World Academy of Science, Engineering and Technology
International Journal of Agricultural and Biosystems Engineering
Vol:3, No:3, 2009

Production of Cellulases by Aspergillus Heteromorphus from Wheat Straw under Submerged Fermentation

Anita Singh, Namita Singh and Narsi R. Bishnoi

***Abstract*—**To investigate the production of cellulases from *Aspergillus heteromorphus*, submerged fermentation was performed using wheat straw as substrate. Optimization of saccharification conditions like pH, temperature and time were studied. Highest reducing sugar was released on 5th day at 5 pH, 30^0 C temperature. When *A. heteromorphous* was grown on wheat straw in submerged fermentation after 5 days incubation at 30 ^{0} C, 3.2 IU/ml and 83 IU/ml, filter paper activity and CMCase activity respectively.

Keywords—Aspergillus heteromorphus, Wheat Straw, Submerged Fermentation, Production of Cellulases

I. Introduction

LIGNOCELLULOSIC biomass is the most abundant organic raw material in the world [1]. Lignocelluloses constitute a major portion of agricultural wastes and forest wastes. Thus they are the most promising feedstock for the production of energy, food and chemicals and their utilization could allow self-sustainable processes and products. The utilization of cellulosic biomass continues to be a subject of worldwide interest in view of fast depletion of our oil reserves and food shortages [2]. The bioconversion of the agro waste material into fuel has received considerable interest during recent years. This lignocellulosic material was in abundant and available in free of cost. Enzymatic hydrolysis of cellulosic biomass is considered as the most efficient and least polluting methods for generating glucose from lignocellulosics, but the production economics of bioethanol is largely dependent on cost of cellulases [3]. Cellulases comprise a complex of enzymes involved in the natural degradation of cellulose, the major polysaccharide of plant cells. The enzymatic complex can convert the cellulose to oligosaccharides and glucose. Microorganisms such as fungi and bacteria are important producers of cellulases. Substrate costs account for a major fraction of the costs of cellulase production, and the use of cheap biomass resources as substrates can help to reduce cellulase prices [4]. The use of agro-industrial residues as the basis for cultivation media is a matter of great interest, aiming to decrease the costs of enzyme production and meeting the increase in awareness on energy conservation and recycling. The conversion of cellulosic mass to fermentable sugars through biocatalyst cellulase derived from cellulolytic organisms has been suggested as a feasible process and offers potential to reduce use of fossil fuels and reduce environmental pollution [5], [6]. Cellulase production by different organisms in submerged state fermentation has received more attention and is found to be cost-prohibitive because of high cost of process engineering. India is an agricultural country and wheat is one of the most important agricultural crop in India. Every year nearly 78.4 Million Tones wheat is produced. With the processesing of wheat grains a large amount of straw is produced which can be used as a substrate for ethanol production. Currently a most important application of cellulases and hemicellulases in the pulp and paper industry is the biobleaching of pulp, the production of dissolving pulp, the treatment of wastewater and the deinking of recycled waste paper. The potential of enzymatic treatments has been assessed and the processes have proved successful [7], [8].

In the present study the production of cellulolytic enzymes by a local isolate of *A. heteromorphus* on lignocelluloses in submerged fermentation is reported.

Anita Singh is with Department of Environmental Science and Engineering, Guru Jambheshwar University of Science and Technology, Hisar-125001, Haryana, India

Namita Singh is with Department of Bio and Nano Technology, Guru Jambheshwar University of Science and Technology, Hisar-125001, Haryana, India. Email: namitasingh71@gmail.com

Narsi R. Bishnoi is also with Department of Environmental Science and Engineering, Guru Jambheshwar University of Science and Technology, Hisar-125001, Haryana, India Email: nrbishnoi@gmail.com

II. Material and Methods

A. Microorganism and Material

Aspergillus heteromorphus MTCC 8624 was used for the present study. The strain was cultivated on potato dextrose agar (PDA) at 28^0 C for 7 days. Wheat straw was used as a substrate for enzyme production. All the experiments were performed in triplicates and the average values were calculated.

B. Fermentation

2 g wheat straw, 50 ml of Mandel and Sternberg's mineral media [9]: 1.4g/l $(NH_4)_2 SO_4$, 2.0 g/l KH_2PO_4, 0.3 g/l $CaCl_2$, 0.3 mg/l $MgSO_4$. $7H_2O$, 5.0 mg/l $FeSO_4$.7 H_2O, 1.6 mg/l $MnSO_4$. H_2O, 1.4 mg/l $ZnSO_4$. $7H_2O$, 2.0 mg/l $CoCl_2$. The initial pH of the medium was adjusted to 5 before being autoclaved at 121^0C for 15min. After cooling the flasks were inoculated with an amount initially containing 2 X 10^6 spores per gram of substrate and the contents, after mixing, were incubated at 30 ^{0}C on an orbital shaking bed at 120 rpm for 7 days and removed after 0,2,3,4,5,6,7 days.

C. Standardization of Conditions for Saccharification

To determine the optimum saccharification conditions of

pH, temperature and time, the above mention reaction mixture was incubated from 3 to 8 pH, from 20 to 45 ^{0}C temperature and for 10 days at 115 ±5 rpm. After this reducing sugar was analyzed by DNS method [10] for optimum conditions.

The percentage saccharification was calculated as:

$$\text{Saccharification (\%)} = \frac{\text{Glucose (mg/ml)} \times 100}{\text{Substrate (mg/ml)}}$$

D. Enzyme Assays

Filter paper assay was used to estimate total cellulase activity and endoglucanase activity (CMCase activity) [11] in the crude enzyme preparation as given below. For filter paper activity Whatman no. 1 filter paper strip of dimension 1.0× 6 cm (50 mg) was placed into each assay tube. The filter paper strip was saturated with 1.0 ml of Na–citrate buffer (0.05 M, pH 4.8) and was temperate for 10 min at 50 °C Half milliliter of an appropriately diluted (in Na–citrate buffer, 0.05M; pH 4.8) enzyme was added to the tube and incubated at 50 °C for 60 min. And in case of endoglucanase activity half milliliter of 1 % carboxymethyl cellulose in 0.05M Na–citrate buffer, pH 4.8 was temperate for 10 min at 50 °C. After that half milliliter of an appropriately diluted enzyme was added to the tube and incubated at 50 °C for 30 min. Appropriate controls were also run along with the test. At the end of the incubation period, tubes were removed from the water bath, and the reaction was stopped by addition of 3 ml of 3, 5-dinitrosalicylic acid reagent per tube. The tubes were incubated for 5 min in a boiling water bath for color development and were cooled rapidly. The reaction mixture was diluted appropriately and was measured against a reagent blank at 540 nm in a UV-VIS spectrophotometer. The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose. One unit of enzyme activity was defined as the amount of enzyme required for liberating 1 μM of glucose per milliliter per minute and was expressed as U/ml.

III. Result and Discussion

The recent thrust in bioconversion of lignocellulosic biomass to chemical feedstock has led to extensive studies on cellulolytic enzymes produced by bacteria and fungi. Though the growth period of bacteria is shorter than that of fungi, their half- backed cellulase system makes them less useful in the industrial production of cellulase. However, high cost of cellulases production hindered use of this enzyme in industry. For the utilization of lignocellulosic biomass, it is necessary step to enhance the cellulose production and reduces its production cost. The use of purified cellulosics as substrate is uneconomical for large scale production of cellulases. Therefore cheaply available agricultural lignocellulose waste wheat straw was tested to find out whether it could support the production of cellulases by *A. heteromorphus* under submerged fermentation. When wheat straw incubated with the cellulolytic enzyme complex, sugar is released. The degree of saccharification was assayed on the basis of release of reducing sugar. Saccharification was affected by many factors like pH, temperature and incubation time. Maximum amount of reducing sugar released on 5th day of incubation period until the end of the saccharification period when it was decreased (Figure 1). Figure 2 and 3 Shows that the optimum pH and temperature for release of reducing sugar was 5 and 30 ^{0}C respectively. The optimum pH, temperature and incubation time for saccharification were optimum for conditions for the synthesis of cellulolytic enzymes by fungus [12]. Their exist a strong influence of initial pH of the medium on enzyme production. The figure 2 shows that low and high pH is not suitable for reducing sugar as for enzyme production. The incubation temperature is also an important factor for enzyme production. Proper cultivation time was also significant for growth and production. Reducing sugar yield is not a linear function of the quantity of enzyme in assay mixture, as discussed by Ghose [11]. Twice as much enzyme will give equal sugar in half the life, while twice the amount of enzyme would not be expected to yield twice the reducing sugar in equal time. Assay mixtures may, in some cases, contain reduced sugars unrelated to hydrolysis of substrate glycosidic bonds by the enzymes [13]. Release of cellulolytic enzymes will lead to initiation of attack on cellulosic components of lignocelluloses. Maximum enzyme activity was observed on 5th days incubation at 30 ^{0} C, cellulase activity 3.2 IU/ml and 83 IU/ml, filter paper activity and CMCase activity respectively. Since comparisons of the results obtained in this study with those obtained by other researchers are difficult, because none of the report available on *Aspergillus heteromorphus* for cellulase production and the yields of each enzyme were presented in Table 1. The table 1 shows that FPase and CMCase activity of the present study was comparable with other studies in case of solid state fermentation. But filter paper assay activity was nearly similar with previously reported on the saw dust by using *Aspergillus niger* as a source of cellulase [14]. And CMCase activity was similar as reported by Muthuvelayudham and Viruthagir [15] using Sugarcane baggase as substrate by *T. reesei* 94.144. Generally, the production of cellulases has been shown to be inducible and was affected by the nature of the substrate used in fermentation. Therefore, the choice of an appropriate inducing substrate is of importance [16]. However, the values of CMC activity were always higher than the filter paper activity during the same interval of the fermentation period. Cellulase levels of CMC are always higher than the FPA. This holds true for all the studies of fungus and using different substrates for fermentation [17]. Difference in titres of enzyme yields in different studies can be attributed to use of different materials as solid matrix, different cultural practices and different organisms. The decrease in both cellulase activities may be due to the accumulative effect of cellulobiose. Cellulobiose is a dimmer of glucose which is known to inhibit both endoglucanase and glucosidase. The time of the highest cellulase activity depends upon the substrate and fungus used as studied by Ojumu [18] and Alam [19] . But in time, it decreases until the end of the fermentation period. As discussed by Hatakka [20] delignification produces aromatic water soluble products which may repress the cellulolytic action of the enzyme.

There were some applications of cellulases and hemicellulases. Considine [21] used the enzyme extracts of solid state fermentation of *P. capsulatum* for the saccharification of beet pulp. According to studies of Gonzalez [22], higher enzymatic activities (FPA and β-

glucosidase) were obtained with growth of *Trichoderma reesei* GM 9414 on wheat straw rather than on solka-floc as carbon source. Okeke and Obi [23] reported that the highest degree of hydrolysis was observed with melon seed shells in the study of saccharification of agro-waste materials by cellulases and hemicellulases. So the possibility of the use of cellulases produced by *A. heteromorphus* and wheat straw as substrate will be vast.

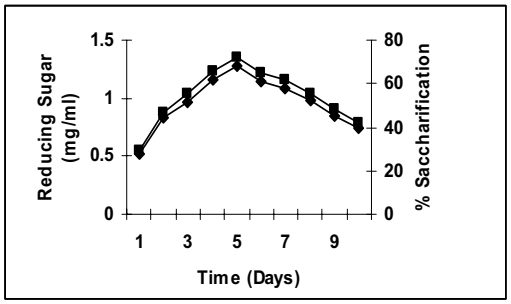

Fig.1 Effect of Time (Days) on saccharification of Wheat straw: (♦) Release sugars (mg/ml); (■) % Saccharification

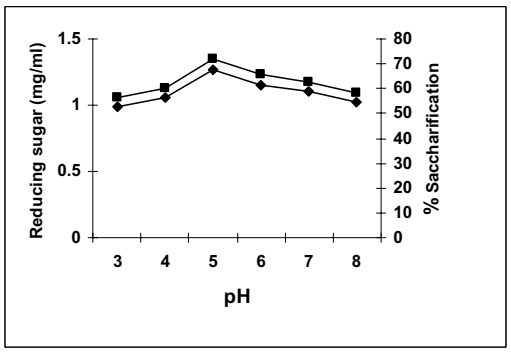

Fig. 2 Effect of pH on saccharification of Wheat straw: (♦) Release sugars (mg/ml); (■) % Saccharification

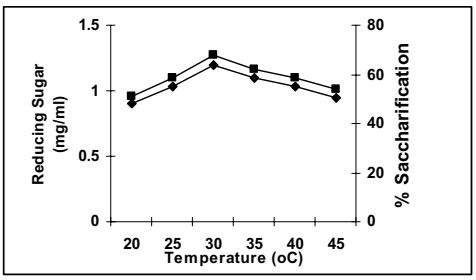

Fig. 3 Effect of Temperature (^{0}C) on saccharification of Wheat straw: (♦) Release sugars (mg/ml); (■) % Saccharification

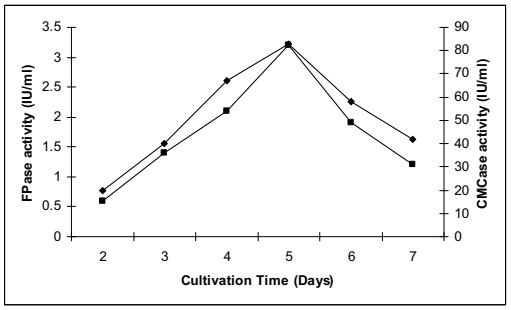

Fig. 4 Time course profile of enzyme production by *A. herteromorphus* on wheat straw under SSF condition (♦) CMCase (IU/ml); (■) FPase (IU/ml)

TABLE I
ENZYME YIELDS BY SUBMERGED FERMENTATION FROM OTHER STRAINS GROWN ON LIGNOCELLULOSIC BIOMASS

Organism	Substrate	Enzyme activities (IU g-1)		Reference
		FP	CMC	
Aspergillus niger	Rice straw	0.96	0.660	Narasimha et al. 2006
	Jowar-straw	1.52	0.627	
	Saw- dust	2.412	0.775	
T. reesei	Sugarcane baggase	5.66	68	Muthuvelayudham and Viruthagir, 2006
	Rice straw	5.26	44	
Aspergillus heteromorphous	Wheat straw	3.2	83	This work

Filter paperase (FPase) is expressed in terms of filter paper units. One unit is the amount of enzyme in the culture filtrate releasing 1 µmole of reducing sugar from filter paper per min.
Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxymethyl cellulose per min.

IV. Conclusion

The production of cellulases on wheat straw under submerged fermentation was studied by *Aspergillus heteromorphus.* Wheat straw is a cheap residue which can be used as a substrate for enzyme production which reduces the cost of enzyme production and enzymatic conversion of carbohydrate part of wheat straw into fermentable sugar.

Acknowledgment

Authors acknowledge University Grant Commission, New Delhi, India, for Financial support.

References

[1] Singh, R., Singh, N., Parkash, A., Poonia, S., 2006. Saccharification studies by a thermophilic fungus Sporotricum thermophile isolated from agriculture waste of Bhopal, Indian Journal of Environment & Ecoplanning, 12 (1) 97-104
[2] Kuhad RC, Singh A, Eriksson KE (1997). Microorganisms enzymes involved in the degradation of plant fiber cell walls. Adv. Biochem. Eng. Biotechnol. 57: 45-125
[3] Reith, J. H., den Uil, H., van Veen, H., de Laat WTAM, Niessen, J. J. & de Jong, E. (2002). Coproduction of bioethanol, electricity and heat from biomass residues. 12th European Conference and Technology Exhibition on Biomass from Energy, Industry and Climate Protection, Amsterdam, The Netherlands, 17–21 June.
[4] Wen, Z., Liao, W., & Chen, S. (2005). Production of cellulase/b-glucosidase by the mixed fungi culture Trichoderma reesei and Aspergillus phoenicis on dairy manure. Process Biochemistry, 40, 3087–3094.
[5] Dale BE (1999). Biobased industrial products: bioprocess engineering when cost really counts. Biotechnol. Prog. 15: 775-776.

[6] Lynd LR, Wyman CE, Gerngross TU (1999). Biocommodity engineering. Biotechnol. Program. 15: 777-793.
[7] Gubitz, G.M., Mansfield, S.D., Bohm, D., Saddler, J.N., 1998. Effect of endoglucanases and hemicellulases in magnetic and flotation deinking of xerographic and laser-printed papers. J. Biotechnol. 65, 209–215.
[8] Bajpai, P., 1999. Application of enzymes in the pulp and paper industry. Biotechnol. Prog. 15, 147–157.
[9] Sternberg D., 1976, Production of Cellulase by Trichoderma. Biotech Bioeng. Symp. (6), 35-53.
[10] Miller GL (1959) Use of dinitrosalicyclic acid reagent for determination of reducing sugar. Analytical Chemistry 31: 426-428
[11] Ghose, T. K. (1987). Measurement of cellulase activities. Pure & Applied Chemistry 59: 257–268
[12] Baig MMV, Baig MLB, Baig MIA & Yasmeen M (2004) Saccharification of banana agro-waste by cellulolytic enzymes. African Journal of Biotechnology 3 (9): 447-450
[13] Khan MMH, Ali S, Razi AF & Alzam MZ (2007) Use of fungi for the bioconversion of rice straw into cellulose enzyme. Journal of Environmental Science and Health Part B. 42: 381-386.
[14] Narasimha G, Sridevi A, Viswanath, B., Chandra M. S., Reddy, R.B. 2006 Nutrient effects on production of cellulolytic enzymes by Aspergillus niger. African Journal of Biotechnology 5 (5), 472-476
[15] Muthuvelayudham, R and Viruthagir, 2006 Fermentative production and kinetics of cellulose protein on Tricoderma reesei ysing sugarcane bagasse and rice straw. African Journal of Biotechnology Vol. 5 (20), pp. 1873-1881, 16 october 2006
[16] Kang, S.W., Park, Y.S., Lee, J.S. , Hong , S.I., Kim, S.W., 2004. Production of cellulases and hemicellulases by Aspergillus niger KK2 from lignocellulosic biomass, Bioresource Technology, 91, 153–156
[17] Pandey A, Selvakumar P, Soccol CR & Nigam P (1999) Solid State Cultivation for the Production of Industrial Enzymes. Current Sci. 77: 149–162
[18] Ojumu TV, Solomon B O, Betiku E, Layokun SK & Amigun B (2003) Cellulase Production by Aspergillus flavus Linn Isolate NSPR 101 fermented in sawdust, bagasse and corncob. African Journal of Biotechnology 2 (6): 150–152.
[19] Alam MZ, Muhammad N & Mahmat ME (2005) Production of cellulase from oil palm biomass as substrate by solid state bioconversion. American Journal of Applied Science 2(2):569-572
[20] Hattaka AI (1983) Biological pretreatment of lignocellulose. Applied Microbiol. Biotechnol. 18:350-357.
[21] Considine PJ, O'Rorke A, Hackett TJ & Coughlan MP (1988) Hydrolysis of beet pulp polysaccharides by extracts if solid state cultures of Penicillium capsulatum. Biotechnol. Bioeng. 31:433– 438.
[22] Gonzalez G, Caminal G & Lopez-Sontin (1986) Cellulase production on lignocellulose materials and performance of a packed-red reactor for enzymatic hydrolysis. Biotechnol. Bioeng. Symp. 17: 535-542.
[23] Okeke BC & Obi SKC (1995) Saccharification of agro-waste materials by fungal cellulases and hemicellulases. Biores. Technol. 51: 23–27.