect of Substrate Concentration and Study of Kinetic Parameters: Effect of substrate concentration on enzyme activity and the affinity of laccase towards substrate was determined by performing activity assay with various concentrations of substrate (Guaiacol). The results obtained

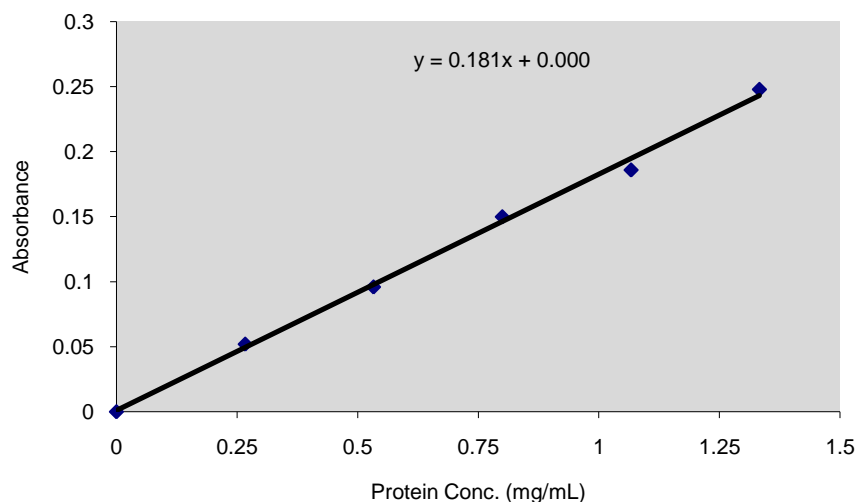


Figure 1. Standard curve drawn using different concentrations of BSA for the determination of protein concentration in the crude enzyme sample and ammonium sulfate partially purified samples.

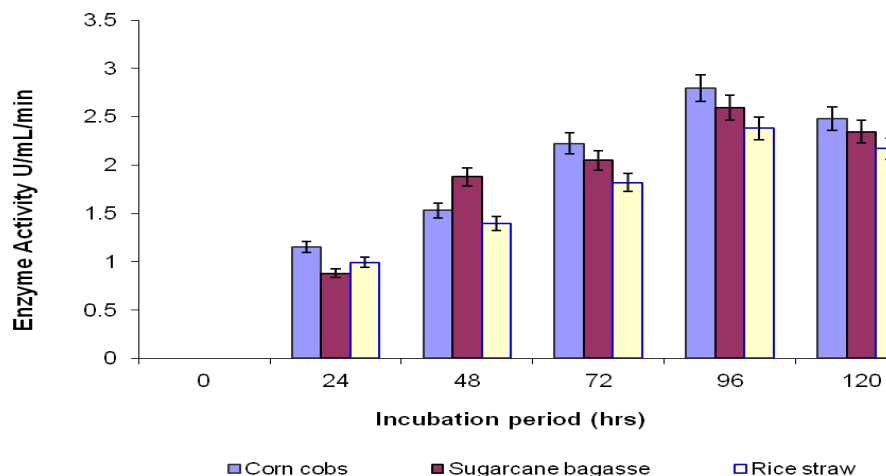


Figure 2. Optimization of fermentation period for the production of laccase using three agro-wastes by *Neurospora sitophila*

were used to determine K_m and V_{max} of laccase.

RESULTS AND DISCUSSION

Fermentation Period: Maximum laccase activities were found to be 2.795 ± 0.03 U/mL/min, 2.595 ± 0.03 U/mL/min and 2.38 ± 0.04 U/mL/min using corn cobs, sugarcane bagasse and rice straw as substrates after 96 hours of fermentation period. Fermentation period of 120 hours showed a decrease in the laccase activities which were found to be 2.48 ± 0.07 U/mL/min, 2.345 ± 0.04 U/mL/min, and 2.17 ± 0.02 U/mL/min (Figure 2). Different optimum fermentation periods (48 hrs to 400

hrs) have been reported for different fungal species (Osama *et al.*, 2007; Niladevi and Perma, 2008; Revankar *et al.*, 2007; Strong, 2011). Maximum laccase production after 96 hr of fermentation was also reported by Galhaup *et al.* (2002) and Viswanath *et al.* (2008).

Moisture Content: Among the various moisture levels tested for the production of laccase, maximum laccase activity was obtained at 70% moisture level for corn cobs and rice straw and 60% for sugarcane bagasse (Figure 3).

The least laccase activities were obtained in case of control having no moisture (0.725 U/mL/min, 0.423 U/mL/min and 0.327 U/mL/min for corn cobs, sugarcane bagasse and rice straw respectively), showing that moisture is important for laccase production. There

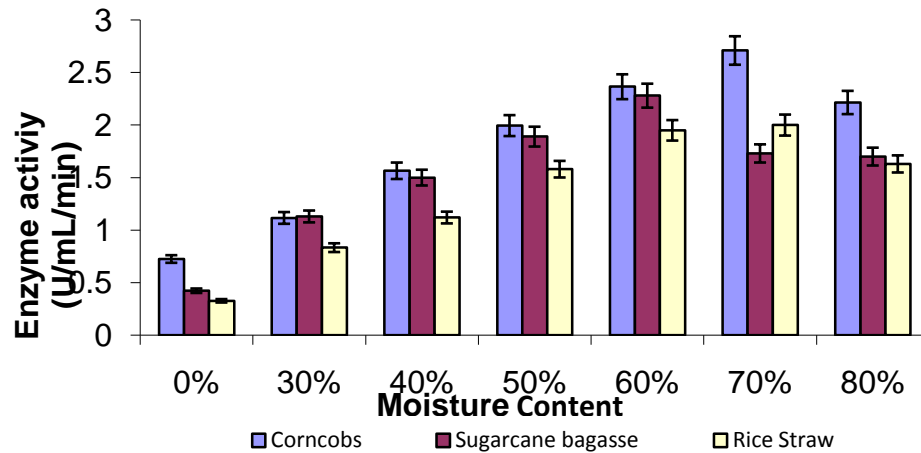


Figure 3. Moisture content optimization for laccase production using three agro-wastes by *Neurospora sitophila*.

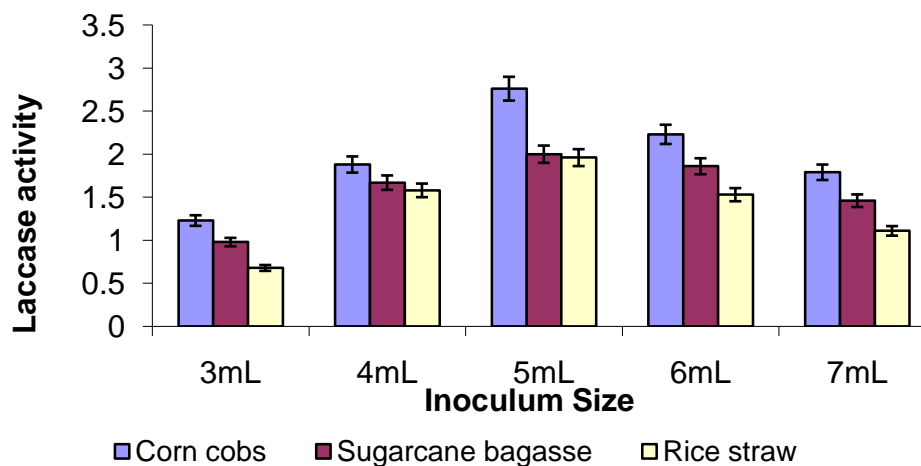


Figure 4. Inoculum size optimization for the production of laccase using three agro-wastes by *Neurospora sitophila*.

was maximum laccase production at 60% of moisture level using sugarcane bagasse as a substrate with decreasing activities at 50% (1.89U/mL/min), 70% (1.73U/mL/min) and 80% (1.70U/mL/min).

Various moisture levels have been reported by different researchers, which range from 60% to 85% for different fungi (Niladevi *et al.*, 2007; Mishra and Kumar, 2008; Patel *et al.*, 2009; Xi and Gang, 2010). Optimum moisture levels of 70.96% and 72-76% for laccase production by different fungi have been reported in previous studies (Saffain *et al.*, 2010; Strong, 2011). While, Niladevi *et al.* (2007) and Patel *et al.* (2009) has reported optimum moisture level for fungal laccase production to be 65% and 60% respectively.

Size of Inoculum: 3mL, 4 mL, 5 mL, 6 mL and 7mL of inoculum containing 10^6 - 10^8 spores/ml were used for the

production of laccase. Results showed that 5mL of the inoculum was the optimum inoculum size for the production of laccase by *Neurospora sitophila* on sugarcane bagasse, corn cobs and rice straw as substrates with laccase activities of 2.08U/mL/min, 2.76U/mL/min and 1.96U/mL/min respectively. After that there was decreased in the production of laccase, showing laccase activities of 2.23U/mL/min, 1.86U/mL/min and 1.53U/mL/min with corn cobs, sugarcane bagasse and rice straw respectively at 6mL of inoculum size (Figure 4). This is possibly due to non availability of substrate with the increasing amount of inoculum.

An increase in the production of laccase up to certain inoculum size and then gradual decrease has been reported by Revankar *et al.* (2007) and Patel *et al.*

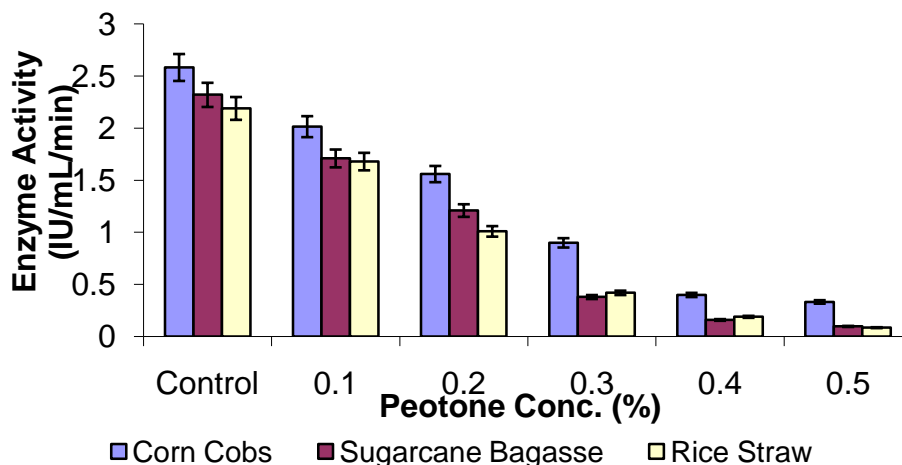


Figure 5. Evaluating the effect of peptone for the production of laccase using three agro-wastes by *Neurospora sitophila*.

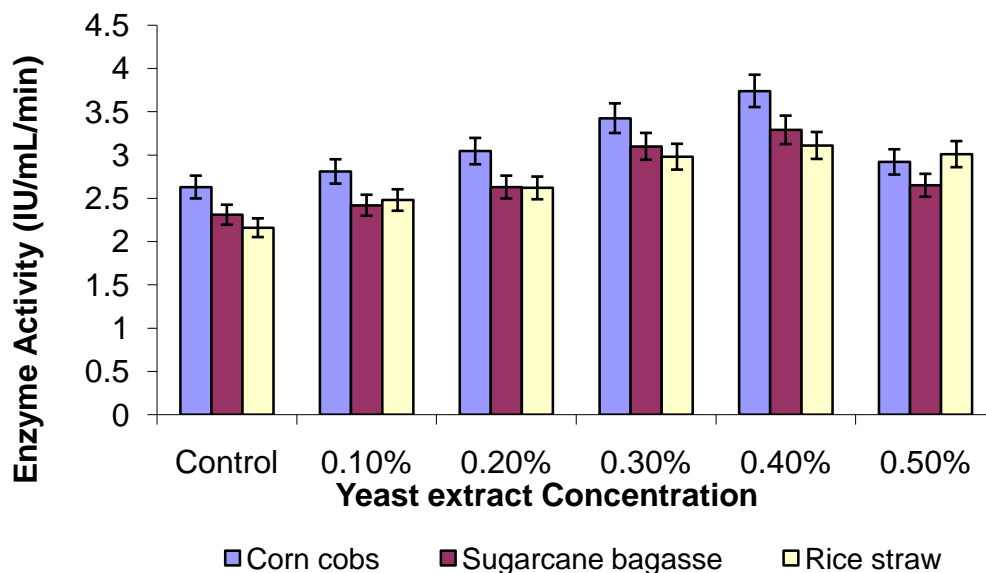


Figure 6. Yeast extract effect on the production of laccase using three agro-wastes by *Neurospora sitophila*.

(2009). This decrease in the laccase production after certain inoculum size is possibly due to the competition between the fungal spores for nutrition and decreased production of laccase (Revankar *et al.*, 2007; Patel *et al.*, 2009).

Peptone Level: All levels of the peptone used showed negative effect on the production of laccase. A decrease in the production of laccase was observed by increasing peptone the concentration of peptone. Maximum production was observed in control having no peptone (2.583, 2.32 and 2.19 IU/mL/min with corn cobs, sugarcane bagasse and rice straw respectively) (Figure 5). Galhaup *et al.* (2002) has reported the decreased laccase activity with the addition of peptone from casein,

supplied by Merk and Fluka.

Yeast Extract: Yeast extract showed good effect on laccase production. Increase in the yeast concentration showed an increase in the laccase production upto 0.4% (3.74, 3.21 and 3.11 IU/mL/min with corn cobs, sugarcane bagasse and rice straw respectively), after that there was decreased in production (Figure 6). Galhaup *et al.* (2002) and Niladevi *et al.* (2009) has reported that there is increase in the production of laccase by the addition of yeast extract because it acts as a good nitrogen source (Niladevi and Perma, 2008).

Tween-20: Laccase production was enhanced by the addition of tween-20. A concentration of 0.2% of tween-20 was found to be the optimum for the production of

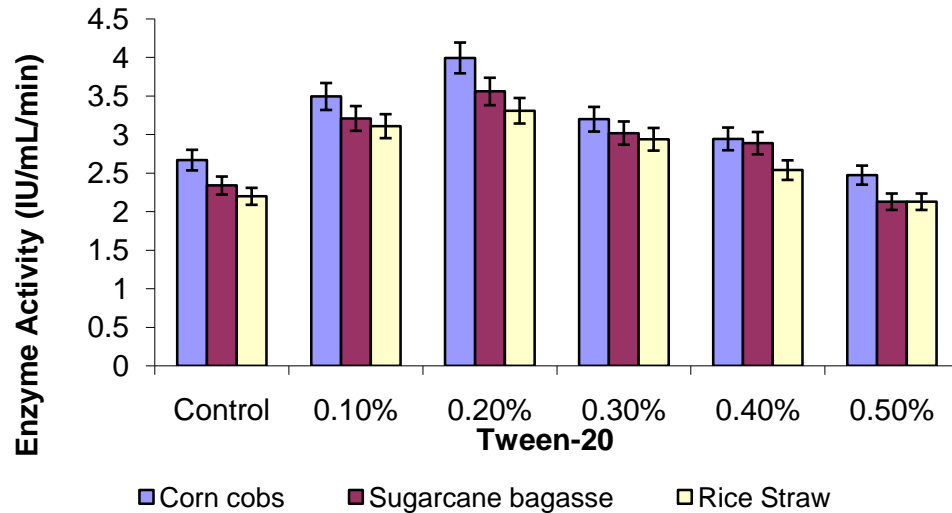


Figure 7: Production of laccase with varying tween-20 conc. by *Neurospora sitophila*.

Table 3. Protein concentrations in the crude and ammonium sulfate purified enzyme samples produced, using rice straw, sugarcane bagasse and corn cobs as substrates by *Neurospora sitophila*.

Substrate	Enzyme Sample (mL)	Vol. of Biuret Reagent	Total Volume (mL)	Absorbance	Protein Conc. mL Enzyme Sample	/0.5 of	Protein Conc. /mL of Enzyme Sample	Mean Protein conc. /mL of Enzyme sample
Corn Cobs	Crude Extract	0.5	1	1.5	0.784	4.31	8.62	8.49±0.183
		0.5	1	1.5	0.761	4.18	8.36	
	Partial purified	0.5	1	1.5	0.241	1.32	2.64	2.58±0.085
		0.5	1	1.5	0.229	1.26	2.52	
Sugarcane Bagasse	Crude Extract	0.5	1	1.5	0.721	3.96	7.92	7.87±0.07
		0.5	1	1.5	0.712	3.91	7.82	
	Partial purified	0.5	1	1.5	0.238	1.31	2.62	2.56±0.085
		0.5	1	1.5	0.228	1.25	2.50	
Rice Straw	Crude Extract	0.5	1	1.5	0.709	3.90	7.80	7.85±0.07
		0.5	1	1.5	0.719	3.95	7.90	
	Partial purified	0.5	1	1.5	0.217	1.19	2.38	2.32±0.085
		0.5	1	1.5	0.207	1.13	2.26	

laccase and showed laccase activity of 3.99 IU/mL/min (corn cobs), 3.56 IU/mL/min (sugarcane bagasse) and

3.31 IU/mL/min (rice straw). But after 0.2% of tween-20 there was a decrease in laccase production (Figure 7).

Table 4. Purification of laccase by the addition of 60% of ammonium sulfate.

Fungal Substrate		Vol. (mL)	Laccase activity (U/mL/min)	Protein conc. (mg/mL)	Total laccase activity	Protein conc. (Total)	Specific activity (U/mg)	Laccase purification (folds)
Corn Cobs	Crude enzyme	200	3.995	12.78	799	2556	0.31	1
	Ammonium sulfate purified enzyme	10	9.06	3.91	90.6	39.1	2.32	7.5
Sugarcane Bagasse	Crude enzyme	200	3.56	11.86	712	2264	0.30	0.96
	Ammonium sulfate purified enzyme	10	8.36	3.87	83.6	38.7	2.16	6.97
Rice Straw	Crude enzyme	200	3.07	11.82	614	1182	0.26	0.85
	Ammonium sulfate purified enzyme	10	7.07	3.52	70.7	35.2	2.01	6.23

Positive effect of tween-20 on laccase production has also been reported by Patel *et al.* (2009), Osama *et al.* (2007) and Saparrat *et al.* (2007). Addition of 0.1% of tween-20 gave the maximum laccase production by fungi (Saparrat *et al.* (2007).

Protein Determination by Biuret Method and Specific Activity Determination: Different concentrations of bovine serum albumin (BSA) were used as standard for protein determination. A standard curve was prepared in Microsoft Excel and a linear regression equation ($y=ax+b$) was also inserted in the curve. Protein concentrations observed in our enzyme samples are shown in the table 2. Specific activities were also determined for the enzyme produced by *N. sitophila* using the three substrates i.e. corn cobs, rice straw and sugarcane bagasse and are shown in the purification chart (Table 3).

Ammonium Sulfate Precipitation: Among different concentrations of ammonium sulfate (30%, 40%, 50%, 60% and 70%) used for the precipitation of laccase, 60% was found to be optimum showing the laccase activity of 9.06, 8.36 and 7.07 IU/mL/min with corn cobs, sugarcane bagasse and rice straw. The protein content decreased after the partial purification, but the specific activity increased (Table 4).

Characterization of the Laccase Enzyme

Optimization of the temperature for laccase activity: Our results indicated 30 °C to be the optimum temperatures for laccase activity. Moreover it was also observed that laccase remained stable between 20 to 40 °C as indicated by the Figure 8. Then there was gradual decrease in laccase activity upto 70 °C due to destruction in the structure of laccase.

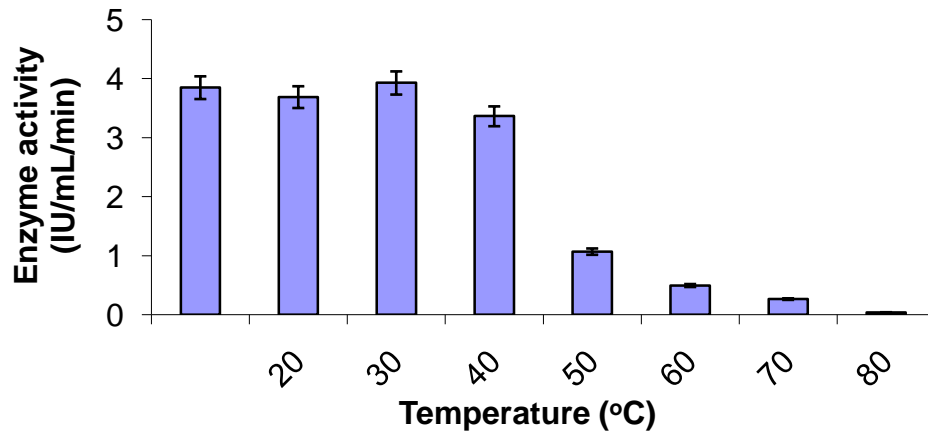


Figure 8. Effect of temperature on laccase activity produced by *Neurospora sitophila*.

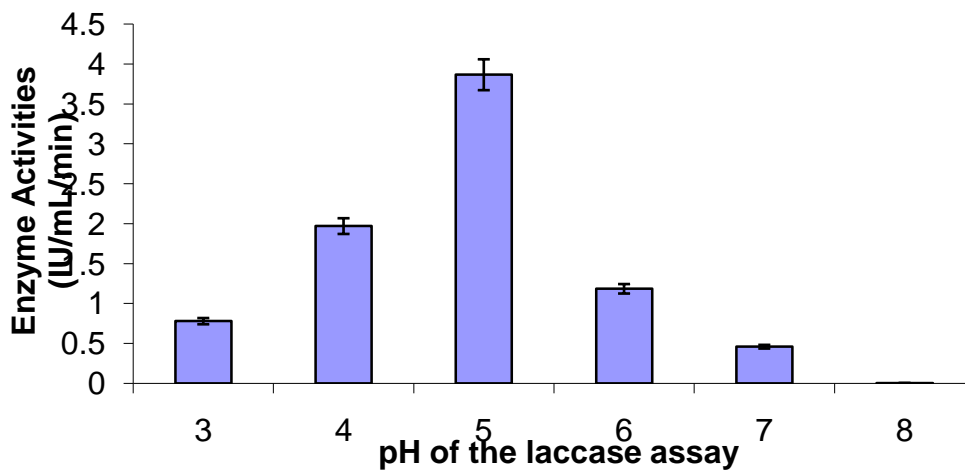


Figure 9. Optimization of pH for the laccase activity.

Laccase obtained from different organisms showed different optimum temperature ranging from 30 °C to 60 °C. Kammoun *et al.* (2009) has reported 55 °C, Sahay *et al.* (2009) 60°C while Dominguez *et al.* and Perez *et al.* (1996) have reported 30 °C as optimum temperature for laccase (Dominguez *et al.*, 2007). This variation suggests that there are different types of laccases produced and used by different fungi.

Optimization of pH for Laccase Activity: The results of current study showed that pH 5 is the optimum pH for the activity of laccase. There was lower laccase activity on the both sides of this pH value (Figure 9). Different pH values are reported as optimum pH for laccase produced by different fungal species. Dong and Zhang (2004) report pH 6 to 9, Perez *et al.* (1996) have reported pH 5 and Rotkova *et al.* (2009) report 3.5 and 5 for laccase

depending upon the type of substrate (ABTS and SGZ) for laccase assay. Various pH values have been reported but most of these are around pH 5.

Effect of Substrate Concentration and Study of Kinetic Parameters: Different concentrations of guaiacol reagent (from 2mM to 10 mM) were prepared to check the effect of substrate concentration on laccase activity and to find the kinetic parameters of laccase. Enzyme velocity (V_0) was calculated by performing laccase assay with each of the concentration and observing the decrease in the concentration of guaiacol. A double reciprocal plot (Line-weaver Burk plot) of $1/V_0$ vs $1/S$ was prepared to get the values of K_m and V_{max} for laccase.

The results indicated that there is linear relation between laccase and its substrate, there is increase in activity with increasing substrate concentration. After certain concen-

tration the rate of increase in the velocity decreased due to occupation of active sites of enzyme by the substrate and finally there was no increase in the rate of reaction. Further addition of the substrate had no effect on laccase activity. The calculated value of K_m and V_{max} for laccase were found to be 0.666 mM and 20.8 $\mu\text{M}/\text{min}$ respectively.

Laccase has different K_m and V_{max} values for different kind of substrates used. Some of the reported K_m values for laccase with different substrate are 480 μM (2,6-

dimethoxyphenol), 350 μM (syringaldize), 320 μM (pyrogallol), 230 μM (catechol) and 210 μM (m-cresol). Sahay *et al.* (2009), Dong and Zhang (2004) have reported 0.001mM K_m for laccase using ABTS as a substrate for one type of laccase and 0.00086mM for the other type of laccase. They have also reported K_m using guaiacol reagent as substrate for laccase and is reported to be 0.405mM for one type of laccase and 0.40mM for other type of laccase (Rotkova *et al.*, 2009; Shradha *et al.*, 2011).

REFERENCES

- Arora DS, Sharma RK (2010). Ligninolytic fungal laccases and their biotechnological application. *Appl. Biochem. Biotechnol.* 160: 1760-1788.
- Birhanli E, Yesilada O (2006). Increased production of laccase by pellets of *Funalia trogii* ATCC 200800 and *Trametes versicolor* ATCC 200801 in repeated-batch mode. *Enzy. Microbial. Technol.* 39: 1286–1293.
- Borkovich KA, Alex LA, Yarden O, Freitag M, Turner GE, Read ND, Seiler S, Pedersen DB, Paietta J, Plesofsky N, Plamann M, Tanrikulu MG, Schulte U, Mannhaupt G, Nargang FE, Radford A, Selitrennikoff C, Galagan JE, Dunlap JC, Loros JJ, Catcheside D, Inoue H, Aramayo R, Polymenis M, Selker EU, Sachs MS, Marzluf GA, Paulsen I, Davis R, Ebbole DJ, Zelter A, Kalkman ER, Rourke RO, Bowring F, Yeadon J, Ishii C, Suzuki K, Sakai W, Pratt R (2004). Lessons from the Genome Sequence of *Neurospora crassa*: Tracing the Path from Genomic Blueprint to Multicellular Organism. *Microbiol. Mol. Biol. Rev.* 68(1): 1-108.
- Davis RH, Perkins DD (2002). Timeline: *Neurospora*: a model of model microbes. *Nat. Rev. Genet.* 3:397–403.
- Dominguez A, Gmez J, Lorenzo M, Sangroman A (2007). Enhanced production of laccase by *Trametes versicolor* immobilized into alginate beads by the addition of different inducers. *World J. Microbiol. Biotechnol.* 23: 367-373.
- Dong JL, Zhang YZ (2004). Purification and Characterization of two laccases isoenzymes from a ligninolytic fungus *Trametes gallica*. *Prep. Biochem. Biotechnol.* 34(2): 179-194.
- Dsouza DT, Tiwari R, Sah AK, Raghukumar C (2006). Enhanced production of laccase by marine fungus during treatment of colored effluents and synthetic dyes. *Enzyme Microbial. Technol.* 38: 504-511.
- Elsayed MA, Hassan MM, Elshafei AM, Haroun BM, Othman AM (2012). Optimization of cultural and nutritional parameters for the production of laccase by *Pleurotus ostreatus* ARC280. *Brit. Biotechnol. J.* 2(3): 115-132
- Galhaup C, Wagner H, Hinterstoisser B, Haltrich D (2002). Increased production of laccase by the wood-degrading basidiomycetes *Trametes pubescens*. *Enzy. Microbial Technol.* 30: 529–536.
- Kammoun MM, Mechichi HZ, Belbahri L, Woodward S, Mechichi T (2009). Malachite green decolorization and detoxification by the laccase from a newly isolated strain of *Trametes sp.* *Int. Biodet. Biodegrad.* 63: 600-606.
- Krishna C (1999). Production of bacterial cellulases by solid state bioprocessing of banana wastes. *Bioresour. Technol.* 69:231-239.
- Kunamneni A, Camarero S, Burgos CG, Plou FJ, Ballesteros A, Alcalde M (2008). Engineering and applications for fungal laccases for organic synthesis. *Microb. Cell Fact.* 7:32 doi:10.1186/1475-2859-7-32.
- Mahmood RT, Asad MJ, Mehboob N, Mushtaq M, Gulfranz M, Hadri SH, Asgher M, Minhas NM (2013). Production, Purification and Characterization of Exoglucanase by *Aspergillus fumigates*. *Appl. Biochem. Biotechnol.* 170(4): 895-908.
- Milner RJ (1997). *Metarhizium flavoviride* (FI-985) as a promising mycoinsecticide for Australian acridids. *Memoirs Entomol. Soc. Can.* 171: 287-300.
- Mishra AS, Kumar S (2008). Application of Box-Benhen experimental design for optimization of laccase production *Coriolus versicolor* MTCC138 in solid-state fermentation. *J. Sci. Indus. Res.* 67: 1098-1107.
- Mohammadian M, Roudsari MF, Mollania N, Dalfard AB (2010). Enhanced expression of recombinant bacterial laccase at low temperature and microaerobic conditions: purification and biochemical characterization. *J. Ind. Microbiol. Biotechnol.* 37: 863-869.
- Niladevi KN, Perma P (2008). Effect of inducers and process parameters on laccase production by *Streptomyces psammoticus* and its application in dye decolorization. *Biosour. Technol.* 99: 4583-4589.
- Niladevi KN, Sukumaran RK, Jacob N, Anisha GS, Prema P (2009). Optimization of laccase production from a novel strain-*Streptomyces psammoticus* using response surface methodology. *Microbiol. Res.* 164: 105-113.
- Niladevi KN, Sukumaran RK, Prema P (2007). Utilization of rice straw for laccase production by

- Streptomyces psammoticus* in solid-state fermentation. J. Ind. Microbiol. Biotechnol. 34: 665-674.
- Osama JF, Saravia V, Herrera JLT, Couto SR (2007). Mandarin peelings: The best carbon source to produce laccase by static culture of *Trametes pubescens*. Chemosphere. 67: 1677-1680.
- Patel H, Gupte A, Gupte S (2009). Effect of different culture conditions and inducers on production of laccase by a *Basidiomycete* fungal isolated *Pleurotus ostreatus* HP-1 under solid state fermentation. Bioresour. 4(1): 268-284.
- Perez J, Martinez J, Rubia TD. (2009). Purification and partial characterization of a laccase from the white rot fungus *Phanerochaete flavid-alba*. Appl Env Microbiol. 4263-4267.
- Poojary H, Mugeraya G (2012). Laccase production by *Phellinus noxius* hpF17: Optimization of submerged culture conditions by response surface methodology. Res. Biotechnol. 3(1): 09-20.
- Revankar MS, Desai KM, Lele SS (2007). Solid-state fermentation for enhanced production of laccase using indigenously isolated *Ganoderma* sp. Appl. Biochem. Biotechnol. 143: 16-26.
- Riva S (2006). Laccases: blue enzymes for green chemistry. Trend. Biotech. 24(5): 219-226.
- Rotkova J, Sulakova R, Korecka L, Zdrzilova P, Jandova M, Lenfeld J, Horak D, Bilkova Z (2009). Laccase immobilized on magnetic carriers for biotechnology applications. J. Magnetism Mag. Mat. 321: 1335-1340.
- Sahay R, Yadav RSS, Yadav KDS (2009). Purification and Characterization of Laccase Secreted by *L. lividus*. Appl. Biochem. Biotechnol. 157: 311-320.
- Saparrat MN, Arambarri AJ, Balatti PA (2007). Growth and extracellular laccase production in liquid cultures of *Minimidochium parvum* LPSC#548 strain. Bol. Soc. Argent. Bot. 42(1-2): 39-44.
- Sathishkumar P, Murugesan K, Palvannan T (2010). Production of laccase from *Pleurotus florida* using agro-wastes and efficient decolorization of Reactive blue 198. J. Basic. Microbiol. 50: 360-367.
- Sharm KK, Kuhad RC (2008). Laccase: enzyme revisited and function redefined. Indian J. Microbiol. 48:309-316.
- Sharma P, Goel R, Capalash N (2007). Bacterial laccases. World J. Microbiol. Biotech. 23: 823-832.
- Shraddha R, Shekher S, Sehgal M, Kamthania, Kumar A (2011). Laccase: Microbial sources, production, purification and potential biotechnological applications (Review article). SAGE-Hindawi Access to res. Doi: 10.4061/2011/217861
- Strong PJ (2011). Improved laccase production by *Trametes pubescens* MB89 in distillery wastewaters. doi:10.4061/2011/379176.
- Suffian M, Annuar M, Murthy SS, Sabanatham V (2010). Laccase production from oil palm industry solid waste: statistical optimization of selected process parameters. Eng. Life Sci. 10(1): 40-48.
- Viswanath B, Chandra MS, Pallavi H, Reddy BR (2008). Screening and assessment of laccase producing fungi isolated from different environmental samples. Afri. J. Biotechnol. 7(8): 1129-1133.
- Xin F, Geng A (2010). Utilization of horticultural waste for laccase production by *Trametes versicolor* under solid-state fermentation. Appl. Biochem. Biotechnol. doi: 10.1007/s12010-010-9033-x.