Int. J. Adv. Res. Biol.Sci. 1(9): (2014): 33–44

International Journal of Advanced Research in Biological Sciences

ISSN : 2348-8069

www.ijarbs.com

Research Article

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

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Abstract

Neurospora sitophila was used for the production of laccase using agro-wastes including rice straw, sugarcane bagasse and corn cobs. Different conditions were optimized for the production of laccase by *Neurospora sitophila* including 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. It was found that peptone had negative effect on the production of laccase at all concentrations while 0.4%yeast extract and 0.2% tween-20 had good effect on the production of laccase and increased its production as compared to control. Ammonium sulfate precipitation was achieved at 60% salt concentration. Biuret assay was used to determine protein concentration in crude extract and ammonium sulfate precipitated enzyme samples. Specific activity was also determined before and ammonium sulfate precipitation. Optimum temperature and pH for the laccase were found to be 30^{o}C and 5 respectively. Km and Vmax for the laccase, using guaiacol were found to be 0.666mM and 20.8µM/min respectively.

Keywords: Laccase, *Neurospora sitophila*, Guaiacol, Solid State Fermentation, Laccase

Introduction

Laccase (E.C. 1.10.3.2; *para*benzenediol: oxygen: oxidoreductase) belongs to a group of Cu containing polyphenol (PP) oxidases, known as multicopper (MC) oxidases [1,2,3]. This enzyme causes the oxidation of many phenolic compounds with the help of molecular oxygen (O_2), which act as the acceptor of electrons [4] and reduces this oxygen to water [5]. Laccase has low specificity towards substrate and can degrade many of xenobiotic compounds including industrial colored effluents [6, 7].

Laccase has been identified in different fungal species, plants [8, 2], insects [3], bacterial species [9]. The presence of laccase is limited in higher plants than in fungi. Laccase has been reported in turnip, pears, mango, peach, prune [2] pine, lacquer, mung bean, cabbages, potatoes, apples and other vegetable [10].

The utilization of laccase in various fields has been ignored in last few years because of its non-availability for commercialization [6]. This enzyme has so many industrial and biotechnological applications due to its ability of nonspecific oxidation of many phenolic and non-phenolic compounds [11]. 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 [12, 2].
Neurospora sitophila is a specie belonging to genus *Neurospora* and kingdom fungi and is well known model organism for experiments [13]. *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 [14].

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 [5]. Various cultural conditions can be optimized in research laboratory to increase the yield oflaccase [2] as its yield is highly dependent on these cultural conditions [15]. Furthermore the cost of the yield can also be reduced by using agro-wastes [16].
Considering the above described facts the current study was planned and carried out for enhancing laccase production by optimizing various cultural and nutritional conditions. Agro wastes including rice straw, sugar cane bagasse and corn cobs were used in solid state fermentation. Laccase was partially purified by ammonium sulfate precipitation and its specific activity and kinetic parameters like *Km* and *Vmax* were also determined.

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 ^{0}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 forsolid 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), $(NH_4)_2SO_4$ (0.02g), Calcium chloride (0.005 gm), Magnesium sulfate.$7H_2O$ (0.005 gm), potassium dihydrogen phosphate (0.02 gm) and distill water to make total volume of 100mL. The pH of the medium was maintained at 5.5. Fungal Slants were incubated in incubator at 30 ^{o}C for 96 hours [17].

Inoculum

Inoculum was prepared to preserve the fungal spores for future use. Erlenmeyer flasks of 500 mL were used for it preparation with following composition; glucose (2.0g), $(NH_4)_2SO_4$ (0.02g), Calcium chloride (0.005 gm), Magnesium sulfate.$7H_2O$ (0.005 gm), potassium dihydrogen phosphate (0.02 gm) and distill water to make total volume of 100mL [18].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 ^{0}C for 72 hours. Number of spores was adjusted between 10^7-10^8 spores/mL [19].

Solid State Fermentation

The grounded agro wastes were poured in Erlenmeyer flasks of 500mL capacity [20]. These were then moistened with mineral salts solution having composition of; KH_2PO_4 (0.5%); $(NH_4)_2SO_4$ and$MgSO_4{\cdot}7H_2O$ (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 ^{0}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 [21]. 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 ^{0}C to remove all spores and other impurities. 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

H_2O_2, 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 H_2O 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.

Protein Estimation By Biuret Assay Method

Biuret method was used for the estimation of protein in the sample [22] (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 SulphatePrecipitation

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
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Characterization of the enzyme

Partially purified Laccase was then characterized for optimum pH, temperature, substrate affinity and kintics parameters like *Km* and *Vmax*.

Temperature and pH Characterization

Sanyal*et al* method described in 1988 was used to determine optimum temperature of laccaseactivity.Laccase assay was performed at various temperatures ranging from 20 ^{0}C to 80 ^{0}C with the difference of 10 ^{0}C. 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 were used to determined*Km* and *Vmax*of laccase.

Results and Discussion

Fermentation Period

Maximum laccase activities were found to be 2.795±0.03U/mL/min, 2.595±0.03U/mL/min and

2.38±0.04U/mL/min using corn cobs, sugarcane bagasse and rice straw as substrates after 96 hours of fermentation period. Fermentation period of 120hours 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 ± 0.02U/mL/min (Fig. 2).

Different optimum fermentation periods (48 hrs to 400 hrs) have been reported for different fungal species [15, 23, 24, 25, 26, 27, 28]. Maximum laccase production after 96 hr of fermentation was also reported by Galhaup*et al.* andViswanath*et al.*[23, 27].

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 laccaseactivities were obtained in case of control having no moisture (0.725U/mL/min, 0.423U/mL/min and 0.327U/mL/min for corn cobs, sugarcane bagasse and rice straw respectively), showing that moisture is important for laccase production. There 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[15, 29, 30, 31]. Optimum moisture levels of 70.96% and 72-76% for laccase production by different fungi have been reported in previous studies [32]. While, Niladevi*et al.*[30]and Patel *et al.*[15] 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*Neurosporasitophila*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.* [29]and Patel *et al.*[15]. This decrease in the laccase production after certain inoculum size is possibly due to the competition between the fungal spores for nutritionand decreased production of laccase [15, 29, 33]

Peptone Level

All levels of the peptone used showed negative effect on the production of laccase. Adecrease 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.* [23] and Hess *et al.* [34] hasreported 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). Hess *et al.* [34], Galhaup*et al.* [23] andNiladevi*et al.* [35] 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[28].

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 laccase and showed laccase activity of 3.99IU/mL/min (corn cobs), 3.56IU/mL/min (sugarcane baggase) and 3.31 IU/mL/min (rice straw). But after 0.2% of tween-20 there was a decrease in laccase production (Figure

7).Positive effect of tween-20 on laccase production has also been reported by Patel *et al.* [15], Osama *et al.* [26] andSaparrat*et al.* [36].Addition of 0.1% of tween-20 gave the maximum laccase production by fungi [36].

Protein Determination by Biuret Method and Specific Activity Determination

The standard curve for protein concentration with the BSA was prepared and simple linear regression equation was also inserted. 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 LaccaseEnzyme

Optimization of the temperature for laccase activity

Our results indicated 30 ^{o}C to be the optimum temperatures for laccase activity. Moreover it was also observed that laccaseremained stable between 20 to 40 ^{o}C as indicated by the figure 8. Then there was gradual decrease in laccase activityupto 70 ^{o}C due to destruction in the structure of laccase.

Laccase obtained from different organisms showed different optimum temperature ranging from 30 ^{0}C to 60 ^{0}C. Kammoun*et al.* [37] has reported 55 ^{o}C, Sahay*et al.*[38] 60^{o}C while Dominguez *etal.*andPerez *et al.*has reported 30 ^{o}C as optimum temperature for laccase [39, 40]. 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 [41]report pH 6 to 9, Perez *et al.* [40] have reported pH 5 andRotkova*et al.*[42] 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/Vo vs 1/S was prepared to got the values of*Km* and *Vmax* 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 concentration 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 Km and Vmax for laccase were found to be 0.666 mMand 20.8µM/min respectively. Laccase has different Km and Vmax values for different kind of substrates used. Some of the reported Km values for laccase with different substrate are 480µM (2,6-dimethoxyphenol), 350µM (syringaldize), 320µM (pyrogallol), 230µM (catechol) and 210µM (m-cresol). Sahay*et al.* [38]), Dong and Zhang [41] have reported 0.001mM *Km* 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 *Km* 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 [41, 43].

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	$CuSO_4.5H_2O$	3.0 g
3	KI	5.0 g
4	Sodium Potassium Tartarate	96.0 g
5	$D.H_2O$	Up to 1000 mL

Table 2 Protein estimation by Biuret assay

Sample No.	D. H_2O (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

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 *Neurosporasitophila*

Substrate		Enzyme Sample (mL)	Vol. of Biuret Reagent	Total Volume (mL)	Absorbance	Protein Conc. /0.5 mL of Enzyme Sample	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	

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)
	Crude enzyme	200	3.995	12.78	799	2556	0.31	1
Corn Cobs	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

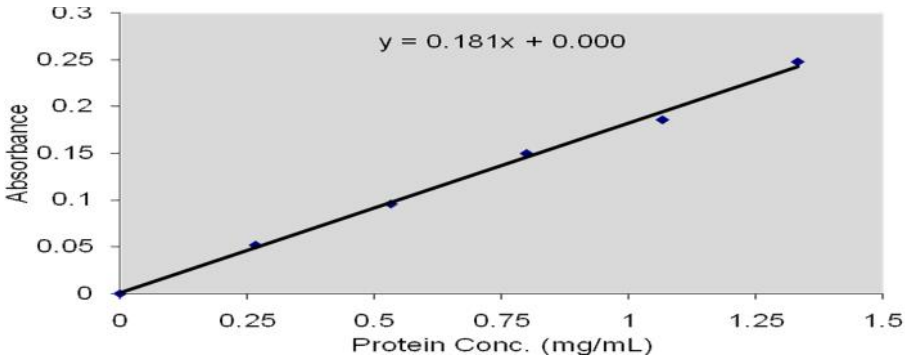

Figure 1: Standard curve drawn using different concentrations of BSA for the determination of protein concentration in the crude enzyme sample and ammonium sulfate partially purified samples

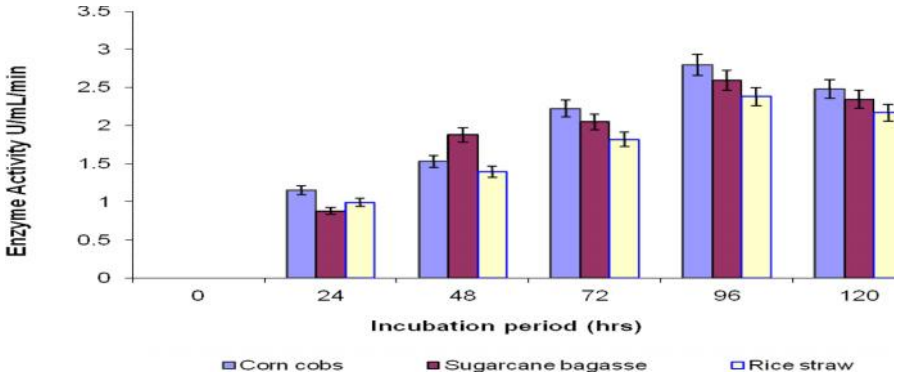

Figure 2: Optimization of fermentation period for the production of laccase using three agro-wastes by *Neurospora sitophila*

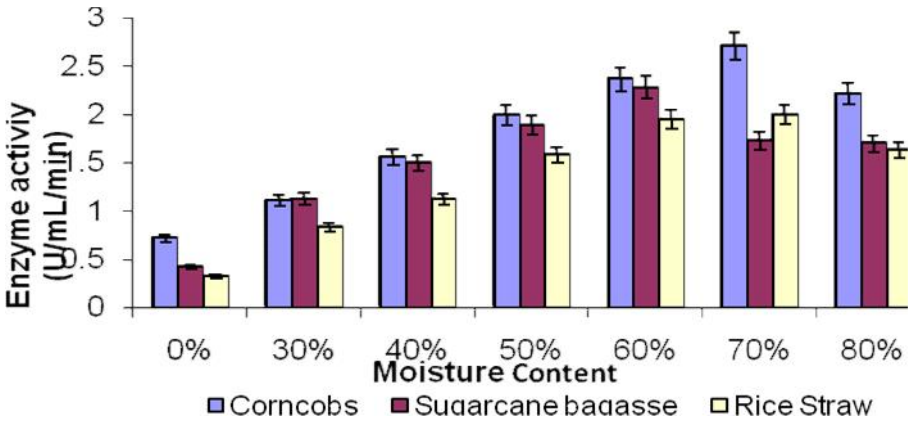

Figure 3: Moisture content optimization for laccase production using three agro-wastes by *Neurospora sitophila*

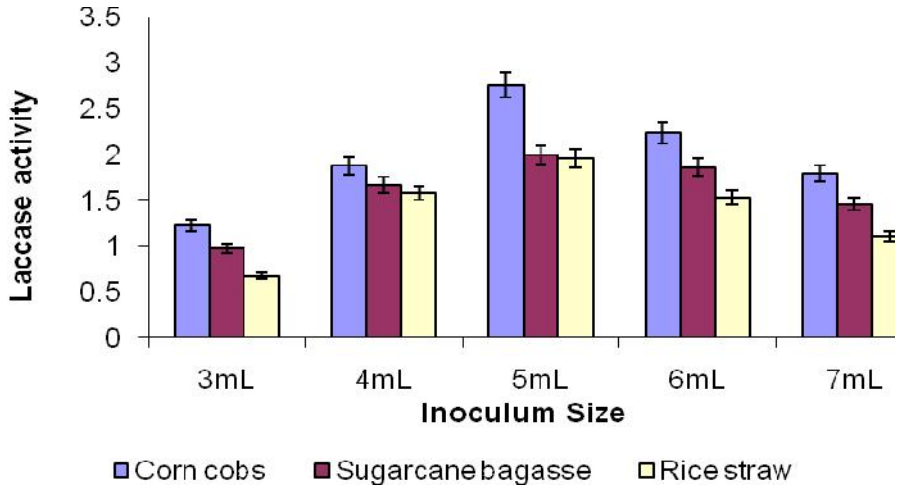

Figure 4: Inoculum size optimization for the production of laccase using three agro-wastes by *Neurospora sitophila*

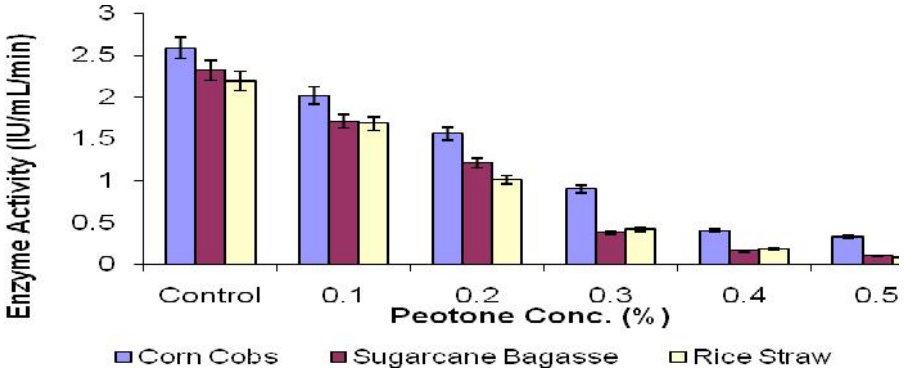

Figure 5: Evaluating the effect of peptone for the production of laccase using three agro-wastes by *Neurospora sitophila*

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

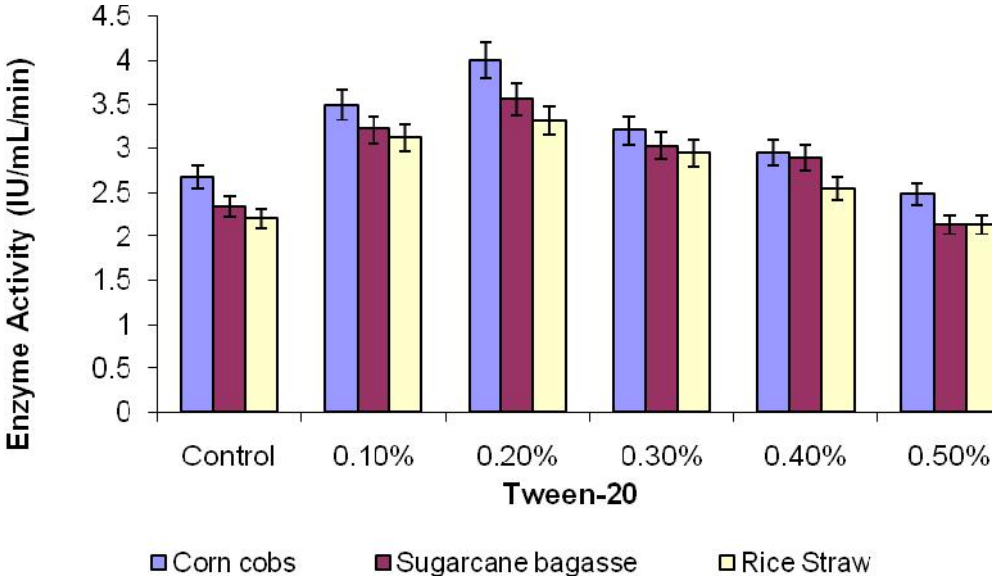

Figure 7: Production of laccase with varying tween-20 conc. by *Neurosporasitophila*

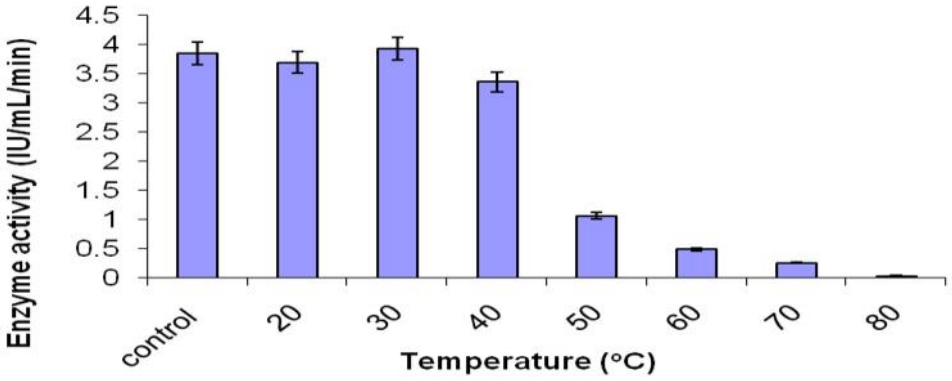

Figure 8: Effect of temperature on laccase activity produced by *Neurosporasitophila*

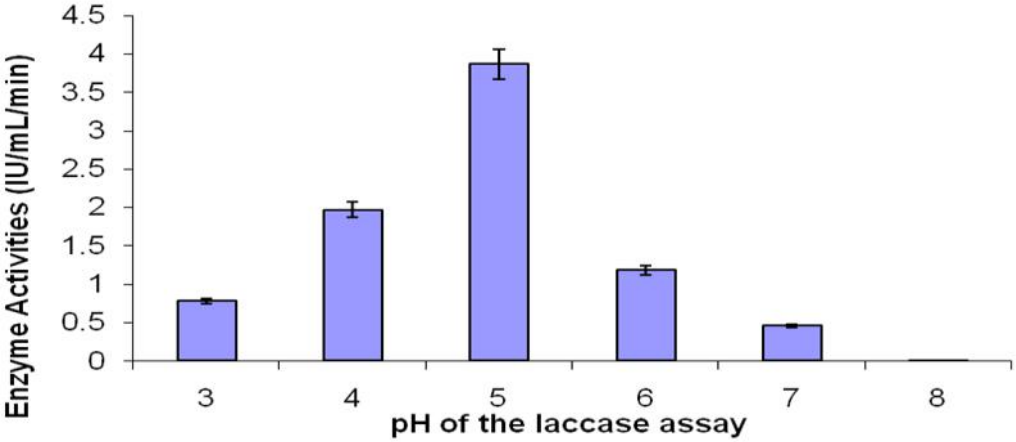

Figure 9: Optimization of pH for the laccase activity

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