Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

ISSN: 0972-6268	Vol. 14	No. 1	pp. 47-52	2015

Original Research Paper

Evaluation of Lignocellulosic Agro Wastes for the Enhanced Production of Extracellular Cellulase and Xylanase by *Trichoderma harzianum*

S. Karthick Raja Namasivayam, M. Babu* and R. S. Arvind Bharani
Department of Biotechnology, Sathyabama University, Chennai 600 119, T.N., India
*Department of Chemical Engineering, Sathyabama University, Chennai 600 119, T.N., India

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com
Received: 3-3-2014
Accepted: 22-5-2014

Key Words:
Agro wastes
Trichoderma harzianum
Cellulase
Xylanase
Enzyme activity

ABSTRACT

In the present study, various lignocellulotic agro-wastes paddy straw, coir pith, sugarcane bagassae and dried leaf litter were evaluated for cellulase and xylanase enzyme production adopting solid state fermentation with different moisture levels ranging from 0 to 50% by soil isolate of *Trichoderma harzianum*. The crude enzyme obtained was partially purified by ammonium sulphate precipitation and DEAE cellulose column chromatography. All the wastes supported growth and enzyme production. Maximum cellulase and xylanase production was recorded in paddy straw followed by sugarcane bagassae with 25% moisture content. Both the enzymes retained maximum activity at 60°C and pH 8.0. The partially purified enzyme by ammonium sulphate precipitation at 60% saturation followed by DEAE cellulose column chromatography yields pure xylanase with the molecular weight of 56 and 61 KD.

INTRODUCTION

Microorganisms are the major source of industrial enzyme production and the production of enzyme from microbes is highly appreciated because of ease of cultivation, high yield and utilization of various cheapest sources. Biological wastes contain several reusable substances of high value such as soluble sugars and fibre. Direct disposal of such wastes to soil or landfill causes serious environmental problems (Namasivayam & Nirmala 2011). These biological wastes can be used as support-substrates in solid-state fermentation (SSF) to produce industrially relevant metabolites with great economical advantage (Chutmanab 2008). Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source. In addition to the conventional applications in food and fermentation industries, microbial enzymes have attained significant role in biotransformations involving organic solvent media, mainly for bioactive compounds (Chahal & Young 1982). This system offers numerous advantages over submerged fermentation (SmF) system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipment, etc. (Pandey et al. 1994, Selvakumar et al. 1997). Agro-industrial residues are generally considered the best substrates for the SSF processes, and use of SSF for the production of enzymes is no exception to that. A number of such substrates have been employed for the cultivation of microorganisms to produce host of enzymes. Some of the substrates that have been used included sugar cane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soyhull, sago hampas, grapevine trimmings dust, saw dust, corncobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, aspen pulp, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch, etc. (Mitra et al.1994, Babu & Sathyanarayana 1994). The selection of a substrate for enzyme production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate, and thus may involve screening of several agro-industrial residues. In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as an anchorage for the cells. The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate. However, some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates. In such cases, it would become necessary to supplement them externally with these. It has also been a practice to pre-treat (chemically or mechanically) some of the substrates before using in SSF processes (e.g. lignocellulose), thereby making them more easily accessible for mi-

crobial growth (Tengerdy 1998, Liu et al. 2007). In the present study, various agro wastes such as paddy straw, coir pith, sugarcane bagasse and leaf litter were utilized for cellulase and xylanase production adopting solid state fermentation with different moisture levels. The effect of pH and temperature on enzyme activity was also studied. Purification of the enzymes by ammonium sulphate precipitation and column chromatography was also carried out.

MATERIALS AND METHODS

Isolation of *Trichoderma harzianum*

Trichoderma harizanum was isolated from the agricultural field soil adopting soil dilution method (Clark 1997) and the isolated fungi was identified based on cultural characteristics and microscopic examination of fungal spores with lactophenol cotton blue. The pure culture was maintained on potato dextrose agar slant.

Screening of Enzyme Production

Xylanase: Primary inoculum media (peptone 1%, yeast extract, 0.5 % and KH_2PO_4) supplemented with 1% hinchwood xylan was sterilized by autoclaving. After sterilization the media was poured into sterile Petri plates, purified isolates of respective fungi cultured on oat spelt xylan agar medium and incubated at 32°C for 4 days. The plates were then flooded with absolute ethanol and left for 16 h at room temperature to precipitate xylan. Colonies producing xylanase enzyme were surrounded by clear zones against an opaque background of non-hydrolyzed media. Positive results were confirmed in a repeat test. The fungi which showed maximum zone of clearance were selected for further study.

Cellulase: Cellulase screening was carried out in primary inocula media described earlier with 1.5 % carboxyl methyl cellulose. Respective fungal culture was inoculated on the sterile media, the seeded plates were incubated at 32°C. After the incubation period, the plates were flooded with congo red solution followed by successive washing with saline solution. Colonies producing cellulase enzyme were surrounded clear zones against an opaque background of non-hydrolyzed media. Positive results were confirmed in a repeat test. The fungi which showed maximum zone of clearance were selected for further study.

Isolation of crude enzyme: Among the different isolates, *Trichoderma harzianum,* which shows maximum zone of clearance, was inoculated into 100 mL of mineral salt medium supplemented with 0.5% of soluble oat spelt xylan (xylanase) and carboxyl methyl cellulose (cellulase) in 250 mL of conical flask at the spore concentration of 108 spores/mL. The seeded flasks were incubated at 30°C with 100 rpm for four days in orbital shaker. After the incubation the culture contents were filtered through a 0.45µm pore size filter (HA type; millipore) and the collected filtrate was used as crude enzyme for further study.

Enzyme activity assay: Assays for crude xylanase and cellulase were performed using 0.5% soluble oat spelt xylan (xylanase) and carboxyl methyl cellulose (cellulase) in 50 mM sodium phosphate buffer, pH 7. The reaction mixture was composed of 1.8 mL substrate and 0.2 mL crude enzyme. The mixture was incubated in a water bath at 60°C for 15 min. The released reducing sugar was measured by the 3, 5-dinitrosalicylic acid (DNSA) method in which the reaction was stopped by adding 3 mL of DNSA acid.

Evaluation of agro wastes for enzyme production: Paddy straw, sugarcane bagasse, coir pith and leaf litter were selected for the present study. Respective collected wastes were soaked overnight in 0.1N NaOH solution and the treated wastes were allowed to shade dry. After drying, 100g of the respective wastes were transferred to the 250 mL of conical flask and the moisture content was adjusted to 0, 5, 10, 25 and 50% with distilled water. The flasks were sterilized by autoclaving. After the sterilization, 0.1 mL of the spore suspension (10^8 spores/mL), originally obtained from seven days fungal slant, was flooded with tween 80 and scrapped well with sterile glass rod. The slurry thus obtained was filtered through crude filter paper and the filtrate obtained was used as source of inoculum, the inoculated flasks were incubated at 32°C for 7 to 10 days.

Extraction of the crude enzymes and enzyme activity: After the incubation period, the respective flasks were mixed with double the volume of distilled water containing 1% tween 80, mixed well and kept in the shaker at room temperature for 2 hours. The suspension was filtered through cheese cloth to remove the debris. The collected filtrate thus obtained was used as source of crude enzyme. Enzyme activity was determined by DNS method as described earlier.

Purification: Culture filtrate was submitted to ammonium sulphate precipitation. The fraction from 60% saturation, which contained 60% of the total enzyme activity, was dialysed for 24 hours at 5°C against three changes of 2 litres of distilled water. The dialysate was lyophilized and stored at -20°C. Twenty milligram of this material was applied to a Bio-gel P-70DEAE cellulose column (Bio-Rad laboratories, California), eluted with 0.05 M tris hydrochloric acid -0.1 MKCl (pH 7). Fractions showing xylanase activity were dialysed against 0.001 M tris hydrochloride buffer (pH 7) for 24 hours at 5°C, lyophilized and stored at -20°C.

SDS-PAGE: SDS-PAGE was carried out to assess the molecular weight of the protein. The gel was polymerized from

Table 1: Generic composition of fungi isolated from soil.

S.No	Fungi	composition (%)
1	*Trichoderma*	45.5
2	*Fusarium*	20.5
3	*Phoma*	17.0
4	*Pencillium*	10.0
5	*Alternaria*	7.0

Table 2: Xylanase (X) and cellulase (C) production by *Trichoderma harzianum* (SK 01) strain in MSM media.

S.No.	Fermentation time (Hour)	Enzyme activity (U/mL)	
		X	C
1	6	0.0	0.29
2	12	0.21	0.45
3	18	0.48	0.74
4	24	0.61	0.81
5	30	0.93	1.03
6	36	1.03	2.01
7	42	1.54	1.97
8	48	2.21	1.43
9	54	2.01	1.12
10	60	1.87	0.97
11	66	1.21	0.76
12	72	1.03	0.64
13	78	0.63	0.41
14	84	0.42	0.24
15	92	0.12	0.19

Table 3: Effect of purification on xylanase and cellulase activity (U/mL).

S.No.	Purification	Enzyme activity (U/mL)	
		X	C
1	Crude enzyme	2.26	2.13
2	Ammonium sulfate fractionation (60%)	3.01	2.76
3	DEAE column chromatography	4.12	3.12

Table 4: Effect of pH on xylanase (X) and cellulase (C) activity.

S.No.	pH	Enzyme activity (U/mL)	
		X	C
1	4.0	0.51	0.32
2	5.0	2.62	0.51
3	6.0	3.71	2.72
4	7.0	1.01	1.01
5	8.0	0.21	0.52
6	9.0	0.06	0.07

the mixture of 17.5 mL of 30% acrylamide-0.8% methylene bisacrylamide-17.5mL of 1.5M tris hydrochloride (pH 8.8)-35 mL of distilled water.

Effects of pH and temperature on enzyme activity: The range of buffers were used at 50 mM in preparing 0.5% (w/v) xylan solution for detection of xylanase activity and 0.5 % of carboxyl methyl cellulose was aceto-acetate buffer (pH 4, 5 and 6), phosphate buffer (pH 7), tris buffer (pH 8 and 9). The reaction conditions were as mentioned before using the culture filtrate as the enzyme source. The pH value giving the highest enzyme activity was used in further enzyme assays. The optimal temperature for enzyme activity was determined by performing the standard assay procedure at 40, 50, 60, 70, 80 and 90°C to determine the effect of temperature.

Table 5: Effect of temperature on xylanase (X) and cellulase (C) activity .

S.No	Temperature (°C)	Enzyme activity (U/mL)	
		X	C
1	40	1.12	0.91
2	50	2.19	1.21
3	60	3.14	2.17
4	70	1.23	1.21
5	80	0.11	0.12
6	90	0.09	0.04

X- xylanase ; C- Cellulase

RESULTS AND DISCUSSION

Generic composition of fungi: A total of 86 fungi isolates belonging to five different genera *viz., Fusarium, Trichoderma, Alternaria, Phoma* and *Penicillium* were isolated. All the isolates were identified by cultural and morphological characteristics. As shown in the Table 1 *Trichoderma* was the predominant genera (45.5%) followed by *Fusarium* (20.5%), *Phoma* (17.0), *Penicillium* (10.0%) and *Alternaria* (7.0%).

Xylanase and cellulase production in mineral salt media: Among the different soil isolates, xylanase and cellulase activity was recorded in all the isolates of *Trichoderma harzianum,* and *Trichoderma harzianum* SK 3 strain was further selected because of the maximum zone of clearance was observed in oat spelt xylan and CMC agar medium inoculated with *Trichoderma harzianum* SK 3. When *Trichoderma harzianum* (SK 01) strain grown in mineral salt medium supplemented with 0.5 % of soluble oat spelt xylan and carboxyl methyl cellulose, xylanolytic and cellulolytic activity was appeared rapidly in the medium. Supernatants of *Trichoderma harzianum* (SK 3) strain showed xylanolytic and cellulolytic activity during tenth and six hour of fermentation time and maximum enzyme production was recorded during 48 and 36 hour and the enzyme production was gradually decreased in successive time periods (Table 2).

Enzyme production from agrowastes: All the wastes used in the present study supported growth and enzyme produc-

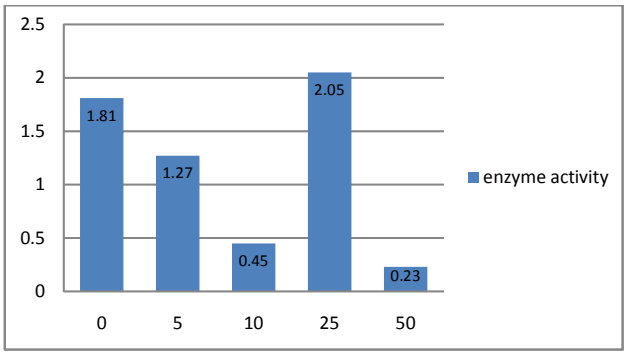

Fig. 1: Cellulase production on paddy straw.

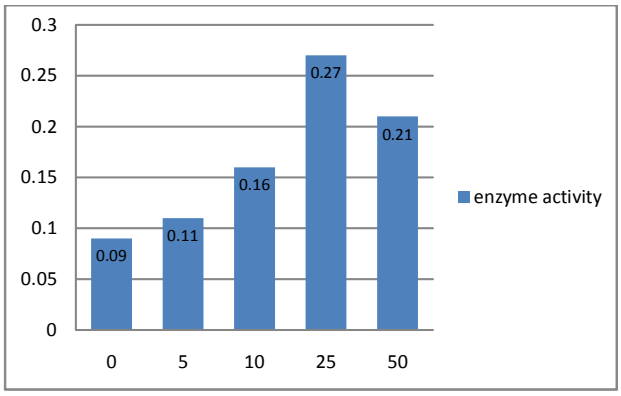

Fig. 2: Cellulase production on sugarcane baggasse.

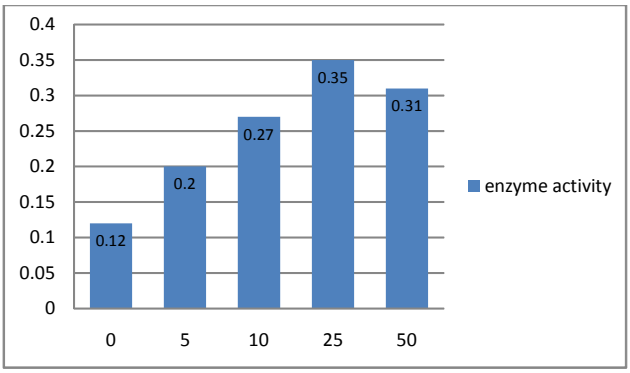

Fig. 3: Cellulase production on coir pith.

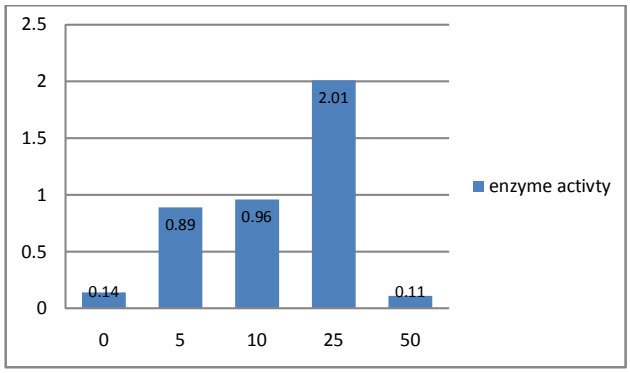

Fig. 4: Cellulase production on dried leaf litter.

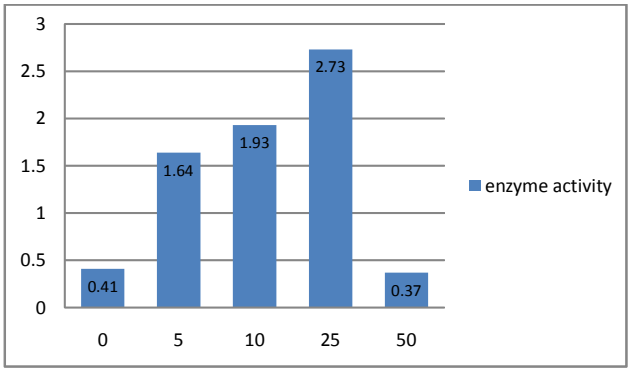

Fig. 5: Xylanase production on paddy straw.

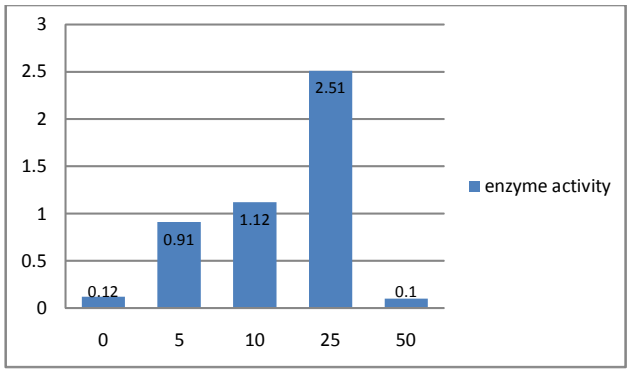

Fig. 6: Xylanase production on sugarcane baggasse.

tion. But the enzyme production was highly influenced by moisture content (Fig. 1). In the case of cellulase, maximum enzyme production was recorded in paddy straw with 2.75 U/mL at 25% moisture content (Figs. 1 & 2). At 0, 5, 10% moisture content, 0.41, 1.64, 1.93 U/mL enzyme activity was recorded. Enzyme production was highly reduced in 50% moisture content and 0.37 U/mL of enzyme activity was recorded. Followed by paddy straw, sugarcane bagassae revealed maximum production with 2.05 U/mL at 25% moisture content followed by 1.81, 1.27, 0.45 and 0.21 U/mL enzyme activity at 0, 5, 10 and 50% of moisture content. Dried leaf litter and coir pith also supported cellulase production. 0.12, 0.20 ,0.27 ,0.31, 0.35 U/mL and 0.09, 0.11, 0.16, 0.21, 0.27 U/mL of enzyme activity was recorded in the respective moisture content (Figs. 3 & 4) Chutmanop et al. (2008) evaluated various agroindustrial wastes for the production of protease. Liu et al. (2007) also studied solid state fermentation of cellulase with lignocellulosic wastes by *Tri-*

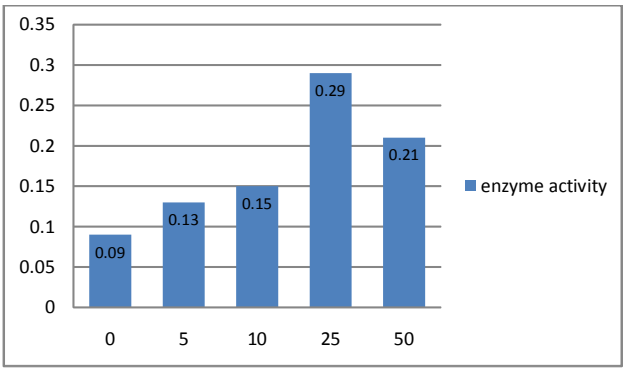

Fig. 7: Xylanase production on coir pith.

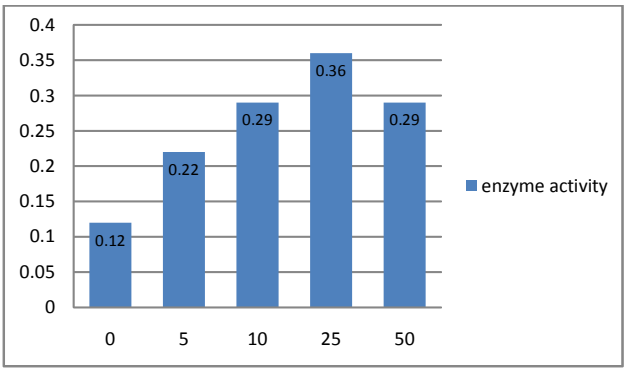

Fig. 8: Xylanase production on dried leaf litter.

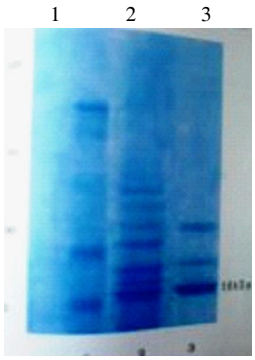

Fig. 9: SDS image of purified xylanase. Lane 1- Protein marker Lane 2 Ammonium sulphate precipitates of culture supernatant Lane 3 Fraction from DEAE cellulose column

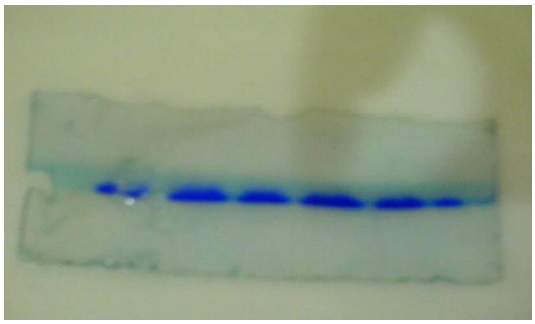

Fig. 10: SDS-PAGE image of purified cellulase after DEAE chromatography.

choderma koningii. Production of cellulase by *Aspergillus niger* using water hyacinth blend (Usama et al. 2008) and groundnut shell wastes by *Aspergillus terreus* (Vyas & Vyas 2005) has also been reported. Among the different wastes, xylanase production was found to be maximum in sugarcane bagasse and paddy straw treated at 25% of the moisture content with 2.51 and 2.01 U/mL respectively (Figs. 5, 6) 0.12, 0.91, 1.12, 0.1 U/mL enzyme activity was recorded in sugarcane bagasse with 0, 5, 10, 50% moisture content. Enzyme activity in paddy straw was found to be 0.10, 0.63, 0.95 U/mL at 0, 5 and 10% moisture content. Dried leaf litter with the respective moisture content revealed xylanase production with 0.12, 0.22, 0.29, 0.36 and 0.29 U/mL enzyme activity (Fig. 7). Least enzyme production was observed in coir pith 0.09, 0.13, 0.15, 0.29 and 0.25 U/mL enzyme activity at respective moisture content (Fig. 8). Allen & Roche (1989) using SSF process, isolated a thermostable cellulase-free xylanase produced by *T. lanuginose* using various agro wastes and the used agro wastes supported enhanced xylanase production.

Purification: Table 3 shows the summary of the purification steps. Ammonium sulphate precipitation of filtered culture supernatant showed that cellulase and xylanase fractionated at 60% saturation. The fractions were submitted to Bio-gel P-70 DEAE cellulose column. Cellulase and xylanase activity of all the fractions collected from DEAE cellulose column was obtained with a high purity since a single major band appeared after SDS-PAGE which revealed a monomer with a molecular weight of 56 and 61 KD (Figs. 9 and 10). Enzyme was lyophilized and stored at -20°C and no reduction in activity was observed after 25 days. Similar purification step was reported for xylanases from *Bacillus stearothermophillus* T-6 and *B. subtilis* (Khasim 1993, Bernier 1983).

Effect of pH and temperature on enzyme activity: Xylanase activity was found to be maximum at the pH of 6.0 (3.71U/mL) followed by 5.0 (2.62 U/mL) and the activity was significantly reduced at pH 8.0 and 9.0 (Table 4). The enzyme could retain the maximum activity at the temperature of 60°C (3.4 U/mL) followed by 50°C (2.19) and the enzyme activity was significantly reduced at 70, 80 and 90°C (Table 5). Cellulase activity was found to be maximum at pH 6.0 and 60°C with 2.79 and 2.17 U/mL respectively. Effect of pH and temperature on xylanase activity revealed that the enzyme could retain maximum activity at pH 6 and 60°C. Tuohy & Coughlan (1991) compared ther-

mostable xylanase production on various substrates by a strain of *Talaromyces emersonii* in liquid culture and SSF systems and they observed that xylanase from SSF showed higher enzyme activity than xylanase from submerged fermentation. Namasivayam & Malikeswaran (2012) obtained thermostable and acidophilic xylanase from endophytic fungi *Phoma europyrena*. In the present study, xylanase and cellulase from *Trichoderma harzianum* (SK 01) strain produced xylanase in a low cost solid media and being active at acidic pH and high temperature. These are desirable properties used in large scale production of xylanase, cellulase and application in paper, pulp and food industries.

REFERENCES

Allen, A.L. and Roche, C.D. 1989. Effects of strain and fermentation conditions on production of cellulase by *Trichoderma reesei*. Biotechnology and Bioengineering, 33(5): 650-656.

Babu, K. R. and Satyanarayana, T. 1996. Production of bacterial enzymes by solid state fermentation. J. Sci. Ind. Res., 55: 464-467.

Bernier, R., Desrocher, M., Jurasek, L. and Paice, M.G. 1983. Isolation and identification of xylanase from *Bacillus subtilis*. Appl. Environ. Microbiol., 65: 511-514.

Chahal, D.S. 1991. Production of *Trichoderma reesei* cellulase system with high hydrolytic potential by solid-state fermentation. In: Enzymes in Biomass Conversion. ACS Symp. Series, Vol. 460, ed. G.F. Leatham & M.E. Himmel. American Chemical Society, Washington, pp. 111-122.

Chutmanop, J., Sinsupha Chuichulcherm, Yusuf Chisti and Penjit Srinophakun 2008. Protease production by *Aspergillus oryzae* in solid-state fermentation using agroindustrial substrates. J. Chem. Technol. Biotechnol., 83: 1012-1018.

Clark, F.E.1997. Method of Soil Analysis. Part 2. Chemical and Microbiological Properties. Mudson Int., New York, pp. 1460-1466.

Khasin, A., Alchanati, I. and Shoham, Y. 1993. Purification and characterization of a thermostable xylanase from *Bacillus stearothermophilus* T-6. Applied and Environmental Microbiology, 59(6): 1725-1730.

Liu, J. and Yang, J. 2007. Cellulase production by *Trichoderma koningii* as 3.4262 in solid-state fermentation using lignocellulosic waste from the vinegar industry. Food Technol. Biotechnol., 45(4):420-425.

Mitra, P., Chakraverty, R. and Chanda, A.L. 1996. Production of proteolytic enzymes by solid state fermentation-An overview. J. Sci. Ind. Res., 55: 439-442.

Namasivayam, S.K.R. and Nirmala, D. 2011. Enhanced production of alpha amylase using vegetable wastes by *Aspergillus niger* SK 01 strain. Indian Journal of Marine Sciences, 40(1): 130-133.

Namasivayam, S.K.R. and Malikeswaean, N. 2012. Studies on the effect of nutrient sources on xylanase production by endophytic fungi *Phoma europyrena* SK 01 strain. International Journal of Institutional Pharmacy and Life Science, 2: 1.

Pandey, A., Selvakumar, P., and Ashakumary, L. 1994. Glucoamylase production by *Aspergillus niger* on rice bran is improved by adding nitrogen sources. World J. Microbiol. Biotechnol., 10(3): 348-349.

Selvakumar, P., Ashakumary, L. and Pandey, A. 1998. Biosynthesis of glucoamylase from *Aspergillus niger* by solid-state fermentation using tea waste as the basis of a solid state. Biores. Technol., 65: 83-85.

Tengerdy, R. P. 1998. Advances in Biotechnology. Educational Publishers and Distributors, New Delhi, pp. 13-16.

Tuohy, M. G. and Coughlan, M. P. 1992. Production of thermostable xylan degrading enzymes by *Talaromyces emersonii*. Biores. Technol., 39: 131-137.

Usama, F.A. and Hala, S. 2008. Production and partial purification of cellulase complex by *Aspergillus niger* and *A. nidulans* grown on water hyacinth blend. Journal of Applied Sciences Research, 4(7): 875-891.

Vyas, A. and Vyas, D. 2005. Production of fungal cellulases by solid scale bioprocessing of groundnut shell wastes. Journal of Scientific and Industrial Research, 64: 763-770.