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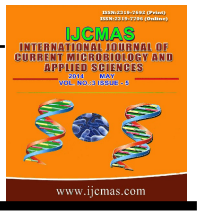

Original Research Article

Production of thermostable lignolytic enzymes by *Thermoascus aurantiacus* MTCC 375 using paddy straw as substrate

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Lignolytic enzymes, *Thermoascus aurantiacus* MTCC 375, Mandel's medium, Digested Biogas Slurry Medium.

A B S T R A C T

The present study reports the production of thermostable lignolytic enzymes (laccase, lignin peroxidase and manganese peroxidase) by *Thermoascus aurantiacus* MTCC 375 using paddy straw as substrate under solid state fermentation in two different media (viz., Mandel's medium and Digested Biogas Slurry Medium (DBS). Both the media were inoculated with two bits (5mm dia) of *Thermoascus aurantiacus* MTCC 375 and activity of laccase, manganese peroxidase and lignin peroxidase were measured for the incubation period of 2, 4, 6 and 8 days. Maximum production of laccase, manganese peroxidase and lignin peroxidase in DBS were found as 785.0 IU/g, 225.0 IU/g and 9187.5 IU/g respectively, where as in the Mandel's medium 65.0 IU/g, 125.0 IU/g and 500.0 IU/g were found respectively. Since all the three activities were higher in DBS as compared to Mandel's medium, which means that this medium is suitable for lignolytic enzyme production as compared to Mandel's medium. No addition of expensive media is required and is thus economically advantageous. Thus, because of the resulting high levels of enzyme production, this medium could be used to scale up the production of lignolytic enzymes.

Introduction

Huge quantity of agro-industrial biomass is produced worldwide annually, that is including about 900 million tonnes of rice straw (RS) of which more than 90% are produced in Asia (Jahromi *et al* 2011) and India has been reported to produce about 150 million tonnes of paddy straw during 2011-12 (Anonymous 2012). Only a minor portion of this major fraction is used as animal feed and household fuel while the remaining paddy straw is disposed off by burning. Paddy straw is lingo-cellulosic material that contains cellulose, hemicellulose and lignin as major components. Cellulose is a linear homopolymer of glucose units linked with

β-1,4-glucosidic bonds. Hemicelluloses are heteropolysaccharides consisting of short branched chains of hexoses, e.g. mannose units in mannans and pentoses such as xylose units in xylans (Kuhad *et al* 1997). After cellulose lignin is the second most abundant renewable biopolymer in nature. It is an essential part of the plant cell wall, imparting rigidity and protecting the easily degradable cellulose from attack by pathogens. It is synthesized from phenyl propanoid precursors by polymerization in higher plants. The lignin precursors *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol consist of an aromatic ring and a 3-carbon side chain (Brown 1985). In the lignin molecule the precursors form 3 types of subunits: hydroxyphenol- (H-type), guaiacyl- (G-type) and syringyl subunits (S-type). A typical finding for the lignin polymer is that there is no single repeating bond between the subunits but a random distribution of at least 10 different types of bond, the most common being the β-aryl ether (*β*-O-4) bond (Argyropoulos and Menachem 1997).

Lignin breakdown is thought to occur by concomitant action of ligninolytic enzymes. The main extracellular enzymes participating in lignin degradation are heme-containing lignin peroxidase (ligninase, LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13) and Cu-containing laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) (Hatakka 2001). A new group of ligninolytic heme-containing peroxidases, combining structural and functional properties of the LiPs and MnPs, are the versatile peroxidises (VPs). The lignin-degrading enzymes known so far are extracellular and nonspecific, participating in different oxidative reactions where the aromatic structure of lignin and bonds between the basic units are broken (Kuhad *et al* 1997). The resulting small-molecular-weight compounds can then be transported inside the cell for further breakdown by fungi and also bacteria. Cell-free mineralization of synthetic 14C-labelled and natural lignin by one of the ligninolytic peroxidases MnP, was first reported by Hofrichter *et al.* (1999). This may point to the extracellular mineralization (outside the fungal cell) of lignin.

The thermophiles have more stable enzymes as compared to mesophiles. Thermophilic enzymes are also active at low temperature. Thermophiles develop more rapidly to higher peaks as compared to mesophiles and stability of obligate thermophiles increases with process temperature. Thermozymes can be used in several industrial processes, in which they replace mesophilic enzymes or chemicals (Li *et al* 2005).Some thermophilic fungal strains such as *Talaromyces emersonii*, *Thermoascus aurantiacus*, *Thielavia terrestris*, *Sporotrichum thermophile*, *Chaetomium thermophilum* and *Corynascus thermophilus* can produce thermostable enzymes which are stable and active at elevated temperatures (>60°C) well above their optimum growth temperature (30°C to ~55°C) (Maheshwari *et al* 2000).

Thermoascus aurantiacus MTCC 4890, a lignocellulolytic thermophilic fungus, the lignolytic enzymes of which can be used for pretreatment of paddy straw for increasing paddy straw digestibility. The present study was proposed to achieve the production of lignolytic enzymes employing two different media.

Materials 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 (Mandel *et al* 1976) and digested biogas slurry (DBS) supplemented with powdered paddy straw were used for solid state fermentation.

Fungal strain

Thermoascus aurantiacus MTCC 375 was procured from Microbial Type Culture Collection,Institute of Microbial Technology Chandigarh. Stock culture was maintained on potato dextrose agar by monthly transfers.

Solid state fermentation

Solid state fermentation experiment was performed using Mandel's medium (Mandel *et al* 1976) and digested biogas slurry (DBS). For solid state fermentation, 10g of paddy straw was taken in an Erlenmeyer flask (500ml) and mixed with 40ml of digested biogas slurry i.e. in 1:4 (w/v) ratio. Similarly Mandel's medium was taken for other set of experiment separately. 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 (5mm dia) of *Thermoascus aurantiacus* MTCC 375. The inoculated flasks were incubated at 45±2ºC in a BOD incubator. These flasks were removed at a regular interval of 48 hours for a period of eight days and crude enzyme was extracted. The enzyme was extracted from the substrate using 0.1 M citrate buffer (pH 6.2) in the ratio of 1:10 and shaking the contents for 30 min in shaking incubator (250 rpm) at 20˚C. The extract was filtered with muslin cloth to obtain a clear filtrate. The filterate was centrifuged in a cooling centrifuge at 10,000 rpm for 20 minutes at 4˚C and supernatant was used for estimation of laccase, lignin peroxidase and manganese peroxidase activity by spectrophotometric method (Hitachi 2800 UV-Vis Spectrophotometer). The experiment was performed in triplicate.

Enzyme Assay

Laccase estimation was carried out according to the method described by Shandilya and Munjal (1983), manganese peroxidase activity was determined by method of Paszczynski *et al* (1985) and lignin peroxidase activity was determined by method given by Tien and Kirk (1988). Extracellular protein content of enzyme filtrate was estimated according to the method given by Lowry *et al* (1951).

Results and Discussion

Lignolytic enzyme production from *Thermoascus aurantiacus* MTCC 375 was studied under solid state fermentation in two different media viz., Mandel's medium, Digested Biogas Slurry Medium (DBS). Incubation time plays an important role in substrate utilization and enzyme production. The effect of incubation time was evaluated by checking enzymes activities after two day interval for eight days of incubation at 45°C and results are depicted in Tables (1).

Enzyme production in DBS medium:

Maximum lignolytic enzyme activities (laccase 785 U/g; Manganese peroxidase 225 U/g; Lignin peroxidase 9187.5 U/g) were found in DBS medium.While comparing the enzyme activities of substrate after different days of fermentation in DBS medium, it was found that the LiP activity was maximum on 4th day (9187.5 U/g) followed by 8th day (7000 U/g), in case of MnP and LiP two peaks were observed in each, ist on 2nd day (175 U/g, 750 U/g) and 2nd on 8th day (225 U/g, 785 U/g). The high enzyme activity in this medium might be due to the high lignin content as both paddy straw (substrate) and digested biogas slurry itself. As lignin content is higher, so will be the higher concentration of aromatic compounds. According to Elisashvili *et al* (2006), aromatic compounds regulate the ligninolytic enzyme synthesis although their effect is very specific depending on the physiological peculiarities of fungi. No addition of expensive chemicals are required and the use of inexpensive digested biogas slurry will have important economic advantages.

Enzyme production in Mandel's medium

In Mandel's medium, increasing trend was found in laccase activity till 8th day where 65 U/g of enzyme units were observed. MnP was found to increase upto 4th day (125 U/ g), thereafter, it decreased continuously. LiP activity was found to increase continuously upto 6th day (500 U/g) afterwhich it started decreasing. In case of DBS medium, laccase activity increased on 2nd day (750 U/g) then it started declining and on the 8th day (785 U/g) it was found to have increased as compared to 2nd day by 35 U/g, similarly MnP activity was also higher on 2nd day (175 U/g) then it abruptly decreased and on the 8th day again peak activity was noticed (225 U/g), LiP activity was found to be increasing upto 4th day (9187.5 U/g) then decreased abruptly but again it increased sharply on 8th day (7000 U/g) but comparatively less than 4th day. Enzyme activity reported in 0 hr inoculated media (control) in all cases indicates that the enzymes for laccase, lignin peroxidase and manganese peroxidase from *Thermoascus aurantiacus* MTCC 375 may be constitutive in nature.
Our findings are in confirmation with Elisashvili and Kachlishvili (2009) who screened the laccase activity of basidiomycetes and reported that the carbon source and lignocellulosic substrate play a crucial role in enzyme production. Laccase activity of *Pseudotrametes gibbosa* varied from 0.3 Uml^{-1} (Avicel) to 13.7 Uml^{-1} (lactose), while the substitution of wheat bran with walnut pericarp increased *Cerrena unicolor* manganese peroxidase yield from 0.7 Uml^{-1} to 8.3 Uml^{-1}.

The enzyme yield is species-dependent and strain-dependent and selection of new organisms with tremendous synthesis of these enzymes is possible. Vikeneswary *et al* (2006) reported that the productivity of laccase in solid state fermentation of selected agro-residues by *Pycnoporus sanguineus* reached a maximum of 48.7 Ug^{-1} while *Fomes sclerodermeus* grown on wheat straw produced 270 Ug^{-1} of laccase reported by Papinutti *et al* (2003). Bajwa and Arora (2009) studied the lignolytic enzyme production from *Phanerochaete chrysosporium* and *Pycnoporus sanguineus* fungi in malt extract supplemented mineral salts broth (MSB) for a total period of 20 days and reported that LiP appeared earlier than MnP in both

Table.1 Lignolytic enzyme profile of *Thermoascus aurantiacus* MTCC 375 under solid state fermentation in 2 different media

Media	Parameters		Incubation time (days)					CD (5%)
			Control[#]	2	4	6	8	
Mandel's medium	Enzyme activity (U/g)	Laccase	5±0.60 (0.20)	30±0.60 (0.86)	50±0.88 (1.53)	60±1.01 (2.09)	65±0.88 (2.52)	2.6
		MnP	3±0.56 (0.17)	75±0.88 (2.16)	125±1.45 (3.83)	100±1.15 (3.48)	50±1.30 (1.95)	3.8
		LiP	8±1.59 (0.46)	156±2.33 (4.49)	400±2.03 (12.27)	500±1.76 (17.42)	475±0.58 (18.48)	6.8
	Protein (mg/g)		17.3±0.75	34.7±0.87	32.6±0.57	28.7±1.12	25.7±0.33	0.6
Digested biogas slurry	Enzyme activity (U/g)	Laccase	100±1.20 (3.32)	750±1.76 (15.27)	490±0.88 (11.11)	495±0.60 (9.59)	785±0.73 (16.53)	7.5
		MnP	65±1.45 (2.16)	175±0.88 (3.56)	75±0.81 (1.7)	100±0.44 (1.94)	225±1.04 (4.74)	3.8
		LiP	119±1.15 (3.95)	3162.5±1.30 (64.4)	9187.5±0.93 (208.33)	2675±1.45 (51.84)	7000±2.60 (147.37)	5.3
	Protein (mg/g)		30.1±1.01	49.1±0.99	44.1±0.72	51.6.0± 0.88	47.5±0.55	1.8

Control is 0 hr inoculated medium; MnP: Manganese peroxidase; LiP: Lignin peroxidase
Cultural conditions: 20 ml Mandel's medium containing 5g paddy straw; 20 ml Digested Biogas Slurry (DBS) containing 5 g paddy staw ; temperature 45°C; pH 6; ± values indicate standard error for triplicate data; the data represents the mean of three determinations each. CD: Critical difference at 5% level. values in parenthesis indicate specific activities (Umg $^{-1}$ of protein).

the fungi, achieving maximum yields on day 6 and day 10 in *P. chrysosporium* and *P. sanguineus*, respectively. A second LiP peak was noted on day 16 to day 18 in both fungi. Rate of MnP production was relatively slow in *P. sanguineus*, and the highest level was achieved only on day 18. In *P. chrysosporium*, however, MnP production peaked on day 6. Similar to the production of LiP, laccase was produced to detectable levels by *P. sanguineus* on day 2 itself and peaked on day 14.

In the present investigation, specific activities of lignolytic enzymes: laccase (16.53 U/mg of protein), MnP (4.74 U/mg of protein) and LiP (147.37 U/mg of protein) in digested biogas slurry medium were quite higher than the reported ones as above as well as the commercially available ones like laccase from *Trametes versicolor* (15 U/mg); MnP from *Nematoloma frowardii* (≥4.2 U/mg) and LiP from *Phanerochaete chrysosporium* (≥20 U/mg) (www.sigma-chem.com.au), thus indicating that the fungus *Thermoascus aurantiacus* MTCC 375 has the commercial potential for lignolytic enzymes.

Very high yields of laccase (785 U/g), MnP activity (225U/g) and LiP (9187.5 U/g) were obtained on a simple medium i.e. digested biogas slurry by using

Thermoascus aurantiacus MTCC 375. No addition of expensive chemicals is required and is thus economically advantageous. Thus, because of the resulting high levels of enzyme production, this medium and fungi could be used to scale up the production of lignolytic enzymes.

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