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Effect of pH and Temperature on Lignolytic Enzyme Production by *Coriolus versicolor* MTCC 138 Using Paddy Straw as Substrate

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Abstract: The present study reports the effect of pH and temperature on lignolytic enzyme production by *Coriolus versicolor* MTCC 138 using paddy straw as substrate. The effect of pH and temperature on lignolytic enzyme stability was also studied. Results showed that a maximum of 2550.6 U/g of lignin peroxidase was produced at 25°C and 532.0 U/g & 3366.3 U/g of laccase and manganeses peroxidase, respectively were produced at 30°C. A pH of 5.0 was found to be optimum for laccase, lignin peroxidase and manganese peroxidase showing acitivities of 560.6 U/g, 2300.8 U/g and 2906.6 U/g, respectively. Results also revealed that laccase and lignin peroxidase were stable at 50°C for 60 minutes, whereas manganese peroxidase was found to be heat labile. All the three lignolytic enzymes i.e. laccase, lignin peroxidase and manganese peroxidase were found to be stable in a pH range of 5.0-6.0.

Keywords: *Coriolus versicolor*; Lignolytic enzymes; Paddy straw; Solid state fermentation

Introduction

Paddy being the major cereal crop is produced in large quantities in India. The production of rice in India, which was 74.29 million tones in 1990-1991, goes up to 102 million tones in 2011-2012. About 110.0 lakh tones of rice was produced only in Punjab during 2011-2012 [1], which produced 100-150 lakh tones of paddy straw. From such a large quantity of paddy straw only a minor portion is used as animal feed and household fuel while the remaining residue is burnt in field.

Hydrolysis of agricultural biomass to simple fermentable sugars is required prior to energy conversion process [5]. However, high lignin (6-14 %) content in the cell wall of paddy straw hinders the accessibility of cellulase to cellulose and hemicellulase to pentoses, thereby reducing the hydrolysis efficiency significantly. In addition to high lignin content, high concentration of silica (8-12 %) in its epidermal surface acts as a physical barrier preventing microbial attachment for hydrolytic process [24].

Lignin after cellulose and hemicellulose, is the most abundant aromatic polymer on earth consisting of three different phenyl propane units namely p-coumaryl, coniferyl, sinapyl alcohol. The inter unit bonds of lignin are not hydrolysable. Therefore, it is very difficult to degrade lignin chemically or biologically.

Lignin degrading microorganisms and their enzymes have become very attractive because they may provide environment friendly technologies for biofuel industry. The major enzymes associ-

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ated with lignin-degrading ability of white rot fungi are lignin peroxidase, manganese peroxidase and laccase. Most of the research concerning biodegradation of lignin has been focused on *Phanerochaete chrysosporium, Streptomyces viridosporus, Pleurotus eryngii, Trametes trogii and Fusarium proliferatum* [17]. Wood-rotting basidiomycetous fungi that cause white rot in wood are the most efficient lignin degraders in nature [8] and they are perhaps nature's major agents for recycling the carbon of lignified tissue. Phutela and Sahni [15] studied the effect of *Coriolus versicolor* MTCC 138 on paddy straw and found that *C. versicolor* enhanced the paddy straw digestibility by producing ligno-cellulolytic enzymes. The biogas production was found to be increased by 26.2%. The lignolytic enzymes production by *Coriolus versicolor* using paddy straw as a substrate through solid state fermentation showed enhanced activities as compared to submerged fermentation [9].

Enzymatic processes, which hold several advantages, are now substituting the chemical, physical and microbial processes. The efficiency of enzymatic process is quite high and the mild process conditions require neither expensive materials nor high process energy. Enzymatic hydrolysis not only economizes energy on account of the relatively mild reaction conditions, but also avoids using toxic and corrosive chemicals.

Bourbonnais and Paice [3] studied laccase and veratryl alcohol oxidase (VAO) enzyme production from *Pleurotus sajor caju* in agitated mycological broth culture. They found maximum activity of laccase after 15 day of incubation at 25°C and maximum VAO production after 28 day of incubation at 25°C. Shanmugam and Yadav [19] reported the extracellular lignin peroxidase enzyme by *P. sajor caju*. The optimum pH and temperature required for enzyme activity was 9.0 and 50-60°C. Km value of enzyme was 400 mm using veratryl alcohol as substrate.

Keeping above facts in mind, the present paper reports the effect of pH and temperature on lignolytic enzyme production by *Coriolus versicolor* MTCC 138 and their effect on enzyme stability.

Material and methods

Paddy straw-substrate for enzyme production

Paddy straw was procured from the research field of Punjab Agricultural University, Ludhiana after the harvesting of crop in the month of October and November. The dried paddy straw was powdered with a grinding machine and was stored in polythene bags at room temperature. The Mandel's medium [11] was supplemented with powdered paddy straw is used for solid state fermentation.

Maintenance of culture

Standard culture of *Coriolus versicolor* MTCC 138 was procured from MTCC (Microbial Type Culture Collection), Institute of Microbial Technology, Chandigarh and was maintained on glucose yeast extract agar slants at 27±2°C by monthly transfers. The culture was stored in refrigerator after sub-culturing.

Lignolytic enzyme production from paddy straw through solid state fermentation

For solid state fermentation, 10 g of paddy straw was taken in a Erlenmeyer flask (500 ml) with 40 ml of Mandel's medium i.e. in 1:4 (w/v) ratio. The flasks were autoclaved at a pressure of 1.1 kg/cm² for 20 minutes. Flasks were cooled at room temperature and inoculated with two bits (5 mm dia) of *Coriolus versicolor* MTCC 138. The inoculated flasks were incubated at required temperature. These flasks were removed at a regular interval of 24 hours for a period of seven days and crude enzyme was extracted. The enzyme was extracted from the substrate using 0.1 M citrate buffer (1:10 w/v ratio) and shaking the contents for 30 min in shaking incubator (250 rpm) at 20ºC. The extract was filtered with muslin cloth to obtain a clear filtrate. The filtrate was centrifuged in a cooling centrifuge at 10,000 rpm for 20 minutes at 4ºC and supernatant was used for estimation of laccase, lignin peroxidase and manganese peroxidase activity by spectrophotometric method. The experiment was performed in triplicate.

Effect of incubation temperature on enzyme production

The effect of incubation temperature on enzyme

production was examined by keeping the inoculated flasks containing 10g of substrate with 40 ml of Mandel's medium of pH 5.0 at different temperature starting from 20 to 35°C with 5°C intervals i.e. 20, 25, 30 and 35°C for 96 h. After an incubation period of 4 days the crude enzyme was extracted and used for analyzing laccase, lignin peroxidase and manganese peroxidase activities. The experiment was performed in triplicate.

Effect of incubation pH on enzyme production

The effect of initial pH on enzyme production was investigated by adjusting the initial pH of Mandel's medium to 3.0, 4.0, 5.0 and 6.0. The flasks containing 5 g of substrate and 20ml of sterile Mandel's medium were inoculated with 2 bits (5 mm dia) of standard culture of *Coriolus versicolor* MTCC 138 and were incubated at 27±2°C in a BOD incubator. After an incubation period of 4 days the crude enzyme was extracted and used for analyzing laccase, lignin peroxidase and manganese peroxidase activities. The experiment was performed in triplicate.

pH stability and thermostability studies of crude enzyme extract

To study pH stability and temperature stability, lignolytic enzyme extract was prepared using extraction buffer i.e. citrate buffer of different pH ranging from 4.0-7.0 (4.0, 5.0, 6.0 and 7.0). The crude enzyme extract of different pH was kept at different temperatures of 40°C, 50°C, 60°C and 70°C for one hour. Samples were taken periodically for enzyme estimation at 15 minute interval. The experiment was performed in triplicate.

Estimation of lignolytic enzymes

Laccase (EC.1.10.3.2)

Laccase estimation was carried out according to the method described by Shandilya and Munjal [18].

Estimation of lignolytic enzymes

One ml of the enzyme filterate was put in a cuvette and was placed in the UV spectrophotometer (Hitachi UV- VIS U-2800) at 495 nm, to adjust light absorbance at zero. The cuvette was taken out and dispensed with 3 ml of guaiacol solution and immediately transferred back to spectrophotometer. Change in light absorbance was recorded for every 15 sec upto 180sec. The change in absorbance between the 30 and 150 sec was taken and results were expressed as change in absorbance/min (ΔA_{495}nm/min). An increase in the activity by 0.001 in 60sec was taken as 1 unit of laccase enzyme.

Manganese peroxidase (MnP) (EC.1.11.1.13)

Manganese peroxidase activity was determined by method of Paszczynski *et al.*[14].

Assay procedure

Enzyme filterate 0.2 ml, guaiacol solution 3 ml, $MnSO_4$ 0.2 ml and H_2O_2 0.2 ml was put in a cuvette, mixed and change in light absorbance was recorded for every 15 sec upto 180sec at 465nm against a blank without H_2O_2. An increase in O.D. by 0.001 in 60 sec was taken as 1 unit. The specific activity was expressed as ΔA_{465} min per mg protein.

Lignin peroxidase (LiP) (EC.1.11.1.14)

Lignin peroxidase activity was determined by method given by Tien and Kirk [23].

Assay Procedure

One ml of 10 mM veratryl alcohol, 1.5 ml of phosphate-citrate buffer, 0.4 ml of the enzyme filterate and 0.1 ml of H_2O_2 was put in a cuvette, mixed and change in light absorbance was recorded for every 15 sec upto 180sec at 310 nm against a blank without H_2O_2. Enzyme unit for LiP activity was defined as activity of an enzyme that catalyzes the conversion of 1 µmol of veratryl alcohol to veratrylaldehyde per minute.

Estimation of extracellular protein

Extracellular protein content of enzyme filtrate was estimated according to the method given by Lowry *et al*. [10].

Assay procedure

Tubes containing 1 ml of enzyme filtrate and 5 ml of reagent C were incubated in duplicates for 20 minutes at room temperature. Then added 0.5 ml of reagent D and incubated for another 10 minutes again at room temperature. Light absorbance was measured at 520 nm in the UV- spec-

trophotometer. A control was run where enzyme was replaced with distilled water. A standard curve for protein estimation was prepared under same conditions as described above using standard solution of bovine serum albumin (BSA) at a concentration range of 0.1 to 1mg.

Resuls and discussion

Effect of two operational parameters such as pH and temperature on lignolytic enzyme production and also their effect enzyme stability were studied.

Effect of incubation temperature on lignolytic enzyme production

Incubation temperature not only influences the growth of microorganisms but also their biological activities and it is one of the important parameters that determine the success of solid state fermentation system. To know the optimum temperature for the production of laccase, lignin peroxidase and manganese peroxidase enzymes by *Coriolus versicolor* MTCC 138 on paddy straw, a temperature range between 20-35°C was studied and it was observed that lignin peroxidase activity was maximum at 25°C (2550.9 U/g) and that of laccase and manganese peroxidase was maximum at 30°C (532.0 U/g of laccase and 3366.3 U/g of manganese peroxidase). Further increase in temperature resulted in decrease in production of enzymes **(Table 1).** Maximum specific activity of laccase (152 U/mg) and lignin peroxidase (1109 U/mg) was observed at 25°C and that of manganese peroxidase (765 U/mg) was at 30°C. Minimum activity for laccase (95.6 U/g), lignin peroxidase (375.1U/g) and manganese peroxidase (300.6 U/g) was obtained at 20°C. Minimum specific activity for laccase (34 U/mg), lignin peroxidase (134 U/mg) and manganese peroxidase (107 U/mg) was also obtained at 20°C. Lesser activity of fungal enzymes at low (20°C) and high temperature (35°C) as compared to 30°C might be due to slow growth at low temperature and inactivation of the enzyme at high temperature. Pointing *et al*.[16] too reported that the optimum temperature for laccase production is between 25°C and 30°C. When fungi were cultivated at temperatures higher than 30°C the activity of enzyme was reduced [25].

Dritsa and Rigas [4] studied laccase and manganese peroxidase production from basidiomycetes sp. namely *Pleurotus ostreatus sp.*, *Pleurotus pulmonarius, Polyporus iliates*, *Polyporus brumalis*, *Polyporus* sp., and *Ganoderma austral* at temperature range of 15-30°C and reported that maximum laccase and manganese peroxidase was expressed by all fungi at 25°C and 30ºC respectively. Jiang *et al*.[6] also reported 30°C to be an optimum temperature for a standardized fed-batch fermentation process for recombinant manganese peroxidase (rMnP) production in *Pichia pastoris*.

Effect of initial pH on lignolytic enzyme production

The negative logarithm of H_3O^+ ions concentration in moles per liter is called as pH. Hydro-

Table 1. Effect of incubation temperature on lignolytic enzyme production

Incubation temperature (°C)	Enzyme activity (U/g of paddy straw)			Protein (mg/g)
	Laccase	Lignin peroxidase	Manganese peroxidase	
20	95.6±5.8 (34)	375.1±25.7 (134)	300.6±16.9 (107)	2.8
25	350.4±12.5 (152)	2550.9±121.6 (1109)	1066.3±46.1 (463)	2.3
30	532.0±23.1 (120)	1683.5±114.4 (383)	3363.3±78.7 (765)	4.4
35	206.6±19.3 (115)	1520.6±116.9 (884)	1100.3±34.6 (611)	1.8

Medium: 40 ml mineral medium/10 g of substrate; incubation time: 4 days; pH: 5.0.
The data represents the mean of three determinations each; ± values indicate standard error.
The values in parenthesis indicate specific activity (U/mg). CD (5 %) – for A (incubation time): 46.9; for B (enzyme activity): 40.7; for AB (enzyme acitvity incubation time): 81.3

gen ion concentration plays significant role in the various physiological processes of the microorganisms. The effect of pH on lignolytic enzyme production was studied by varying the pH of Mandel's medium to 3.0, 4.0, 5.0 and 6.0 for solid state fermentation of paddy straw by *Coriolus versicolor* MTCC 138. From the results presented in Table 2, medium pH 5.0 was found to be optimum for laccase, lignin peroxidase and manganese peroxidase activity. Maximum enzyme activity reported at pH 5.0 after an incubation period of 4 days was 560.6 U/g of laccase, 2300.8 U/g of lignin peroxidase and 2960.6 U/g of manganese peroxidase. Minimum enzyme activities for laccase (220.6 U/g), lignin peroxidase (980.6 U/g) and manganese peroxidase (710.6 U/g) were obtained at pH 3.0. Maximum specific activities for laccase (280 U/mg), lignin peroxidase (1150 U/mg) and manganese peroxidase (1480 U/mg) were also obtained at pH 5.0. Minimum specific activity of laccase (123 U/mg) and lignin peroxidase (544 U/mg) was observed at pH 3.0, but that of manganese peroxidase (364 U/mg) was at pH 4.0. Similarly, Thurston [22] observed that initial pH between 4.5 and 6.0 is suitable for enzyme production. It was reported that the pH significantly influenced the extracellular protein content and laccase activity in *Ganoderma* sp. The fungus was able to release a maximum protein content of 40 µg/ml at pH 5.5 and laccase activity of 0.24 U/ml at pH 6.0 [20]. Jiang *et al.* [6] also reported optimum pH for a standardized fed-batch fermentation process for recombinant manganese peroxidase (rMnP) production in *P. pastoris* to be 6. Enzymes had optimal activities at pH values between 4.5 and 6.0 and were stable under pH range of 4.0 to 7.0 [13]. Change in pH from the optimum to extreme levels results in in activation of the enzymes of the organisms which hinder saccharification of the substrate [5].

Effect of temperature and pH on enzyme stability

For temperature stability studies, crude enzyme extract produced under optimum conditions were stored at different temperatures (30, 40, 50, 60 and 70°C). Results from Table 3 showed that laccase and lignin peroxidase were stable at 50°C temperature for 60 minutes where as there was decrease in enzyme activities at 60°C and inactivation at 70°C. Manganese peroxidase was observed to be heat labile as its enzyme activity started decreasing at 40°C and enzyme get completely inactivated after 15 minutes incubation at 50°C. Results are similar with the findings of Junga *et al.*[7] who reported that laccase from fungus *Trichophyton rubrum* LKY-7 was very stable below 50°C, but it was inactivated very rapidly at temperatures over 70°C, and completely inactivated within 10 min at 80°C. It was also reported by Maria *et al*.[12] that the laccase from *Trametes versicolor* was inactivated at 65°C after incubation time of 1 hour. Reports by Baborova *et al.* [2] showed that MnP was active at 5-70°C with an optimum between 50-60°C. At temperatures above 65°C, the enzyme rapidly lost activity.

Table 2. Effect of initial pH on lignolytic enzyme production

Initial pH	Enzyme activity (U/g of paddy straw) Laccase	Lignin peroxidase	Manganese peroxidase	Protein (mg/g)
3	220.6±11.6 (123)	980.6 ±13.5 (544)	710.6 ±24.9 (395)	1.8
4	350.5±12.3 (159)	1720.5±125.3(782)	800.0±34.2 (364)	2.2
5	560.6±23.1 (280)	2300.8±132.6 (1150)	2960.6±86.2 (1480)	2.0
6	240.6±9.8 (127)	2030.8±134.2 (1068)	1230.3±55.8 (647)	1.9

Medium: 40 ml mineral medium/10 g of substrate; incubation time: 4 days; temeprature: 27±2°C.
The data represents the mean of three determinations each; ± values indicate standard error.
The values in parenthesis indicate specific activity (U/mg). CD (5%) – for A (incubation time): 9.7; for B (enzyme activity): 8.4; for AB (enzyme acitvity incubation time): 16.8

To check pH stability, crude enzyme was extracted in citrate buffer of pH 4,5,6 and 7 and results are presented in Table 4. All the three enzymes were found to be stable in a pH range of 5.0-6.0. Further increase or decrease in the pH leads to decrease in the enzyme activities. The pH optimum for white-rot fungus *Irpex lacteus* was relatively broad, from 4.0 to 7.0 with a peak at pH 5.5 [1].

Table 3. Effect of temprature on laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP) stability

Enzyme activity (U/g of paddy straw)		Temperature (°C) 30	40	50	60	70
Laccase	15 min	498.0±12.6	496.7±11.2	452.3±15.6	317.3±11.2	106.0±9.6
	30 min	495.4±14.4	451.6±15.3	440.2±14.8	212.0±11.5	65.3±3.5
	45 min	490.2±17.5	444.5±12.7	425.3±11.8	99.3±8.3	34.6±1.6
	60 min	480.4±19.3	439.3±14.3	416.3±11.0	68.6±9.2	14.3±1.2
LiP	15 min	2169.4±79.3	2110.0±60.3	2092.5±92.2	1115.8±56.0	510.0±15.3
	30 min	2152.2±57.4	2094.2±79.6	1990.0±49.6	910.8±66.2	326.6±15.3
	45 min	2124.0±78.3	2085.0±11.2	1771.2±99.0	618.3±87.0	122.5±14.0
	60 min	2096.0±78.3	1976.7±86.0	1664.8±76.0	257.5±21.0	12.0±0.6
MnP	15 min	3025.9±112.0	3015.0±98.6	2525.0±89.5	1023.2±56.2	312.2±24.3
	30 min	3011.0±102.0	2562.0±56.3	2005.0±102.3	512.9±49.3	120.0±5.3
	45 min	2979.4±79.5	2236.4±84.2	1503.1±90.5	312.0±36.5	65.0±3.5
	60 min	2957.3±67.8	1963.2±78.6	1025.8±21.6	93.0±16.8	15.0±3.5

± values indicate standard error

Table 4. Effect of initial pH on lignolytic enzyme stability

Buffer pH	Extraction Enzyme activity (U/g of paddy straw) Laccase	Lignin peroxidase	Manganese peroxidase	Protein (mg/g)
3	220.6 ± 11.6	980.6 ± 13.5	710.6 ± 24.9	1.8
4	350.5 ± 12.3	1720.5 ± 125.3	800.0 ± 34.2	2.2
5	560.6 ± 23.1	2300.8 ± 132.6	2960.6 ± 86.2	2.0
6	240.6 ± 9.8	2030.8 ± 134.2	1230.3 ± 55.8	1.9

Medium: 40 ml mineral medium/10 g of substrate; incubation time: 4 days; temeprature: 4°C; ± values indicate standard error; CD (5 %) – for A (pH): 9.7; for B (enzyme activities): 8.4; for AB (enzyme acitvities X pH): 16.8

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