Toxicology International, *Vol 25(2), 93-99, April-June 2018, DOI: 10.18311/ti/2018/v25i2/22222*

Original Research
ISSN (Print) : 0971-6580
ISSN (Online) : 0976-5131

Microbial Production and Purification of Cellulase Enzyme on Waste as a Substrate

Mohit Nigam, Shoaa Muzaffar Husain and Garima Awasthi*

Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow Campus, Lucknow – 227105, Uttar Pradesh, India; gawasthi@lko.amity.edu

Abstract

The cellulase is of industrial importance and there is a high demand to produce low cost cellulase. The cellulase production was carried out by *Aspergillus niger* on wastes like saw dust and wheat straw, as it is a need to reduce and reuse waste from the environment. The production was optimized at 72 hrs for saw dust and wheat straw with enzyme activity of 249.10 U/ml/min and 338.02 U/ml/min respectively. The produced enzyme was purified, the specific enzyme activity of Cellulase after precipitation at 80% $(NH_4)_2SO_4$ was observed as 4.71 U/min/mg for saw dust and 6.91 U/min/mg for wheat straw respectively. After ion-exchange chromatography best specific enzyme activity was observed in saw dust at 0.6 M NaCl with 3498.04 U/min/mg and for wheat straw at 0.4 M NaCl with 1692.6 U/min/mg. The industrial importance is to produce cellulase at low cost which can be achieved using waste as substrate as well as reduces toxicity of waste.

Keywords: *Aspergillus niger*, Cellulase, Fermentation, Purification

1. Introduction

High demand and the increased prices of cellulase have lead to explore alternative ways, so as to reduce the production and processing cost and also to meet the market demand[1]. Cellulase is an industrially important enzyme, which hydrolysis cellulose molecule into monosaccharide or small molecules[2]. Cellulase belongs to E.C 3.2.1.4 group of enzyme that hydrolysis 1, 4 β-D glycosidic linkages in cellulose component[3]. Cellulases effectively hydrolysis complex celluloses and based on their mode of action they are classified as endocellulase: hydrolyse internal bond, exocellulase: break end of exposed chain, cellobiase or beta glucosidase: hydrolyses exocellulase product into monosaccharide, oxidative cellulases and cellulose phosphorylases: depolymerize cellulose[4–6].

Hence cellulase have several applications in textile industry for the polishing of fabrics to increase its softness and brightness, pulp and paper industry, coffee processing, pharmaceutical industry, biofuel industry, improving the nutritional quality and digestibility of animal feeds and also important for the conversion of renewable cellulosic biomass into commodity chemicals[7-8].

Cellulase enzyme is an inducible bioactive compound, which can be synthesized by wide range of microorganisms like fungi, bacteria, and protozoans as they grow well on cellulosic matters. Although, only a few of these can produce significant quantities of cell-free bioactive compounds that can completely hydrolyze crystalline cellulose in vitro[1]. After numerous studies on the degradation of cellulosic materials, only few microorganisms had met the industrial requirement[9]. Fungi are the main cellulase-producing microorganisms, preferred for commercial enzyme production as the enzyme production is higher than those from yeast and bacteria[9-10]. *Aspergillus sp.*, *Penicillium sp.* and *Trichoderma spp*. have been reported to be efficient source for the production of cellulases, though maximum species of *Aspergillus* have a great potential for producing cellulase enzyme[11]. The enormous work on production of cellulose using various agricultural waste and industrial residues such as coir waste, rice straw, saw dust, vegetable waste, wheat straw and wheat bran as sub-

*Author for correspondence

strates has been carried out, but still more work is needed to identify cheaper substrates and high yielding fermentation and purification process to develop more economic and cost effective product of high purity[12–17]. The current work was focused to increase the production of cellulase using waste material and also to study the purification folds and recovery of cellulase enzyme[18].

2. Material and Methods

The *Aspergillus niger* was previously isolated in our laboratory and was maintained on slant culture media having composition of Potato infusion 200g, Dextrose 20g, Agar 20g (PDA) in 1 liter distilled water and pH 5.6 ± 0.2. The incubation temperature was maintained at 28°C for 96 hrs[19].

2.1 Inoculum Preparation

A homogeneous suspension used for inoculum was prepared by growth of pure culture of fungus *Aspergillus niger* in potato dextrose (PD) media. The media composition (in mg/l) for inoculum was potato infusion 200, dextrose 20 and pH 5.6 ± 0.2. The 3-3 ml inoculums was prepared for each production setup, by transferring loop full of *Aspergillus niger* aseptically in each 3 ml PD media, which was incubated at 28°C for 72 h. These prepared inoculums were aseptically transferred into production media for microbial growth and production formation. The inoculums help in increasing product yield and reducing lag phase.

2.2 Pre-Treatment of Substrate

Saw dust was pre-treated with 2N NaOH and Wheat straw was pre-treated with 0.1% sulphuric acid.

2.3 Production Media Preparation

The production media used was Mineral Salt Media (MSM) and was designed as g/l Potassium chloride (KCl) 0.8, Sodium chloride (NaCl) 0.8, Calcium chloride ($CaCl_2$) 0.1, Glucose 8.0, Ferrous sulphate ($FeSO_4$) 0.1, Ammonium chloride (NH_4Cl) 2.0, Disodium phosphate (Na_2HPO_4) 2.0, Magnesium sulphate ($MgSO_4$) 0.2. The media was then autoclaved for 15 minutes at 121°C. The MSM is enrichment medium, it is well buffered and provides conditions for recovery of injured cells and it is also free from inhibitors. This media supplement mineral to the microbe for increasing its growth and product yield.

2.4 Fermentation Conditions

The experiment was carried in 500 ml Erlenmeyer flask. 20g pre-treated substrate wheat straw and saw dust was added to the 100 ml - 100ml of production media and again it was autoclaved for 15 minutes at 121 °C. After cooling the flask was inoculated with 3-3 ml previously prepared 24 hr old inoculums. The flasks were then put on incubation for 6 days at 28°C in shaker incubator at 100 rpm. The shock treatment of +2°C was given to the microorganism at the interval of 24hr.

2.5 Kinetics Study of Cellulase Production

The kinetics study of Cellulase production was done at the interval of 24 hrs, for which protein estimation was done by Lowry's method[20], glucose estimation by DNS method[21] and CMCase activity in enzyme was determined by DNS method[22].

2.6 Purification of Cellulase Enzyme

Purification was done to see the purification fold and percentage yield of the Cellulase enzyme produced from the waste material. The minimum possible steps were used to recover the enzyme of high purity.

2.6.1 Removal of Insolubles

Purification was carried out by first removing the insolubles by filtration using a muslin cloth and then centrifugation was done at 5000 rpm for 5 minutes.

2.6.2 Isolation of Protein

Precipitation of protein was done by salting out method using 80% ammonium sulphate solution[23].

2.6.3 Purification of Cellulase

The isolated enzyme was subjected to ion exchange chromatography with DEAE column. The column was equilibrated with 50 mM phosphate buffer pH 7.0 and bound proteins were eluted using gradient of 0.1-1.0 M NaCl elution buffer[24, 25].

3. Result and Discussion

3.1 Kinetic Study of Substrate Utilization and Product Formation

Cellulase was produced by solid state fermentation process using saw dust and wheat straw as substrate. The kinetic study for substrate utilization and product formation was carried for both the substrates. This study was carried out for 6 days at 24 hrs, 48 hrs, 72 hrs till 144 hrs. The maximum enzyme activity was observed at 72hrs in both saw dust (249.10 U/ml/min) and wheat straw (338.02 U/ml/min). The enzyme activity was 244.88 U/ml/min and 141.63 U/ml/min at 24 hrs in saw dust and wheat straw respectively, which gradually increased upto 72 hrs, whereas after 72hrs it started decreasing. The specific enzyme activity of saw dust was 14.92 U/min/mg after 24hrs and slightly decreased to 13.63 U/min/mg till 72 hrs, where as it was increasing in wheat straw from 66.92 U/min/mg after 24 hrs to 115.92 U/min/mg after 72 hrs of incubation as shown in Table 1.

The substrate was consumed as the concentration was gradually decreasing simultaneously till 144 hrs of incubation. The percentage consumption of substrate was very high in case of wheat straw as compared to saw dust. The enzyme produced by the wheat straw was higher than the saw dust as both the substrates were taken in equal concentrations (20gm in 100ml production media). The highest protein content was observed 18.27 mg/l in saw dust as compared to 2.92 mg/l in wheat straw at 72 hrs incubation, as shown in Table 2.

The enzyme activity was highest at 72 hr incubation in both the substrate wheat straw (338.02 U/ml/min) and saw dust (249.10 U/ml/min) as shown in Figure 1. The enzyme activity was reduced in 144 hr incubation as the substrate concentration was reduced.

3.2 Purification of Cellulase Enzyme

The steps involved in purification of cellulase enzyme were removal of insoluble by filtration and centrifugation, isolation by protein precipitation (salting out by ammonium sulphate) and purification by ion exchange chromatography.

3.2.1 Purification after Removal of Insoluble

The enzyme activity after removing the cell debris by filtration and centrifugation was observed 93.36 U/ml/min in saw dust and 14.12 U/ml/min in wheat straw. The specific activity was observed 8.44 U/min/mg and 6.25 U/min/mg in saw dust and wheat straw respectively.

Table 1. Enzyme activity and specific enzyme activity in saw dust and wheat straw samples

S.No	Incubation time (hr)	Enzyme Activity (U/ml/min)		Specific Enzyme Activity (U/min/mg)	
		Saw Dust	Wheat Straw	Saw Dust	Wheat Straw
1.	24	244.88	141.62	14.92	66.92
2.	48	246.14	253.10	14.36	100.59
3.	72	249.10	338.02	13.63	115.92
4.	144	188.61	97.57	9.61	35.34

Table 2. Kinetics study for protein and glucose content in saw dust and wheat straw samples

S.No	Incubation time (hr)	Protein content (mg/ml)		Glucose Content (mg/ml)	
		Saw Dust	Wheat Straw	Saw Dust	Wheat Straw
1.	24	16.41	2.12	1.16	1.42
2.	48	17.14	2.52	1.05	1.32
3.	72	18.27	2.92	0.89	1.26
4.	144	19.63	2.76	0.84	0.21

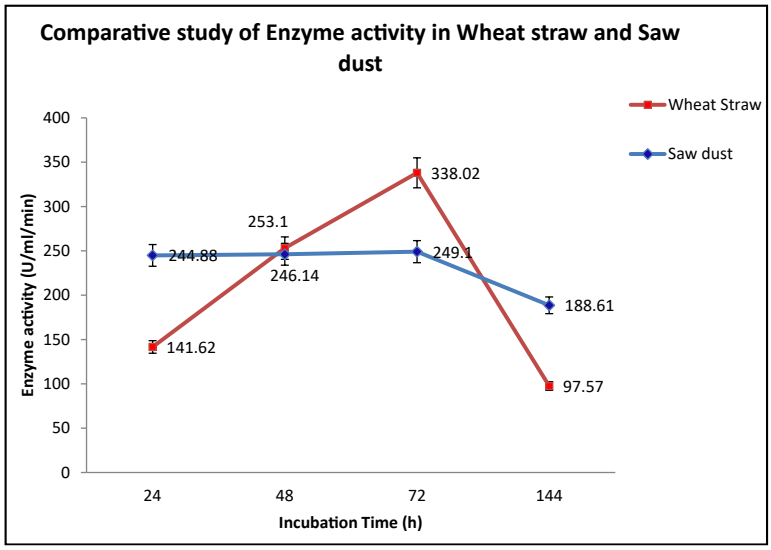


Figure 1. Comparative study of Enzyme activity in wheat straw and saw dust

3.2.2 Purification after Protein Precipitation at 80% Ammonium Sulphate

The enzyme activity observed was 6.32 U/ml/min in saw dust and 2.32 U/ml/min in wheat straw. The specific activity was observed 4.71 U/min/mg and 6.91 U/min/mg in saw dust and wheat straw respectively.

The fold purification observed was 0.56 in saw dust and 1.11 in wheat straw. The percentage yield was observed 6.77 and 16.42 in saw dust and wheat straw respectively.

3.2.3 Purification after Ion-Exchange Chromatography

The ion-exchange chromatography was carried out and the isolated samples were run from 0.1-1.0 M NaCl concentration gradient of elution buffer. The best enzyme activity was observed at 0.6 M NaCl gradient for Saw Dust and 0.4 M NaCl gradient for Wheat Straw. The purification folds were highest 414.34 at 0.6 M NaCl for saw dust where as it was 270.69 at 0.4 M NaCl for wheat straw. The percentage yield was 7.25 for saw dust and 46.40 for wheat straw. The specific activity of enzyme was 3498.04 U/min/mg for saw dust and 1692.6 U/min/mg for wheat straw.

The enzyme activity was also observed 7.86 (U/ml/min) in 0.1 M NaCl concentration for saw dust whereas the protein concentration was 0.025 mg/ml which was giving low specific enzyme activity of 320.70 U/min/mg hence the purified fraction of enzyme can be reported on 0.1 M NaCl concentration also. Similarly, the enzyme activity was also observed 6.33 U/ml/min in 0.5 M NaCl concentration for wheat straw whereas the protein concentration was 0.007 mg/ml which was giving low specific enzyme activity of 892.46 U/min/mg hence the purified fraction of enzyme can be reported on 0.1 M NaCl concentration. This data can be referred from Tables 3 and 4. These results indicate that the cellulases produced during the fermentation process of different classes are purified at different concentration of elution buffers.

4. Discussion

The maximum CMCase activity wheat straw was 338.02 U/ml/min and that of saw dust was 249.10 U/ml/min in present study, where as the maximum CMCase activity in wheat straw was 0.024U/ml/min, and saw dust was 0.018U/ml/min reported by previous researcher[17]. This increase in CMCase activity was due to the increased concentration of MSM production media and secondly the heat shock treatment given to *Aspergillus niger* during production process. Currently the major research work is emphasized on production of cellulases on waste substrate, where as very few work is reported on purification of cellulase. In the previously reported work cellulase enzyme was purified and fractionated using a size exclusion chromatography, they determined molecular weight of purified cellulose was 48 kDa as revealed by SDS-PAGE and zymogram analysis[26]. In our work the fractionation of saw dust was done by ion exchange chromatography. The enzyme produced from saw dust was eluted at 0.1M NaCl, 0.3M NaCl, 0.6M NaCl and 0.9M NaCl strength buffer, where as

Table 3. Purification steps of cellulase produced from Saw Dust showing Specific Activity, Purification fold and Percentage yield after each step

S.No.	Purification steps	Concentration of reagents used for purification	Protein Content (mg/ml)	Enzyme Activity (U/ml/min)	Specific Activity	Fold Purification	%age Yield
1.	**Removal of insolubles**	by filtration and centrifugation	11.06	93.36	8.44	-	-
2.	**Protein precipitation**	at 80% Ammonium Sulphate	1.34	6.32	4.71	0.56	6.77
3.	**Ion-exchange chromatography**	[0.1 M NaCl]	0.025	7.86	320.70	37.99	8.42
		[0.2 M NaCl]	0.0071	1.97	276.97	32.81	2.11
		[0.3 M NaCl]	0.003	5.46	1692.6	200.49	5.85
		[0.4 M NaCl]	0.004	0.44	112.84	13.37	0.47
		[0.5 M NaCl]	-	-	-	-	-
		[0.6 M NaCl]	0.002	6.77	3498.04	414.34	7.25
		[0.7 M NaCl]	-	-	-	-	-
		[0.8 M NaCl]	-	-	-	-	-
		[0.9 M NaCl]	0.006	3.60	620.62	73.51	3.86
		[1.0 M NaCl]	-	-	-	-	-

Table 4. Purification steps of Cellulase produced from Wheat Straw showing enzyme activity, specific activity, purification fold and percentage yield after each step

S.No.	Purification step	Concentration of reagents used for purification	Protein Content (mg/ml)	Enzyme Activity (U/ml/min)	Specific Activity	Fold Purification	%age Yield
1.	**Removal of insolubles**	by filtration and centrifugation	2.26	14.12	6.25	-	-
2.	**Protein precipitation**	at 80% Ammonium Sulphate	0.34	2.32	6.91	1.11	16.42
3.	**Ion-exchange chromatography**	[0.1 NaCl]	0.006	1.31	225.68	36.09	9.28
		[0.2 NaCl]	-	-	-	-	-
		[0.3 NaCl]	0.005	5.2416	1160.64	185.62	37.12
		[0.4 NaCl]	0.004	6.552	1692.6	270.69	46.40
		[0.5 NaCl]	0.007	6.33	892.46	142.73	44.86
		[0.6 NaCl]	0.003	2.84	880.15	140.76	20.11
		[0.7 NaCl]	0.006	4.37	752.27	120.31	30.94
		[0.8 NaCl]	-	-	-	-	-
		[0.9 NaCl]	-	-	-	-	-
		[1.0 NaCl]	-	-	-	-	-

the fractionation enzyme produced from wheat straw was eluted at 0.3M NaCl, 0.4M NaCl, 0.5M NaCl, 0.6M NaCl and 0.7M NaCl strength buffers. The maximum purification fold was observed in saw dust and wheat straw was 414.34 and 270.69 respectively, after the third step purification. These results of fractionation indicate that the cellulases produced during the fermentation process are of different classes, which are purified at different strength of elution buffers. The purification of cellulase enzyme can be achieved in such a way to reduce cost input, with the increase in percentage yield at industrial level. To accomplish this target, the purification fold was studied.

5. Conclusion

The wheat straw and saw dust are the promising waste material as substrates for the production of Cellulase enzyme. The yield of cellulase enzyme was high while making slights changes in production process. The use of increased concentration of production media and thermal shock treatment gave promising results of cellulase production. The best enzyme activity was observed in wheat straw compared to saw dust as earlier reported. The major focus was to purify the Cellulase enzyme produced from the waste substrate applying least possible steps. The best percentage yield was obtained in wheat straw, whereas saw dust is also giving good purification folds after three steps purification process. The selected purification steps gave effective purification folds and high product yield. The selection of waste material, optimization of production process and application of effective least purification steps can come up with the production of industrially important cellulase enzyme at low cost.

6. Acknowledgement

We wish to acknowledge Amity Institute of Biotechnology, Amity University Utter Pradesh, Lucknow Campus, Lucknow, U.P., for providing laboratory facilities to conduct this research work. I would also like to thank the management, staff and those who directly or indirectly helped us to perform this series of experiments.

7. References

1. Kubicek CP, Messner R, Guber F, Mach RL, Kubicek-Pranz EM. The *Trichoderma* cellulase regulatory puzzle: From the interior life of a secretory fungus. Enzyme and Microbial Technology. 1993; 15: 90–5. https://doi.org/10.1016/0141-0229(93)90030-6
2. Lee SM, Koo YM. Pilot-scale production of cellulase using *Trichoderma reesei* Rut C-30 in fed-batch mode. Journal of Microbiology and Biotechnology. 2001 April; 11(2): 229–33.
3. Singh J, Kaur P. Optimization of process parameters for cellulase production from *Bacillus sp.* JS14 in solid substrate fermentation using response surface methodology. Brazilian Archives of Biology and Technology. 2012; 55(4). https://doi.org/10.1590/S1516-89132012000400004
4. Walsh G. Industrial enzymes: Proteases and carbohydrases. Proteins; Biochemistry and Biotechnology, John Wiley and Sons. Ltd; 2002.
5. Himmel ME, Baker JO, Overend RP. Approaches to cellulase purification. Enzymatic Conversion of Biomass for Fuel Production, ACS symposium series 566; 1994.
6. Jahangeer S, Khan N, Jahangeer S, Sohail M, Shahzad S, Ahmad A, Khan SA, Screening and characterization of fungal cellulases isolated from the native environmental source. Pakistan Journal of Botany. 2005; 37(3): 739–48.
7. Gong CS, Cao NJ, Tsao GT. Ethanol production from renewable resources. Advances in Biochemical Engineering/ Biotechnology. Recent Progress in Bioconversion of Lignocellulosics, Berlin: Springer- Verlag. 1999; 65: 207–41. https://doi.org/10.1007/3-540-49194-5_9
8. Himmel ME, Ruth MF, Wyman CE. Cellulase for commodity products from cellulosic biomass. Current Opinion in Biotechnology. 1999; 10: 358–64. https://doi.org/10.1016/S0958-1669(99)80065-2
9. Bai S, Kumar MR, Kumar DJM, Balashanmugam P, Kumaran MDB, Kalaichelvan PT. Cellulase production by *Bacillus subtilis* isolated from cow dung. Archive of Applied Science Research. 2012; 4(1): 269–79.
10. Bakri YP, Jacques P, Thonart. Xylanase production by *Penicillum canescens* 10-10c in solid state fermentation. Applied Biochemistry and Biotechnology 2003; 108(1-3): 737–48. https://doi.org/10.1385/ABAB:108:1-3:737
11. Peij N, Gielkens MMC, Verles RP, Visser K, Graff LH. The transcriptional activator X in R regulates both xylanolytic endoglucanase gene expression in *Aspergillus niger*. Applied and Environmental Microbiology. 1998; 64: 3615–17. PMid:9758775 PMCid:PMC106473
12. Abdullah JJ, Greetham D, Pensupa N, Tucker GA, Du C. Optimizing cellulase production from Municipal Solid Waste (MSW) using Solid State Fermentation (SSF). Journal of Fundamentals of Renewable Energy and Applications. 2016; 6: 3. https://doi.org/10.4172/2090-4541.1000206
13. Bairagi S. Optimization of cellulase enzyme from vegetable waste by using *Trichoderma atroviride* in solid state fermentation. IOSR Journal of Environmental Science, Toxicology and Food Technology. 2016 May; 10(5): 68–73.
14. Jadhav AR, Girde AV, More SM, More SB, Khan S. Cellulase Production by utilizing agricultural wastes. Research Journal of Agriculture and Forestry Sciences. 2013 Aug; 1(7): 6–9.
15. Singhania RR, Sukumaran RK, Patel AK, Larroche C, Pandey A. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme and Microbial Technology. 2010; 46: 541–9. https://doi.org/10.1016/j.enzmictec.2010.03.010
16. Goyal M, Kalra KL, Sareen VK, Soni G. Xylanase production with xylan rich lignocellulasic waste by a local soil isolatee of *Trichoderma viride*. Brazilian

Journal of Microbiology. 2008; 39: 535–41. https://doi.org/10.1590/S1517-83822008000300025. PMid:24031262. PMCid:PMC3768426

17. Khan JA, Singh SK. Production of cellulase using cheap substrates by solid state fermentation, International Journal of Plant Animal and Environmental Sciences. 2011; 1(3): 179–87.
18. UdoHölker, JürgenLenz, Solid-state fermentation - are there any biotechnological advantages? Current Opinion in Microbiology. 2005; 8(3): 301–6. https://doi.org/10.1016/j.mib.2005.04.006. PMid:15939353
19. Atlas RM, Parks LC. Handbook of Microbiological Media. Florida CRC Press; 1996.
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin-Phenol reagents. Journal of Biological Chemistry. 1951 May 28; 193 :265–75. PMid:14907713
21. Dubois M, Gilles KA, Hamilton JK, Rebers PK, Smith F. Colorimetric method for determination of sugar and related substrate. Analytical Chemistry. 1956; 28: 350–6. https://doi.org/10.1021/ac60111a017
22. Mandels M, Weber J. The production of cellulases. American Chemical Society, Advances in Chemistry, ACS publishers; 1969; 95: 391–414.
23. Dunn MJ. Protein determination of total protein concentration. Harris ELV, Angal S, editors, Protein Purification Methods, Oxford: IRL Press. 1992; 59: 209–12.
24. Gaur R, Tiwari S. Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. BMC Biotechnology. 2015 Mar 19; 15(19): 1–12. https://doi.org/10.1186/s12896-015-0129-9
25. Garg N, Ashfaque M. Mango peel as substate for production of extracellular polygalacturonase from *Aspergillus fumigatus*. Indian Journal of Horticulture. 2010 Mar; 67(1): 140–3.
26. Sriariyanun M, Tantayotai P, Yasurin P, Pornwongthong P, Cheenkachorn K. Production, purification and characterization of an ionic liquid tolerant cellulase from Bacillus sp. isolated from rice paddy field soil. Electronic Journal of Biotechnology. 2016; 19: 23–8. https://doi.org/10.1016/j.ejbt.2015.11.002