RESEARCH PAPER | Microbiology | Volume : 6 | Issue : 3 | March 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

Enhancement of cellulase production by substrate manipulation in three Aspergillus sp.

KEYWORDS: Cellulase; Pretreatment; Mesh Size; Wheat straw.

Divyani Agrawal
Softvision College, Sector F, Scheme No. 54, Vijay Nagar, Indore 452010.

Dr. Ketna Matkar
Softvision College, Sector F, Scheme No. 54, Vijay Nagar, Indore 452010.

ABSTRACT *Aspergillus fumigatus, Aspergillus ellipticus and Aspergillus fumigatus Fersenius were employed for production of cellulase under solid state fermentation conditions using 10 mesh size wheat straw. There was significant increase in cellulase production upon pretreatment of substrate using a combination of 0.75% (v/w) H2SO4 and 1.5% (w/v) NaOH at 10% (v/v) loading for 90 min and 120 min respectively. There was a 1.18-fold increase in filter paper activity upon pretreatment which was further increased by 2.21-fold upon mesh size reduction. There was an overall increase of 4.76, 3.62 and 1.40 fold in exoglucanase, endoglucanase and β-glucosidase activity upon optimization. This proves that significant increase in cellulase efficiency can be achieved by manipulation of substrate.*

Introduction

The most important challenge which is faced by the world is to have a promising and sustainable alternative source of energy which can help to reduce the dependency on fossil fuels (Turhan et al., 2015). Bioethanol is being recognized as the sustainable potential replacement for the fossil fuel with many advantages as a transportation fuel. Efficient bioethanol production needs a good, sustainable and cost effective source of fermentable sugars. This can be easily achieved through saccharification of lignocellulosic biomass. Different lignocellulosic biomass feedstocks have been used for the purpose e.g. wheat straw, rice straw, sugarcane bagasse, sweet sorgum bagasse etc. Wheat straw has been used as substrate for cellulase production in this study due to its easy availability and abundance in Madhya Pradesh, India.

The saccharification of lignocellulosic biomass is achieved through the hydrolytic action of cellulases which converts the complex carbohydrates to simple fermentable sugars that can be utilized for production of ethanol (Maurya et al., 2013). To increase the saccharification efficiency, manipulation of dependent key parameters can be taken into consideration, of which cellulase is the most important. The existing commercial preparations of cellulase employed for bioconversion are not economically sustainable. Therefore, the target is to reduce the cost of cellulase by manipulation of various parameters like: substrate, production technology, engineering of cellulases or by optimizing growth conditions (Singhania, 2010).

The present study is targeted to increase cellulase production by optimizing pretreatment conditions and particle size reduction.

Materials and methods

The fungal cultures used

The cultures of *Aspergillus ellipticus* and *Aspergillus fumigatus* were obtained from Prof. Datta Madamwar (S. P University-Bioscience lab). *Aspergillus fumigatus* Fresenius was obtained from Prof. Sridhar Patil (Life Science Department, D. A.V.V). The other cultures used for initial screening were *Trichoderma reesei* NCIM 1186, *Aspergillus sydowii* MTCC 11504 and *Fusarium oxysporum* MTCC 1755. All the cultures were known for production of cellulase.

Inoculum preparation

The stock cultures were maintained on potato dextrose agar (PDA) slants. Spores from eight day old culture were transferred in modified Mandels medium with 1% lactose as carbon source (Matkar et al., 2013). The flask was incubated at 26 ± 2°C at 100 rpm for 12 h.

Production medium preparation

Modified Mandels medium containing wheat straw moistened with salt solution in (1:5 w/v) ratio was used for production of cellulase. A 10% (v/v) of inoculum medium was added to the production medium and incubated for 8 days at 26 ± 2°C under static condition.

Enzyme extract preparation

The contents of flask were mixed with 50 ml of citrate buffer (0.05 M, pH 4.8) and kept on shaker at 100 rpm for 20 min. It was then subjected for extraction through moistened cheese cloth and subsequently squeezed thoroughly and centrifuged at 10,000 x g for 10 min to get the clear supernatant as enzyme sample for assay and protein estimation.

Soluble protein was measured by Folin-Lowry method (Lowry, 1951) using 0.2 mg/ml bovine serum albumin as standard. Reducing sugar was estimated using 1mg/ml glucose as standard by Dinitro salicylic acid (DNSA) method (Miller, 1959).

Enzyme assays

One unit of filter paper activity (Ghose, T. K. 1987), endoglucanase and exoglucanase (Mandels, 1975) is defined as amount of enzyme liberating 1µmol of glucose per min under the assay conditions One unit of β-glucosidase is defined as the amount of enzyme liberating 1 µmol of p-nitrophenyl per min under the assay conditions (Kubicek, 1982). The enzyme activities are reported as IU/g/min.

Pretreatment optimization

Various pretreatment methods have been employed for wheat straw so far (Talebnia et al., 2010) but the present investigation refers to the modification of the one adopted by Govumoni et al. (2013) using 0.75% (v/v) H_2SO_4 and 1.5% (v/v) NaOH.

Volume : 6 | Issue : 3 | March 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

50 g of dried wheat straw was treated with 0.75% (v/v) sulphuric acid at 85°C placed in a water bath for varying time periods (30 min, 60 min, 90 min and 120 min) at 10% (v/w) concentration. After acid hydrolysis, the residual substrates were neutralized and dried. The acid treated wheat straw was then subjected to alkali treatment using 1.5% (v/v) sodium hydroxide at 100°C at a 10% (v/w) concentration. The ratio for time (min) of treatment in dilute acid and alkali were 30:30, 60:60, 90:90, 60:120, 90:120 and 120:120. The contents were squeezed using cheese cloth and thoroughly washed with tap water until the pH came to 7. The last few rinses were done with distilled water and then the wheat straw was sun dried.

Particle size optimization

Substrate of desired mesh size was obtained by first grinding the pretreated and sun dried wheat straw which was then subjected to a sieving procedure employing sieves of mesh-size: 10 (2.00 mm), 20 (0.85 mm), 40 (0.425 mm) and 60 (0.25 mm). These sieved wheat straw fibers were classified into different diameter sizes according to the sieves mesh-size that retained the fiber.

Screening of six fungal cultures for production of cellulase was performed under unoptimized conditions using 10 mesh size untreated wheat straw. The isolates producing higher proportions of enzyme components were selected. Further enhancement in cellulase production was attempted in two steps wherein optimization of pretreatment of wheat straw and mesh size were sequentially undertaken.

Results and discussion

Screening of fungal isolates for cellulase production

Six fungal isolates were screened for cellulase production by SSF under static conditions employing untreated wheat straw of 10 mesh size at 25°C, pH 5.5 with incubation for 8 days.

Maximum cellulase activities were exhibited by *A. ellipticus, A. fumigatus and A. fumigatus* Fresenius and therefore these isolates were considered for further work (**Figure 1**).

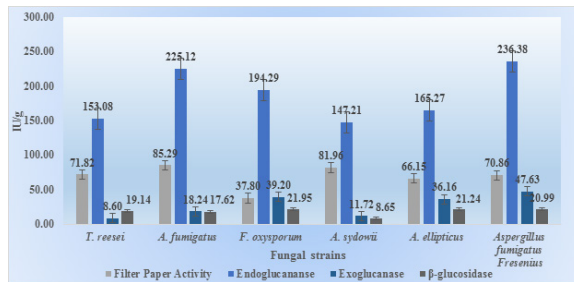

Figure 1 Comparative evaluation of crude cellulase extract obtained by SSF under static condition using 10 mesh size untreated wheat straw at 25°C, pH 5.5 for 8 days.

Optimization of pretreatment conditions for cellulase production

A pretreatment optimization study was undertaken for cellulase enzyme production under SSF. Combination of dilute acid (0.75% v/w H_2SO_4) and dilute alkali solution (1.5% v/w NaOH) when used at 10% (v/w) concentration loading for 90 min and 120 min time duration with temperature standing of 85°C and 100°C respectively gave best results (**Table 1**).

Table 1 Enzyme activity after pretreatment using 10 mesh size wheat straw

Pretreatment by 0.75% (v/v) H_2SO_4 and 1.5% (w/v) NaOH at for various time durations						
Time duration	**30:30 min**	**60:60 min**	**90:90 min**	**60:120 min**	**90:120 min**	**120:120 min**
Endoglucanase	**IU/g**					
A. fumigatus	100.80	104.83	149.98	165.08	216.83	212.58
A. ellipticus	95.39	96.34	100.09	103.22	182.25	180.44
A. fumigatus Fresenius	136.33	139.06	204.83	222.21	261.27	258.69
Time duration	**30:30 min**	**60:60 min**	**90:90 min**	**60:120 min**	**90:120 min**	**120:120 min**
Filter paper activity	**IU/g**					
A. fumigatus	83.85	87.20	93.31	96.11	99.95	97.99
A. ellipticus	64.30	68.16	71.57	73.00	75.28	74.53
A. fumigatus Fresenius	79.00	80.15	80.95	85.00	86.77	84.24
Time duration	**30:30 min**	**60:60 min**	**90:90 min**	**60:120 min**	**90:120 min**	**120:120 min**
β-glucosidase	**IU/g**					
A. fumigatus	17.89	18.25	18.43	19.72	19.92	18.97
A. ellipticus	20.06	20.86	21.28	21.49	23.21	22.98
A. fumigatus Fresenius	19.24	20.20	21.01	21.64	22.07	20.63
Time duration	**30:30 min**	**60:60 min**	**90:90 min**	**60:120 min**	**90:120 min**	**120:120 min**
Protein	**IU/g**					
A. fumigatus	108.01	109.09	120.00	122.40	124.85	123.61
A. ellipticus	105.38	107.49	108.57	110.74	114.06	109.67
A. fumigatus Fresenius	88.06	90.71	93.43	97.17	105.91	102.83
Time duration	**30:30 min**	**60:60 min**	**90:90 min**	**60:120 min**	**90:120 min**	**120:120 min**
Exoglucanase	**IU/g**					
A. fumigatus	39.00	39.78	41.37	42.61	46.45	41.85
A. ellipticus	70.65	75.59	77.86	79.42	83.39	69.49
A. fumigatus Fresenius	75.26	77.52	78.30	82.21	88.79	78.58

Volume : 6 | Issue : 3 | March 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

The increase in activities of all enzyme components was significant specifically exoglucanase which showed a 2.4 fold increase. Dilute acid pretreatment removes hemicellulose content and also releases essential nutrients which enhance the downstream fermentation (Zhang et al., 2012). Dilute NaOH loosens structure of biomass and decreases degree of polymerization and crystallinity thereby disrupting structure of lignin (Fan et al., 1987). Govumoni (Govumoni et al. 2013) reported better results for saccharification by allowing the treatment at 100°C for 2 h.

A further increase in cellulase production was desired and therefore mesh size optimization was performed.

Particle size optimization for cellulase production

Pretreated wheat straw with 10, 20, 40 and 60 mesh size was used to determine the cellulase activities, filter paper activity and protein estimation. There was a very large increase in cellulase activities upon reduction in particle size from 10 to 20 mesh size (**Table 2**).

Smaller particle size provides larger surface area for growth but too small size may lead to poor growth due to interference with microbial respiration and aeration. Substrate with optimal particle size allows better nutrient absorption, gas exchange and heat transfer thereby leading to increase in enzyme production. Bahrin (Bahrin et al., 2011) obtained higher cellulase production for particle size between 0.42 mm-0.60 mm (40-60 mesh size). Usually a particle size of 1-2 mm (18-10 mesh) is considered suitable for cellulase production (Golan, 2011).

Table 2 Enzyme activity after mesh size optimization using pretreatment wheat straw

Mesh Size optimization				
Mesh Size	**10**	**20**	**40**	**60**
Endoglucanase		**IU/g**		
A. fumigatus	216.83	743.61	619.68	563.34
A. ellipticus	182.25	725.11	483.41	360.75
A. fumigatus Fresenius	261.27	747.37	574.90	479.08
Mesh Size	**10**	**20**	**40**	**60**
Filter paper activity		**IU/g**		
A. fumigatus	99.95	215.07	174.85	142.16
A. ellipticus	75.28	177.25	133.27	109.24
A. fumigatus Fresenius	86.77	185.15	130.39	117.47
Mesh Size	**10**	**20**	**40**	60
β-glucosidase		**IU/g**		
A. fumigatus	19.92	25.83	23.27	21.15
A. ellipticus	23.21	29.65	27.45	22.88
A. fumigatus Fresenius	22.07	28.32	25.98	23.41
Mesh Size	**10**	**20**	**40**	**60**
Protein		**IU/g**		
A. fumigatus	124.85	122.92	108.78	97.12
A. ellipticus	114.06	113.60	94.67	83.78
A. fumigatus Fresenius	105.91	103.70	91.77	82.68
Mesh Size	**10**	**20**	**40**	**60**
Exoglucanase		**IU/g**		
A. fumigatus	46.45	132.10	110.08	84.68
A. ellipticus	83.39	138.06	114.10	91.28
A. fumigatus Fresenius	88.79	153.55	139.59	109.05

Conclusion

Cellulase is a major factor deciding the economics of bioethanol production, contributing around 20% to the total cost (Wooley et al., 1999). The major impediment is either because of lower titers or lower efficiencies of the cellulases (Singhania et al., 2014). The present study has shown significant increase in the enzyme activities making contribution to cost effective enzyme technology. This would help in reducing the enzyme loadings for further saccharification work.

Acknowledgement: The authors are thankful to MPCST for the financial assistance and to Softvision college for the infrastructural support.

Reference

1. Bahrin, E. K., Seng, P. Y., & Aziz, S. A. (2011). Effect of Oil Palm Empty Fruit Bunch Particle Size on Cellulase Production by *Botryosphaeria sp.* Under Solid State Fermentation. *Australian Journal of Basic and Applied Sciences*, 5, 276-280.
2. Fan, L. T., Gharpuray, M. M., & Lee, Y. H. (1987). In: cellulose hydrolysis biotechnology monographs. 1987. *Springer, Berlin*, p.57.
3. Ghose, T. K. (1987). Measurement of Cellulase activities. *Pure and Applied Chemistry*, 59, 257-268.
4. Golan, A. (2011). Cellulase Types and Action, Mechainsm and Uses. New York, NY: Nova Science Publishers.
5. Govumoni, S. P., Koti, S., Kothagouni, Y. S., Venkateshwar, S., & Linga, V. R., (2013). Evaluation of pretreatment methods for enzymatic saccharification of wheat straw for bioethanol production. *Carbohydrate Polymers*, 91, 646-650.
6. Kubicek, C. P. (1982). β-glucosidase excretion by *T. pseudokoningii* correlation with cell wall bound β-1, 3-glucanase activities. *Archives of Microbiology*, 132, 349-354.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of General Microbiology*, 131, 3017-3027.
8. Mandels, M. (1975). Microbial sources of cellulase. *Biotechnology and Bioengineering Symposium*, 5, 81-105.
9. Matkar, K., Chapla, D., Divecha, J., Nighojkar, A., & Madamwar, D. (2013). Production of cellulase by a newly isolated strain of *Aspergillus sydowii* and its optimization under submerged fermentation. *International Biodeterioration and Biodegradation*, 78, 24-33.
10. Maurya, D. P., Vats, S., Rai, S., & Negi, S. (2013). Optimization of enzymatic saccharification of microwave pretreated sugarcane tops through response surface methodology for biofuel. *Indian Journal of Experimental Microbiology,* 51, 992-996.
11. Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31, 426-428.
12. Singhania, R. R., Saini, J. K., Saini, R., Adsul, M., Mathur, A., Gupta, R., & Tuli, D. K. (2014). Bioethanol production from wheat straw via enzymatic route employing *P. janthinellum* cellulases. *Bioresource Technology,* 169, 490-495.
13. Singhania, R. R., Sukumaran, R. K., Patel, A. K., Larroche, C., & Pandey, A. (2010). Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. *Enzyme and Microbial Technology*, 46, 541-549.
14. Talebnia, F., Karakashev, D., & Angelidaki, D. (2010). Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. *Bioresource Technology,* 101, 4744-4753.
15. Turhan, O., Isci, A., Mert, B., Sakiyan, O., & Donmez, S. (2015). Optimization of Ethanol Production from Microfluidized Wheat Straw by Response Surface Methodology. *Preparative Biochemistry and Biotechnology*, 45-8, 785-795.
16. Wooley, R., & Ruth, M. (1999). World Energy Statistics. International Energy Agency. Paris: France; 2010.
17. Zhang, Y., Liu, Y. Y., X. U., J. L., Yuan, Z. H., Qi, W., Zhuang, X. S., & He, M., C. (2012). High solid and low enzyme loading based saccharification of agricultural biomass. *BioResources*, 7, 345-353.