

Investigation and Optimization of Extracellular Cellulase Production by *Trichoderma harzianum*

Tabasom Naseripour (PhD)

Department of plant protection,
Faculty of Plant Production, Gorgan
University of Agricultural Sciences
and Natural Resources, Golestan, Iran

Saeed Nasrollah Nejad (PhD)

Department of plant protection,
Faculty of Plant Production, Gorgan
University of Agricultural Sciences
and Natural Resources, Golestan, Iran

Samira Shahbazi (PhD)

Nuclear Agriculture Research School,
Nuclear Science and Technology
Research Institute (NSTRI), Atomic
Energy Organization of Iran (AEOI),
Alborz, Iran

Kamran Rahnama (PhD)

Department of plant protection,
Faculty of Plant Production, Gorgan
University of Agricultural Sciences
and Natural Resources, Golestan, Iran

Corresponding author: Tabasom
Naseripour

Tel: +98-9183391945

Email: naseripour1391@gmail.com

Address: Gorgan University of
Agricultural Sciences and Natural
Resources, Golestan, Iran

Received : 04 Apr 2015

Revised: 10 May 2015

Accepted: 17 May 2015

ABSTRACT

Background and Objectives: Cellulose is a major component of plant biomass, which can be converted into biofuels and valuable chemicals. The key step in utilization of this organic material is its hydrolysis into soluble sugars. This study evaluated cellulase production by *Trichoderma harzianum* under different pH values, temperatures and incubation periods with the aim to increase enzyme production and decrease its costs.

Methods: The amount of protein production and the hydrolytic activity of cellulase enzymes including exoglucanase, endoglucanase and β -glucosidase produced by *T. harzianum* were evaluated using various substrates such as avicel, carboxymethyl cellulose, cellobiose, Whatman grade 1 filter paper under different pH values (4, 4.8, 5.5 and 6.5), temperatures (25, 28 and 34 °C) and incubation times (48, 72, 96 and 120 h).

Results: The optimum condition for cellulase production by *T. harzianum* is 120 hours of incubation at 25 °C and pH of 6.5.

Conclusion: *T. harzianum* can be used for the production of all three classes of cellulase. This fungus is suitable for the efficient production of cellulolytic enzymes and reducing the cost of consumables.

Keywords: Cellulose, *Trichoderma harzianum*, Hydrolytic enzymes, Optimization.

INTRODUCTION

Cellulose is an important structural component of the primary cell wall of green plants. Annually, plants produce about 180 billion tons of this organic material. This polysaccharide is considered as one of the largest organic carbon deposits on Earth (1). This long and linear homopolymer is composed of D-glucose units linked by β -1, 4-glycosidic bonds. The number of glucose units varies in the cellulose molecules. The degree of polymerization also varies from 250 to more than 10,000 depending on the source (2). This massive amount of organic matter can be converted into useful products such as biofuels, valuable chemicals, and cheap energy sources for fermentation processes (3). The key step for degradation of cellulose is its hydrolysis into monosaccharides, since the mono-sugars produced can be converted into chemicals and energy. However, enzymatic conversion of cellulose into simple sugars has a major limitation; its high cost for the production of cellulase (4). Cellulase production plays a major role in the conversion of cellulose into simple sugars, and reducing the cost of its production can make this process economically viable (5). Cellulase is composed of at least three classes of enzymes that have synergistic effects (6). In enzymatic degradation of cellulose, endoglucanases (EC.3.2.1.4) first affect the internal β -1, 4-glycosidic bonds and provide a non-reducing end for the activity of exoglucanases (EC.3.2.1.91). Next, exoglucanases degrade crystalline regions by separating cellobiose units from the non-reducing ends. Then, β -glucosidases (EC 3.2.1.21) convert cellobiose into glucose units (7). Many microorganisms such as bacteria, fungi and actinomycetes are capable of cellulose decomposition (8). Filamentous fungi produce most of the cellulolytic enzymes used in the industry. The genus *Trichoderma* is considered as the best producer of this enzyme (9). Although *Trichoderma viride* has been studied extensively and is considered the most powerful producer of this enzyme, the organism produces relatively low amounts of β -glucosidase, which is an important limitation for enzymatic conversion of cellulose to glucose by this fungus. The cellulase produced by *Trichoderma harzianum* is known as the most efficient method for the conversion of cellulose substrates into mono-sugars (10).

MATERIAL AND METHODS

In order to evaluate the suitable conditions for the production of cellulase by *T. harzianum*, production of cellulase was optimized by changing various parameters such as incubation time (48, 72, 96 and 120 hours), pH (4, 4.8, 5.5 and 6.5) and temperature (25, 28 and 34 °C).

Fungal strain, culture media and culture conditions

T. harzianum was isolated from the agricultural lands in Khorasan Province and cultured on selective medium for *Trichoderma* spp. (11). *T. harzianum* was cultured on MYG medium (0.2% malt extract, 0.2% yeast extract, 2% glucose, and 2% agar), and incubated at 28 °C for one week. Then, spores were collected in 1% saline solution (8.5g NaCl in one liter of distilled water). Then, 1 ml of spore suspension (107-108) was cultured in *Trichoderma* complete medium (TCM) (12), and incubated for 24 hours in a shaking incubator at 28 °C. After washing, the mycelia grown on the TCM with 1% saline were centrifuged at 4500×g for 7 min for induction of cellulase expression. Then, 50ml of *Trichoderma* fermentation medium (TFM) (13) with different pH values (4, 4.8, 5.5 and 6.5) were added, and the suspension was placed in shaking incubator at 180 rpm. Enzyme activity was measured after different incubation times (48, 72, 96 and 120 hours). It should be noted that the conditions of this experiment were also optimized at different temperatures (25, 28 and 35 °C).

According to the method described by Dashtani et al. (14), filter paper activity method was used to measure total cellulase activity. First, 1x6 cm Whatman qualitative filter paper No. 1 was used with 0.05% sodium citrate buffer as substrate. Given that cellulase is a heterogeneous enzyme, simultaneous activity of three enzymes of endoglucanase (avicelase), exoglucanase (carboxymethyl cellulose) and β -glucosidase is necessary for completion of the hydrolysis process. In order to measure endoglucanase and exoglucanase activities, carboxymethylcellulose and avicelase were used as substrates, respectively (15). Cellobiose was used as substrate for measuring β -glucosidase activity (16). The level of endoglucanase, exoglucanase and β -glucosidase activities was measured using dinitrosalicylic acid (DNS) method and glucose as standard (11, 17). The reaction

mixture contained 0.5 ml of 0.5% solution (w/v) from each substrate in 0.05M sodium citrate buffer (pH 4.4) and 0.5 ml of the supernatant from TFM. The samples were immersed in warm water bath for 60 min at temperature of 50 °C. The enzymatic reaction was stopped by adding 3 ml of DNS. The samples were well mixed and then placed in a boiling water bath for 5 min, and then cooled immediately. After diluting the mixture, absorbance was read by spectrophotometer at wavelength of 540 nm.

Statistical analysis was performed using SPSS software (Version 17). First, normality of data was assessed. Factorial general linear model was used to evaluate the effect of temperature, time and pH on activity of the enzymes (endoglucanase, exoglucanase, β -glucosidase and total cellulase). A completely randomized design was used to evaluate the significance of interaction of temperature, time and pH with the enzymes. Duncan's test was used to compare the amount of each enzyme separately at different temperatures, incubation times and pH values.

RESULTS

According to the results, temperature, time, pH, and their interactions significantly affected the amount of endoglucanase, exoglucanase, β -glucosidase and total cellulase (Table 1). The obtained data were homogeneous and normally distributed ($P > 0.05$).

Temperature significantly affected the production of endoglucanase, exoglucanase, β -glucosidase and total cellulase. The highest

level of endoglucanase was produced at 25 oC, while the lowest amount of the enzyme was found at 34 oC (Table 2). The highest and lowest levels of β -glucosidase were produced at 25 oC and 34 oC, respectively (Table 2).

The highest amount of total cellulase was observed at 25 and 28 °C, while the lowest amount of the enzyme was detected at 34 °C (Table 2).

As shown in table 3, the amount of endoglucanase, exoglucanase, β -glucosidase and total cellulase was different at different incubation times (48, 72, 96 and 120 hours). The highest amount of endoglucanase was recorded at 48 hours, while the lowest amount was found at 72 hours. The highest amount of exoglucanase was found at 96 hours, and the lowest amount of this enzyme was found at other incubation times. The highest amount of β -glucosidase was found at 48 hours, and the lowest amount was detected at 72 hours. The maximum amount of total cellulase was recorded at 120 hours (Table 3).

As shown in table 4, different pH conditions significantly affected the amount of all enzymes. The highest level of endoglucanase was found at pH 6.5, while the lowest level of the enzyme was found at pH 4. The highest amount of exoglucanase was produced at pH 6.5 and 4.8, while the lowest amount of the enzyme was detected at pH 4 and 5.5. The highest level of β -glucosidase was found at pH 4.5 and 5.5, and the lowest level of the enzyme was found at pH 4. The highest level of total cellulose was found at pH 6.5, while the lowest amount was found at pH 4 and 5.5 (Table 4).

Table 1- Effect of temperature, time and pH on endoglucanase, exoglucanase, β -glucosidase and total cellulase

Variables	Mean square			
	endoglucanase	exoglucanase	β -glucosidase	Total cellulase
Temperature	61.998**	1.544**	10.679**	58.631**
Time	1.430**	0.904**	4.776**	1.016**
pH	37.314**	2.435**	0.101**	53.259**
Temperature x time	0.714*	0.618**	1.978**	4.325**
Temperature x pH	2.901**	1.039**	0.053**	2.449**
Time x pH	1.101**	0.300**	0.037**	1.603**
Temperature x Time x pH	1.315**	0.295**	0.043**	1.679**
Error	0.267	0.082	0.010	0.093

* and ** indicate statistical significance at 0.05 and 0.01, respectively.

Table 2- Comparison of the amount of endoglucanase, exoglucanase, β -glucosidase and total cellulase under different temperatures

Raw	Temperature (°C)	Mean (U/ml) \pm standard deviation			
		endoglucanase	exoglucanase	β -glucosidase	Total cellulase
1	25	5.32 \pm 1.21 ^a	4.99 \pm 0.41 ^b	3.79 \pm 0.080 ^a	7.70 \pm 1.29 ^a
2	28	4.70 \pm 1.39 ^b	5.15 \pm 0.070 ^a	3.66 \pm 0.67 ^b	7.75 \pm 1.67 ^a
3	34	3.10 \pm 0.87 ^c	4.79 \pm 0.28 ^c	2.91 \pm 0.29 ^c	5.81 \pm 0.98 ^c

Table 3- Comparison of the amount of endoglucanase, exoglucanase, β -glucosidase and total cellulase at different incubation times

Raw	Time (hours)	Mean (U/ml) \pm standard deviation			
		endoglucanase	exoglucanase	β -glucosidase	Total cellulase
1	48	4.63 \pm 1.43 ^a	4.86 \pm 0.32 ^b	3.04 \pm 0.34 ^b	7.08 \pm 1.66 ^b
2	72	4.14 \pm 1.74 ^c	4.89 \pm 0.60 ^b	3.26 \pm 0.47 ^b	6.91 \pm 1.31 ^b
3	96	4.42 \pm 1.42 ^{ab}	5.21 \pm 0.50 ^a	3.82 \pm 0.48 ^a	7.04 \pm 1.30 ^b
4	120	4.33 \pm 1.39 ^{bc}	4.94 \pm 0.51 ^b	3.45 \pm 0.67 ^a	7.32 \pm 2.10 ^a

Table 4- Comparison of the amount of endoglucanase, exoglucanase, and at different pH values

Raw	pH	Mean (U/ml) \pm standard deviation			
		endoglucanase	exoglucanase	β -glucosidase	total cellulase
1	4	3.85 \pm 1.29 ^c	4.76 \pm 0.35 ^b	3.38 \pm 0.54 ^b	6.01 \pm 0.90 ^c
2	4.8	4.15 \pm 1.15 ^b	5.16 \pm 0.55 ^a	3.51 \pm 0.62 ^a	7.70 \pm 1.32 ^b
3	5.5	3.64 \pm 1.04 ^c	4.75 \pm 0.50 ^b	3.49 \pm 0.64 ^a	6.14 \pm 0.99 ^c
4	6.5	5.88 \pm 1.37 ^a	5.24 \pm 0.43 ^a	3.44 \pm 0.56 ^a	8.50 \pm 1.60 ^a

The interaction of different variables (temperature, time and pH) on the production of the enzymes was also evaluated. The highest and lowest amount of endoglucanase was produced at 25 °C \times 96 h \times pH 6.5 and 34 °C \times 120 h \times pH 4, respectively. The highest and lowest amount of exoglucanase was produced at 28 °C \times 96 h \times pH 4.8 and 34 °C \times 120 h \times pH 5.5, respectively. The interaction of the variables significantly affected the amount of β -glucosidase. The highest and lowest amount of β -glucosidase was produced at 25 °C \times 120 h \times pH 4.8 and 34 °C \times 48 h \times pH 4, respectively. The highest amount of total cellulase was produced at 25 °C \times 120 h \times pH 6.5, while the lowest amount was produced at 34 °C \times 120 h \times pH 4, respectively.

DISCUSSION

For efficient production of cellulase, an organism is required that can produce the enzyme in large quantities under optimized temperature, time period and pH conditions. *T. harzianum* was used in this study because of its capability for extracellular production of all three classes of cellulase. The study of enzymes' production in fermentation media showed that different enzymes have different optimum conditions. Generally, enzymatic activity was increased at higher pH values (pH 6.5), suggesting that cellulolytic enzymes work better in acidic conditions (18). The optimum pH for cellulase production by fungal species is 3 to 6 (19). Although different

fungal species require different optimum conditions for cellulase production, these conditions are within a certain range in most cases (20). The highest level of β -glucosidase production by *Aspergillus terreus* is in pH range of 4 to 5.5 (21). The highest level of cellulase enzymes production was achieved at temperatures of 25 and 28 °C. Raising the temperature limits the activity of cellulolytic enzymes (22). In this study, 25 °C seems to be the optimum temperature for the production and activity of cellulase since all enzymes had acceptable activity at this temperature. Previous studies have considered 28 °C as the optimum temperature for cellulase production by fungal species (23, 24). The highest total cellulase activity was recorded after 120 hours of incubation. Although the incubation time for different fungal species is within 96-120 h, the effect of incubation time should be further examined by studying longer incubation periods (25). Based on the results of this study, increasing the production of cellulase and reducing the fermentation time are of great importance for reducing the cost of fermentation process in the industrial production of the enzyme.

CONCLUSION

In this study, the native strain of *Trichoderma* shows favorable cellulolytic activity. The results show that maximum total cellulase production is achievable by 120 hours of incubation at 28 °C and pH 6.5.

ACKNOWLEDGMENTS

This article has been supported by a grant for a project entitled "Biofuel production for controlling soil-borne plant pathogens-A88A099". The authors would like to thank the colleagues at the Department of Plant

REFERENCES

1. Lutzen N, Nielsen MH, Oxenboell KM, Schulein M, Stentebjerg-Olesen B. *Cellulases and their application in the conversion of lignocellulose to fermentable sugars*. Biological Sciences, 1983; 300(1100): 283-291.
2. Klemm D, Heublein B, Fink-habil HP, Bohn A. *Cellulose, chemistry and application*. Angew Chem Int Ed. 2005; 44: 3358-3393.
3. Howard RL, Abotosi E, Jansen van Rensburg EL, Howard S. *Lignocellulose biotechnology: issues of bioconversion and enzyme production*. African Journal of Biotechnology. 2004. 2(12): 602-619.
4. Knauf M, Moniruzzaman M. *Lignocellulosic biomass processing: a perspective*. International Sugar Journal. 2004; 106 (1263): 147-150.
5. Garg S, Neelakantan S. *Effect of cultural factors on cellulase activity and protein production by Aspergillus terreus*. Biotechnology and Bioengineering. 1981; 23(7): 1653-1659.
6. Himmel ME, Ruth MF, Wyman CE. *Cellulase for commodity products from cellulosic biomass*. Current Opinion in Biotechnology. 1999; 10(4): 358-364.
7. Galbe, M, Zacchi G. *A review of the production of ethanol from softwood*. Applied microbiology and biotechnology. 2002; 59(6): 618-628. DOI:10.1007/s00253-002-1058-9.
8. Gao J, Weng H, Zhu D, Yuan M, Guan F, Xi Y. *Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal Aspergillus terreus M11 under solid-state cultivation of corn stover*. Bioresource Technology. 2008; 99(16): 7623-7629. doi: 10.1016/j.biortech.2008.02.005.
9. Zhou J, Wang YH, Chu J, Luo LZ, Zhuang YP, Zhang SL. *Optimization of cellulase mixture for efficient hydrolysis of steam-exploded corn stover by statistically designed experiments*. Bioresource technology. 2009; 100(2): 819-825.
10. Ahmed S, Bashir A, Saleem H, Saadia M, Jamil A. *Production and purification of cellulose-degrading enzymes from a filamentous fungus Trichoderma harzianum*. Pakistan Journal of Botany. 2009; 41(3): 1411-1419.
11. Shahbazi S, Askari H, Naseripour T, Mosavi-nasab M, Bakhtiyari M. *The synergistic interactions of Trichoderma spp cellulase enzyme activities in biomass conversion of cellulose Ia, Ib and III*. Intl J Agri Crop Sci. 2014; 7(8): 442-453.
12. Askari H, Shahbazi S, Naseripour T, Moosavi-Nasab M, Bakhtiyari M. *The impact of extracellular enzymes of Trichoderma viride and Trichoderma harzianum on Succinoglycan produced from Agrobacterium radiobacter*. Intl J Agri Crop Sci. 2014; 7(8): 442-453.

production and Food Preservation, the Nuclear Agriculture Research Center.

CONFLICT OF INTEREST

All contributing authors declare no conflicts of interest.

13. Dashtban M, Maki M, Leung KT, Mao C, Qin W. *Cellulase activities in biomass conversion: measurement methods and comparison*. Crit Rev Biotechnol. 2010; 30(4): 202-9. doi: 10.3109/07388551.2010.490938.
14. Shuangqi T, Zhenyu W, Ziluan F, Lili Z, Jichang W. *Determination methods of cellulase activity*. Afr J Biotechnol. 2011; 10(37): 7122-5. DOI: 10.5897/AJB10.2243.
15. Ghose TK. *Mesurement of cellulase activities*. Pure & Appl Chem. 1987; 59(2): 257-268.
16. Zhang YP, Hong J, Ye X. *Cellulase assays. Biofuels methods and molecular biology*. 2009; 581: 213-231.
17. Pandit NP, Maheshwari SK. *Optimization of Cellulase Enzyme Production from Sugarcane Pressmud Using Oyster Mushroom - Pleurotus Sajor-Caju by Solid State fermentation*. J Bioremed Biodegrad. 2012; 3(3): 1-5. doi: 10.4172/2155-6199.1000140.
18. Niranjane AP, Madhou P, Stevenson TW. *The effect of carbohydrate carbon sources on the production of cellulase by Phlebia gigantea*. Enzyme and Microbial Technology. 2007; 40(6): 1464-1468. DOI: 10.1016/j.enzmictec.2006.10.041.
19. Gregg D, Boussaid A, Saddler J. *Techno-economic evaluations of a generic wood-to-ethanol process: effect of increased cellulose yields and enzyme recycle*. Bioresource Technology. 1998; 63(1): 7-12.
20. Ali UF, Saad El-Dein HS. *Production and Partial Purification of Cellulase Complex by Aspergillus niger and A. nidulans Grown on Water Hyacinth Blend*. J Appl Sci Res. 2008; 4: 875-891.
21. Zhou J, Wang YH, Chu J, Zhuang YP, Zhang SL, Yin P. *Identification and purification of the main components of cellulases from a mutant strain of Trichoderma viride T 100-14*. Bioresource technology. 2008; 99(15): 6826-6833.
22. Bara MTF, Lima AL, Ulhoa CJ. *Purification and characterization of an exo-β-1, 3-glucanase produced by Trichoderma asperellum*. FEMS microbiology letters. 2003; 219(1): 81-85.
23. Pushalkar S, Rao K, Menon K. *Production of β-glucosidase by Aspergillus terreus*. Current microbiology. 1995; 30(5): 255-258.
24. Kang S, Park YS, Lee JS, Hong SI, Kim SW. *Production of cellulases and hemicellulases by Aspergillus niger KK2 from lignocellulosic biomass*. Bioresource technology. 2004; 91(2): 153-156.
25. Dedavid e Silva LA, Lopes FC, Silveira ST, Brandelli A. *Production of cellulolytic enzymes by Aspergillus phoenicis in grape waste using response surface methodology*. Applied biochemistry and biotechnology. 2009; 152(2): 295-305. doi: 10.1007/s12010-008-8190-7.

