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Enzyme profiling of lignocellulolytic fungi

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ABSTRACT

Lignocellulolytic fungi produce a variety of lignocellulolytic enzymes which are responsible for the biodegradation of lignocellulosic agro-wastes in nature. These enzymes are also useful for biofuel production, bio-bleaching, bio-pulping etc. We have isolated ecodiversely different seventeen fungi from ecorich soils of Gandhinagar region, Gujarat, India. The objective of this work was to study enzyme production profile by lignocellulolytic fungi, using wheat straw as a model agro-waste by solid state fermentation. Most of these lignocellulolytic fungi have been found to express enzyme activities like filter paper activity (FPase), endoglucanase, exocellulase, β-glucosidase, xylanase, glucoamylase, manganese peroxidase (MnP) and protease. Among these, *A. niger*, *A. oryzae* and *Sporotrichum* sp. produce endocellulase and xylanase in significant amount. *A. niger* and *Sporotrichum* sp. gave 52.47 U/g and 69.441 U/g endocellulase activity, and 48.107 U/g and 112.649 U/g xylanase activity, respectively.

Key words- *Aspergillus niger*, *Aspergillus oryzae*, *Sporotrichum* sp., fungal isolation, solid state fermentation, wheat straw

INTRODUCTION

Agricultural waste contains a high proportion of lignocellulose, which have potentials for bioconversion. Agricultural and agro-industrial waste increased as a result of industrialization, becoming a problem regarding space for disposal and environmental pollution (da Silva et al., 2005). A lignocellulosic plant material contains higher cellulose (23-53%) with lesser but significant amounts of hemicellulose (20-35%) and lignin (10-25%) and other extractable compounds (Knauf and Moniruzzaman, 2004). Cellulose and hemicellulose represent more than 50% of the dry weight of agricultural residues. These polymers can be converted into fermentable sugars by enzymatic hydrolysis, so it can be used as a plentiful and cheap source of liquid fuels, food products or other chemicals of interest (Romero et al., 1999; Kang et al., 2004).

The hydrolytic action of cellulases and hemicellulases is of fundamental importance to obtain fermentable sugars from lignocellulosic biomass (Leite et al., 2007). The enzymatic hydrolysis of cellulose into glucose involves the synergistic action of at least three different enzymes: endocellulase (EC 3.2.1.4), exocellulase (EC 3.2.1.91) and

β-1, 4-glucosidase (EC 3.2.1.21) (Acharya et al., 2008). The enzymatic hydrolysis of xylan requires mainly two enzymes viz. β-1, 4-endoxylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) (Leite et al., 2007). However, for complete hydrolysis of hemicellulose, α-L-arabinofuranosidase (EC 3.2.1.55), endomannanases (EC 3.2.1.78), β-mannosidases (EC 3.2.1.25) and α-galactosidases (EC 3.2.1.22) are also required (Jorgensen et al., 2005). The main extracellular enzymes participating in lignin degradation are lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13) and laccase (EC 1.10.3.2) (Hatakka, 2001).

The enzymatic hydrolysis of lignocellulose represents a special case of enzymology, since the substrate is solid and hydrolysis occurs in the solid phase. Production of enzymes by Solid State Fermentation (SSF) offers several advantages over submerged system, simply because of its low operational and production cost. At the same time, SSF uses inexpensive agro-waste materials as substrates. The use of agro-wastes not only helps to overcome problem of solid waste management but also allows the development of biotechnological processes from cheap natural resources. Enzymes remain most frequently reported metabolites produced via SSF, some of which include cellulases, xylanases, lipases, phytase, protease, lignin degrading enzymes and pectinases (Pang et al., 2006).

Wheat straw is an abundant byproduct from wheat production. The average yield of wheat straw is 1.3-1.4 lb per lb wheat grain (Montane et al., 1998; Saha and Cotta, 2006). Wheat straw contains 35-45% cellulose, 20-30% hemicellulose and 8-15% lignin and can also serve as a low cost attractive feedstock (Saha and Cotta, 2006). In recent years using fungi as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity. Filamentous fungi are attracting greater attention as potential sources of plant cell wall hydrolyzing enzymes, because they secrete high levels of the enzymes into culture medium (Berry and Paterson, 1990).

In our search for fungi capable of efficiently degrading lignocelluloses, some lignocellulolytic fungi were isolated from Gandhinagar region, Gujarat, India. The present study focused on profiling of enzyme production from lignocellulolytic fungi.

MATERIALS AND METHODS

Fungal strains and culture conditions

A total of 13 strains were used in present experiment. These strains were soil inhabitant and isolated from soils of Sadra, Rajpur and Alua hills regions of Gandhinagar (23.22°N and 72.68°E). Soil samples were collected aseptically in UV sterilized plastic bags using sterile specula from top 9 inch layer of soil. In the laboratory, 1g soil was transferred aseptically into 9 mL sterile distilled water and mixed thoroughly. From this, 0.1 mL was spread over Potato Dextrose Agar (PDA) in Petri plates and the plates were incubated at 28 ^{o}C for 5-7 days. After colonial growth pure culture were obtained by repeatedly transferring on PDA plates. Pure cultures were stored on PDA slants at 4 ^{o}C. *Phanerochaete chrysosporium* was used as a model strain for comparative study and was obtained from National Chemical Laboratory, Pune, India.

Media preparation and enzyme production

Enzyme production was carried out using untreated wheat straw as carbon source by solid state fermentation technique. The moisture content was maintained between 60-70% using salt medium (Ekperigin, 2007), which contained 0.01% $MgSO_4$, 0.1% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.7% K_2HPO_4 and 0.05% Na-citrate. pH of media adjusted to 5.0±0.2. Three gram wheat straw was placed in 250 ml conical flask and moistened with 6 ml salt medium in duplication. It was then sterilized by autoclaving at 121 ^{o}C for 15 min followed by cooling and inoculation with 10 discs of 8.0 mm diameter of organism from

PDA culture plates using sterile cork borer. Flasks were incubated at 28 ± 5 ^{o}C for 8 days.

Extraction of extracellular enzymes

The fermented substrate was extracted with 50 ml citrate buffer (pH 4.8; 50mM) by shaking the mixture at 180 rpm for 30 min at 28 ± 2 ^{o}C and filtered through Whatman No. 2 filter paper. The filtrate was used for profiling of various enzymes.

Measurement of enzyme activities

All activity tests were conducted in a 50 mM citrate buffer having pH 4.8. The total cellulase activity was measured as filterpaperase (FPase) against Whatmann No.1 filter paper (Ghose, 1986). The total endoglucanase activity was determined against carboxymethylcellulose (CMC) (Ghose, 1986). The exocellulase activity was determined against cotton. The β-glucosidase activity was determined against salicilin (Chahal, 1985). The Endo-β-1,4-xylanase activity was determined against birchwood xylan (Bailey et al., 1992) and glucoamylase activity was determined against starch (Ghose, 1986). The reducing sugar produced was determined by the DNS method (Miller, 1959). One international unit (U) was defined as the enzymatic activity needed for the release of 1 μmol of glucose equivalents per unit volume and minute of reaction. The protease enzyme was assayed following casein substrate method using L-tyrosine as standard (Anson, 1938). The manganese peroxidase (MnP) activity was determined spectrometrically against phenol (Kuwahara et al., 1984). One international unit (U) was defined as the amount of enzyme that oxidized 1 μmol of dimethoxyphenol per minute.

Statistical analysis

Results were analyzed for standard deviation following the method suggested by Rangaswami (1995).

RESULTS

All the isolates were identified at Agharkar Research Institute, Pune, India. All isolates exhibited endoglucanase, exocellulase, β-glucosidase and filter paper activity (FPase) activities (Table 1). The contents of endoglucanase, β-glucosidase, exocellulase and FPase activities were recorded as 52.47, 6.172, 0.656 and 9.259 U/g in *A. niger*; 69.441, 16.974, 0.712 and 6.794 U/g in *Sporotrichum sp*. and 2.16, 6.172, 0.463 and 6.172 U/g in *A. oryzae*, respectively. Compared to *P. chrysosporium*, *A. niger* and *Sporotrichum* sp. gave 2.8 and 2.1 fold high endoglucanase respectively, and same FPase activity was observed with *A. niger*, while *A. oryzae* and *Sporotrichum* sp. gave 1.5 and 1.4 fold lesser FPase activity. These three organisms gave lesser β-glucosidase activity than *P. chrysosporium*. As shown in Table 1, FPase activity was successfully detected for all species.

Maximum endoxylanase activity (112.649 U/g) was given by *Sporotrichum sp*., while *A. niger* and *A. oryzae* gave 48.107 and 14.802 U/g respectively (Table 1). While *P. chrysosporium* gave 3.4 and 1.5 fold lesser endoxylanase activity than *Sporotrichum sp* and *A. niger* and 2.3 fold higher than *A. oryzae.*

Highest glucoamylase activity (52.466 U/g) was observed in *Sporotrichum sp*. whereas 5.090 and 5.401 U/g glucoamylase activity was determined in *A. niger* and *A. oryzae* respectively. *P. chrysosporium* gave 4.63 U/g glucoamylase activity (Table 1), which was approximately 11, 1.2 and 1.1 fold lesser than *Sporotrichum sp., A. oryzae* and *A. niger*, respectively.

P. chrysosporium gave 3.817 U/l MnP activity, while 2.312 U/l, 0.914 and 0.433 U/l MnP activity were observed with *A. oryzae*, *A. niger* and *Sporotrichum sp*., respectively (Table 1). *P. chrysosporium* gave 2.39 U/ml while *A. oryzae*, *A. niger* and *Sporotrichum sp*. gave 2.450, 3.435 and 0.998 U/ml, protease activity respectively (Table 1). It means enzymes produced by*Sporotrichum sp*. were more stable than those from *P. chrysosporium*, *A. oryzae* and *A. niger*. Phytase activity was also analyzed but unfortunately none of the organisms produced phytase enzyme.

DISCUSSION

Cellulase activity decreased with time which may be due to the accumulative

Table 1: Enzymatic activity of isolated lignocellulolytic fungal strains.

Name of Strain	β-Glucosidase (U/g)	Endocellulase (U/g)	Exocellulase (U/g)	FPase (U/g)	Xylanase (U/g)	Glucoamylase (U/g)	Protease (U/mL)	MnP (U/L)
P.chrysosporium	22.34 ±0.859	24.69±0.988	1.273±0.058	9.259±0.369	33.305±1.259	4.63±0.206	1.243±0.050	3.817±0.191
Rhizopus sp.	6.172± 0.306	15.43± 0.770	1.968±0.078	4.629±0.196	37.005±1.428	5.401±0.262	0.759±0.035	3.28±0.160
Aspergills oryzae	6.172±0291	2.16±0.101	0.463±0.023	6.172±0.227	14.802±0.681	5.401±0.158	1.519±0.161	2.312±0.098
Aspergills niger	9.259±0.394	15.43±0.569	0.424±0.020	4.629±0.126	11.102±0.432	0.772±0.111	0.912±0.035	2.849±0.143
Aspergills niger	6.172±0.159	52.47±1.868	0.656±0.087	9.259±0.354	48.107±1.933	5.09±0.159	2.762±0.123	0.914±0.208
Aspergills oryzae	6.172±0.222	2.16±0.089	0.694±0.077	3.086±0.126	25.903±1.010	7.87±0.274	1.105±0.105	3.71±0.119
Sporotrichm sp.	3.086±0.099	18.52±0.889	1.543±0.129	7.716±0.399	9.399±0.388	4.786±0.191	0.552±0.029	0.376±0.036
Aspergills niger	9.259±0.258	64.811±2.380	0.831±0.092	3.858±0.202	84.87±3.284	21.603±1.081	0.652±0.056	0.108±0.031
Aspergills oryzae	9.259±0.394	67.898±3.241	0.712±0.099	6.172±0.280	61.72±2.019	26.233±1.180	1.441±0.222	0.323±0.051
Sporotrichum sp.	16.974±0.662	69.441±3.646	0.712±0.087	6.794±0.309	112.649±4.506	52.466±1.281	0.998±0.086	0.433±0.036
Aspergills fumigates	0.463±0.023	66.354±2.598	0.949±0.099	9.259±0.351	13.117±0.487	15.431±0.544	1.211±0.154	0.269±0.050
Neurospora crass	12.345±0.581	6.481±0.189	0.823±0.081	6.481±0.288	85.111±3.298	17.623±1.011	0.266±0.041	3.748±0.132
Aspergills nidulus	19.521±0.881	10.108±0.388	1.039±0.108	7.963±0.308	111.015±3.881	19.536±0.969	1.459±0.321	2.498±0.143
Aspergillus sp.	11.265±0.446	14.737±0.589	0.795±0.094	5.509±0.282	74.01±2.556	7.129±0.334	0.286±0.039	14.991±0.443

effect of cellulobiose, which is dimmer of glucose and known to inhibit both endoglucanase and glucosidase (Howell, 1978). The time of the highest cellulase activity depends upon substrate and fungus used (Ojumu et al., 2003). As discussed by Hatakka (1983), delignification produces aromatic water-soluble products which can also repress the cellulolytic action of the enzyme.

For total hydrolysis of lignocellulosic material, synergistically acting enzymes are needed. Xylan is the main carbohydrate found in hemicellulose. Xylanase belong to glycosyl hydrolase families 10 and 11 (Biely et al., 1997). High xylanase production in wheat straw could be attributed to its hemicellulosic nature and favorable degradability as well as the presence of some nutrients in the carbon source (Sonia et al., 2005). Angayarkanni et al. (2006) studied *A. indicus* and *A. flavus* which gave 145.044 and 140.810 IU/ml endoxylanase activity respectively. In lignocellulolytic waste there were traces of starch, on which amylase acted and produced glucose monomers. Glucoamylase consecutively hydrolyzes α-1,4 glycosidic bonds from non-reducing ends of starch, resulting in the production of glucose (Pavezzi et al., 2008).

The multienzyme system involved in lignin degradation and mineralization is constituted of different ligninolytic enzymes combination, being the occurrence of MnP and laccase higher than LiP (Wesenberg et al., 2003). 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 and Anantharaman, 2006). The peroxidases used hydrogen peroxide generated by glyoxal oxidase, glucose oxidase, and cellobiose oxidase to promote the oxidation of lignin to free radicals that then underwent spontaneous reactions with oxygen or water, leading to depolyrmerization (Bennet et al., 2002).

Baldrian and Snajdr (2006) studied the production of lignolytic enzymes by litter-decomposing fungi in HNHC medium. They studied eight different fungal strains and *T. versicolor* gave higher MnP (33.2 U/l) than others at 17th day while *P. ostretus* gave 1.9 U/l at the 21st day. Bonnarme and Jeffries (1990) studied *P. chrysosporium* BKM, which gave 86 U/l at 8th day and *P. chrysosporium HHB6251* gave 29 U/l MnP activities at 7th day.

In addition to lignocellulolytic enzymes the protease activity was also measured because the presence of protease may affect the stability of xylanase during extraction and storage (Gessesse and Mamo, 1999). It was therefore considered essential that the protease titer of selected organisms should be as lower as possible

Conclusion

Isolated fungi exploring from Gandhinagar region, revealed that they can produce lignocellulolytic enzymes directly utilizing abundantly available lignocellulosic waste, wheat straw. Among 13 isolates *A. niger*, *A. oryzae* and *Sporotrichum* sp., were screened as potent lignocellulolytic fungi.

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