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Full Length Research Paper

Activity enhancement of ligninolytic enzymes of *Trametes versicolor* with bagasse powder

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Suspended cultures of white-rot fungus, *Trametes versicolor*, supplemented with bagasse powder showed a concentration dependent enhancement in the ligninolytic enzymes activity in liquid shake cultures. 2% (w/v) bagasse powder improved greater stability to the enzymes. The optimum pH is 3.5 and the optimum temperature is 40°C for maximum lignonolytic enzymatic activity. The optimum shaking speed is 60 rpm for maximum enzymatic activity. The maximum enzymatic activity showed by *T. versicolor* is 495, 440 and 410 μmol/ml.min for LiP, MnP and laccase with bagasse powder at optimum conditions, respectively. Without bagasse powder at optimum conditions, the maximum enzymatic activity for LiP, MnP and laccase is 195, 150 and 170 μmol/ml.min, respectively.

Key words: Bagasse, enhancement, enzymes, optimum, white-rot fungus.

INTRODUCTION

Lignin biodegradation is fundamental to potential applications of biotechnology in the pulp and paper industry. Indeed, the rapid increase during the past decade in understanding how lignin is degraded has provided strong items to the study of possible applications of biotechnology in pulp and paper manufacturer. Research continues on the lignin-degrading enzyme system involved; its nature, production, mechanism of action, and molecular genetics. The current picture of the process is that powerful hemoperoxidases (lignin peroxidases and manganese peroxidases), and in some fungi the blue copper enzyme laccase, act outside the fungal cells to oxidize lignin nonspecifically, generating radical species that undergo further reactions that result in extensive degradation and fragmentation of the polymer (Kirk and Chang, 1980; Kirk and Eriksson, 1981; Plat et al., 1983; Vyas et al., 1994). Laccase is commonly distributed in white-rot fungi and is known to catalyze various reactions. Biochemical and physiological research over the last years was firmly established that peroxidase enzymes produced and secreted by basidiomycete fungi play an important role in the breakdown of lignin (Hatakka, 1994; Kirk and Chang, 1980; Tien and Kirk, 1984; Kirk and Eriksson, 1981; Eriksson et al., 1990). Studies (Hatakka, 1994; Kirk and Chang, 1980; Tien and Kirk, 1984; Kirk and Eriksson, 1981; Eriksson et al., 1990; Vyas et al., 1994) have further shown that these peroxidases are hemecontaining glycoproteins, which may exist and be secreted in multiple forms. Lignin peroxidase (LiP), which is immunological and structural different from manganese II dependent peroxidases (MnP), has been shown to be able to oxidase a member of important bonds in lignin. In contrast, the role played by MnP in lignin degradation has been proposed as indirect by providing H_2O_2 for lignin peroxidase reactions (Kirk and Chang, 1980; Tien and Kirk, 1984; Kirk and Eriksson, 1981; Eriksson et al., 1990). Hossain et al. (2001, 2002, 2003, 2004a, 200b, 2004c, 2004d) showed earlier that the three ligninolytic enzymes; LiP, MnP and laccase are found in lignin biodegradation of sterilized and unsterilized jute stick, rice straw, wheat straw and bagasse with various bioprocess parameters in glucose and l-alanine

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amendment sample using three white-rot fungi *Phanerochate chrysosporium, Lentinus crinitus* and *T. versicolor*.

Thus, there is a wide interest in induction, enhancement and stabilization of ligninolytic enzymes because of their diverse applications in industrial bioprocesses. In recent rears, agro-residues have been experimented for activity enhancement of the ligninolytic enzymes of various white-rot fungi (Schick-Zapanta and Tien, 1992; Adron et al., 1998; Castillo and Ander, 1997; Kaal et al., 1995; Zafar et al., 1996; Plat et al., 1983; Hossain and Das, 2000; Hossain et al., 2001, 2002, 2003, 2004a, 2004b, 2004c, 2004d).

In the present studies, we report the enhancement effects of bagasse powder, a lignocellulosic agro-waste, on the activity of ligninolytic enzymes of white-rot fungus *T. versicolor***.** Attempts were earlier made on enzyme activity of white-rot fungus to optimize bioprocess parameters (Hossain and Das, 2000; Hossain et al., 2001, 2002, 2003, 2004a, 2004b, 2004c, 2004d) like incubation time, pH, amount of bagasse (w/v %), temperature and shaking speed of water bath (rpm) using suspended aerobic batch culture of the fungus.

MATERIALS AND METHODS

Collection of microbe and culture preparation

The freeze-dried white-rot fungus, *T. versicolor*, was collected from Institute of Microbial Technology (CSIR), Chandigarh, India. The slant cultures were prepared with prescribed growth medium (Malt Extract Agar – Blakeslee's formula: malt extract 20.0 g, glucose 20.0 g, peptone 1.0 g, agar 20.0 g, distilled water 1.0 L) for a period of seven days incubation at 25°C temperature in aerobic conditions. The fungus was subcultured regularly within 30 days and stored at 4°C temperature. The slant cultures were used for suspension culture preparation.

Suspension culture preparation

Whenever the suspension cultures are needed, the frozen slant cultures are transferred and are kept in an incubator at 25°C for 7 days for sufficient sporulation. Spore crops are harvested in a culture medium by washing the slant with sterile distilled water. The resulting spore/cell suspension is filtered through several layers of sterile absorbent cotton. The spore densities (Trivedy and Goel, 1986) are counted as 6 x 10^8 numbers per ml (Luckey Drop Method) in the inoculum suspension for the fungus *T. versicolor.*

Culture media preparation

The following constituents were used for culture media preparation for both the fungus per liter: KH_2PO_4, 20 g; $MgSO_4$, 5 g; $CaCl_2$, 1 g; $CuSO_4$, 0.1 g; $ZnSO_4.7H_2O$, 0.1 g; $CuSO_4$, 0.1 g; $AlK(SO_4).12H_2O$, 0.01 g; H_3PO_3, 0.01 g; $Na_2MoO_4.2H_2O$, 0.01 g; Ca-oxalate, 1.5 g; veratryl alcohol, 10% (v/v); 2,2-dimethyl succinate (0.1 M) , 10% (v/v); glucose, 10 g; and l-alanine, 0.1% (w/v).

Preparation of bagasse powder

Bagasse was collected from an indigenous source. It was washed four times with sterilized distilled water to remove any adhering substances, dried at 50°C to constant weight, powdered and passed through 25 mesh sized sieve to get fine uniform size particle.

General method

The ligninolytic enzyme activity was studied in batch bioprocess system. 250 ml of 7 days old suspended inoculum culture (fungal count was 6 x 10^8 cells per ml) supplemented with 25 mesh sized 1.0% (w/v) bagasse powder in 750 ml Erlenmeyer flasks (bioreactor). The initial pH was adjusted to 3.5 by adding 1 M HCl acid and/or 1 M $CaCO_3$ slurry. The bioreactors were placed in a constant temperature water bath maintained at temperature of 30°C with a constant shaking (20 rpm). The aerobic condition in the system was maintained by putting non-absorbent cotton to the mouth of bioreactors. All experiments were conducted aseptically. Same procedures were repeated without bagasse powder (control condition).

Effect of incubation time on activity of ligninolytic enzymes

The general method was repeated accordingly. The bioreactors were taken out after 3, 6, 9, 12, 15, and 18 days of inoculation respectively. The aliquots of culture filtrates were collected on regular interval basis and were stored in capped glass vials at room temperature and analyzed for activity of ligninolytic enzymes.

Effect of bagasse powder dose on activity of ligninolytic enzymes

The general method was repeated for different bagasse powder dose such as 1.5, 2.0, 2.5 and 3.0% (w/v). The aliquots of culture filtrates were collected after 12 days (optimum) of inoculation and were stored in capped glass vials at room temperature and analyzed for activity of ligninolytic enzymes.

Effect of pH on activity of ligninolytic enzymes

The general method was repeated for different pH values such as 2.5, 3.5, 4.5 and 5.5. The bagasse powder dose was maintained at 2.0% (w/v) (optimum). The aliquots of culture filtrates were collected after 12 days (optimum) of inoculation and were stored in capped glass vials at room temperature and analyzed for activity of ligninolytic enzymes.

Effect of temperature on activity of ligninolytic enzymes

The general method was repeated for different temperature values such as 20, 30, 40, and 50°C. The bagasse powder dose was maintained at 2.0% (w/v) (optimum). The pH was maintained at 4.5 (optimum). The aliquots of culture filtrates were collected after 12 days (optimum) of inoculation and were stored in capped glass vials at room temperature and analyzed for activity of ligninolytic enzymes.

Table 1. Effect of time on activity of *Trametes versicolor* ligninolytic enzymes activities.

Enzymes	Enzyme activity (μmol/ml.min)					
	3 days	6 days	9 days	*12 days	15 days	18 days
LiP	85	175	285	335	335	295
	(20)	(80)	(105)	(125)	(125)	(70)
MnP	65	145	265	305	305	250
	(15)	(60)	(85)	(105)	(105)	(80)
Lacasse	65	125	220	290	290	250
	(15)	(45)	(75)	(115)	(115)	(80)

Values in parenthesis () are for cultures without bagasse powder.
*Optimum enzyme activity time.

Table 2. Effect of bagasse powder dose on activity of ligninolytic enzymes activities after 12 days of inoculation of *Trametes versicolor*.

Enzyme activity (μmol/ml.min)	Bagasse powder			
	1.5%	2.0%*	2.5%	3.0%
LiP.	375	415	395	385
	(125)	(125)	(125)	(125)
MnP	330	375	365	320
	(105)	(105)	(105)	(105)
Lacasse	305	350	335	335
	(115)	(115)	(115)	(115)

Values in parenthesis () are for cultures without bagasse powder.
*Optimum bagasse powder dose.

Effect of shaking speed on activity of ligninolytic enzymes

The general method was repeated for different shaking speed of water bath such as 20, 40, 60 and 80 rpm. The bagasse powder dose was maintained at 2.0% (w/v) (optimum). The pH was maintained at 4.5 (optimum). The temperature was maintained at 40°C. The aliquots of culture filtrates were collected after 12 days (optimum) of inoculation and were stored in capped glass vials at room temperature and analyzed for activity of ligninolytic enzymes.

Measurement of enzymes activity

Laccase activity was determined according to Yasdev and Kuhad (1994) in culture filtrates obtained after different days of inoculation using 2,2-amino-bis (3-ethyl benzothiazoline-6-(sulfonate) (ABTS) as substrate. The reaction mixture (1 ml) contained 2 mM ABTS in a 1:1 mixture of 100 mM sodium lactate/sodium succinate buffer (pH 4.5) containing egg albumin (6 mg/ml) and 100-200 μl culture fluid. Changes in absorbance were recorded at 420 nm for up to 3 min using UV- Vis spectrophotometer (Perkin-Elmer, Lambda Bio-40).

Manganese peroxides activity was determined according to Castillo (1994). The reaction mixture (1 ml) contained 0.07 mM 3-methyl-2-benzo-thiazolinone hydrazone (MBTH), 0.99 mM 3-dimethylamino benzoic acid (DMBA), 0.34 mM $MnSO_4$, 100 mM sodium lactate/sodium succinate buffer (pH 5.0), culture fluid and 0.05 mM H_2O_2. The reaction was initiated by addition of H_2O_2. Absorbance change was recorded at 590 nm after 1 min using UV - Vis spectophotometer.

Lignin peroxidases assay was done by oxidation of veratryl alcohol to veratryl aldehyde (Castillo et al., 1997). 1 ml reaction mixture contained 2 mM veratryl alcohol, 0.27 mM H_2O_2 and 100 mM tartarate buffer (pH 3.0). Absorbance was measured at 310 nm using UV- Vis spectrophotometer. Activity of all the enzymes is expressed in μmol/ml.min.

RESULTS

Effect of incubation time on enzyme activity

The results of the effect of time on activity of ligninolytic enzymes of the white-rot fungi are presented in Table 1. All the three enzymes; lignin peroxidase (LiP), manganese peroxidase (Mn P), and laccase attained their peak activity on twelfth day of inoculation both in control (without bagasse) conditions as well as in cultures supplemented with bagasse powder, and began to decline thereafter. Addition of 1.0% (w/v) bagasse powder to the cultures resulted in increase in activity of ligninolytic enzymes of the fungi. The maximum enzyme activities without bagasse powder after 12 days of inoculation were 125, 105 and 115 μmol/ml.min for LiP, MnP, and laccase, respectively. The maximum activities with bagasse powder were 335, 305 and 290 μmol/ml.min for LiP, MnP, and laccase respectively

Table 3. Effect of pH on activity of ligninolytic enzymes activities after 12 days of inoculation of *Trametes versicolor*.

Enzyme activity (µmol/ml.min)	pH			
	2.5	**3.5**	**4.5***	**5.5**
LiP	380	405	430	370
	(125)	(145)	(170)	(140)
MnP	340	365	390	340
	(105)	(115)	(145)	(120)
Lacasse	330	340	365	320
	(115)	(125)	(140)	(120)

Values in parenthesis () are for cultures without bagasse powder.
*Optimum pH value.

Table 4. Effect of temperature on activity of ligninolytic enzymes activities after 12 days of inoculation of *Trametes versicolor*.

Enzyme activity (µmol/ml.min)	Temperature (ºC)			
	20	**30**	**40***	**50**
LiP	420	430	465	405
	(115)	(130)	(170)	(140)
MnP	350	385	415	390
	(105)	(120)	(145)	(120)
Lacasse	330	350	385	365
	(115)	(140)	(155)	(120)

Values in parenthesis () are for cultures without bagasse powder.
*Optimum temperature.

(Table 1). LiP acivity is highest (335 µmol/ml.min) compared to MnP and lacasse with bagasse amendment. Without bagasse, LiP activity was highest (125 µmol/ml.min). Therefore, for further optimization studies on bioprocess parameters, 12 days incubation time was taken as optimum for maximum ligninolytic enzymes activity.

Effect of bagasse powder dose on enzyme activity

The effect of bagasse dose is shown in Table 2. The fungus showed maximum enzymatic activity with 2.0% (w/v) bagasse powder. The maximum activities showed by LiP, MnP, and lacasse were 395, 365 and 335 µmol/ml.min with bagasse powder, respectively. The maximum activities showed by LiP, MnP, and laccase are 125, 105 and 115 µmol/ml.min without bagasse powder, respectively.

Effect of pH on enzyme activity

The effect of pH on acivity of ligninolytic enzymes of the fungi is shown in Table 3. With increase in pH value, the activity of the three enzymes increases for both with and without bagasse powder. LiP is highest (430 µmol/ml.min) with bagasse powder at pH value of 4.5. Beyond and below pH value of 4.5, the activity of ligninolytic enzymes gradually declines (Table 3). LiP, MnP and laccase activities were 160, 130 and 140 µmol/ml.min at pH value of 4.5 without bagasse powder, respectively. For further optimization studies on bioprocess parameters, pH value of 4.5 was taken as optimum for maximum ligninolytic enzymes activity.

Effect of temperature on enzyme activity

The effect of temperature on acivity of ligninolytic enzymes of the fungi is shown in Table 4. With increase in temperature, the activity of the three enzymes increases upto 40^{o}C temperature with and without bagasse powder, and then gradually declines. The maximum enzyme activities were 465, 415 and 385 µmol/ml.min for LiP, MnP and laccase, respectively with bagasse powder at 40^{o}C temperature. Without bagasse, the maximum enzyme activities showed as 170, 145 and 155 µmol/ml.min for LiP, MnP, and laccase, respectively. For further optimization studies on bioprocess parameters, 40^{o}C temperature was taken as optimum for maximum ligninolytic enzymes activity.

Effect of shaking speed on enzyme activity

The effect of shaking speed of water bath (rpm) on acivity of ligninolytic enzymes of the fungi is shown in Table 5. With increase in shaking speed, the activity of the three

Table 5. Effect of shaking on activity of ligninolytic enzymes activities after 12 days of inoculation of *Trametes versicolor*.

Enzyme activity (µmol/ml.min)	Shaking speed (rpm)			
	20	**40**	**60***	**80**
LiP	465 (170)	480 (180)	495 (195)	475 (175)
MnP	415 (145)	428 (145)	440 (160)	425 (150)
Lacasse	385 (155)	390 (155)	410 (170)	390 (160)

Values in parenthesis () are for cultures without bagasse powder.
*Optimum shaking speed.

enzymes increases upto 60 rpm with and without bagasse powder, and then gradually declines. The maximum enzyme activities were 465, 415 and 385 µmol/ml.min for LiP, MnP, and laccase respectively with bagasse powder at 60 rpm. Without bagasse powder, the values were 170, 145 and 155 µmol/ml.min for LiP, MnP and lacasse, respectively.

DISCUSSION

Activity of ligninolytic enzymes was analyzed after 3 days of inoculation. For the full growth and adaptation on the solid substrate, the fungus requires this time. The mycelium has grown over the bagasse, depleted the available nitrogen and the system become ligninolytic. The depletion of limiting nitrogen triggers the development of ligninolytic system and is therefore necessary for activation (Kaal et al., 1995; Zafar et al., 1996). With increase in bagasse powder dose, the activity of ligninolytic enzymes gradually decreases. This can be explained on the basis substrate inhibition. At higher concentration of substrate, the fungus does not follow the Michaelis-Menten enzyme kinetic rate model (Bailey and Ollis, 1981; Shulter and Kargi, 2000) for enzyme kinetics. Therefore, for further optimization studies on bioprocess parameters, 2% (w/v) bagasse dose was taken as optimum for maximum ligninolytic enzymes activity. Ardon et al. (1998) reported that cotton stalk improved the maximum ligninolytic enzymes of *Pleurotus ostreatus* in 8 days incubation time. Castillo et al. (1997) showed lignin and manganese peroxidase activity in extracts from straw solid substrate fermentation in maximum at 6 days of inoculation. Plat et al. (1983) reported increased lignin biodegradation of straw by *P. ostreatus* with increased activity of ligninolytic enzymes.

A pH value of 4.5 was obtained as optimum for maximum ligninolytic enzymes activity. The intact enzyme may contain both positively or negatively charged groups at any given pH. Such ionizable groups are often apparently part of the active site. Variation in the pH of the medium result in change in the ionic forms of the active sites and changes in the activity of the enzymes, and hence the reactions rate. Changes in pH may also alter the three-dimensional shape of the enzymes (Bailey and Ollis, 1981; Shulter and Kargi, 2000; Pclczar et al., 2004). For these reasons, enzymes are very active over a certain pH range.

For many enzymes, denaturation of proteins begins to occur at 45 to 50°C temperatures. Sensitivity of a protein to denaturation at elevated temperatures can vary widely with medium pH, and the influence of various temperature-pH combinations may differ tremendously from enzyme to enzyme (Bailey and Ollis, 1981; Shulter and Kargi, 2000; Pclczar et al., 2004). A temperature of 40°C was obtained as optimum for maximum ligninolytic enzymes activity

With increase in shaking speed, the activity of the three enzymes increases up to 60 rpm with and without bagasse powder, and then gradually declines. Mechanical agitation can disturb the elaborate shape of enzyme molecule to such a degree that deactivation occurs. This characteristic mechanical fragility of enzymes may impose limits on the fluid forces which can be tolerated in enzyme reactors being shakened to increase substrate mass transfer and oxygen transfer rates (Bailey and Ollis, 1981; Shulter and Kargi, 2000; Pclczar et al., 2004).

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