Investigation and Optimization of Extracellular Cellulase Production by Trichoderma harzianum

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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 ℃) 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 ℃ 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.

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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 exogluconases (EC.3.2.1.91). Next, exogluconases 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 oC).

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 oC. 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). Celobiose 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 cellulose. 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	exoglucana	β-glucosida	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 × pH	1.101**	0.300**	0.037**	1.603**
Temperature × Time × 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) ± standard deviation			
		endoglucanase	exoglucanase	β-glucosidase	Total cellulase
1	25	5.32 ±1.21 [a]	4.99 ± 0.41 [b]	3.79 ± 0.080 [a]	7.70 ± 1.29 [a]
2	28	4.70 ± 1.39 [b]	5.15 ± 0.070 [a]	3.66 ± 0.67 [b]	7.75 ± 1.67 [a]
3	34	3.10 ± 0.87 [c]	4.79 ± 0.28 [c]	2.91 ± 0.29 [c]	5.81 ± 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) ± standard deviation			
		endoglucanase	exoglucanase	β-glucosidase	Total cellulase
1	48	4.63 ± 1.43 [a]	4.86 ±0.32 [b]	3.04 ± 0.34 [b]	7.08 ±1.66 [b]
2	72	4.14 ±1.74 [c]	4.89 ± 0.60 [b]	3.26 ± 0.47 [b]	6.91 ± 1.31 [b]
3	96	4.42 ±1.42 [ab]	5.21 ± 0.50 [a]	3.82± 0.48 [a]	7.04 ± 1.30 [b]
4	120	4.33 ± 1.39 [bc]	4.94 ±0.51 [b]	3.45 ± 0.67 [a]	7.32 ± 2.10 [a]

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

		Mean (U/ml) ± standard deviation			
Raw	pH	endoglucanase	exoglucanase	β-glucosidase	total cellulase
1	4	3.85 ± 1.29 [c]	4.76 ± 0.35 [b]	3.38 ± 0.54 [b]	6.01 ± 0.90 [c]
2	4.8	4.15 ± 1.15 [b]	5.16 ± 0.55 [a]	3.51 ± 0.62 [a]	7.70 ± 1.32 [b]
3	5.5	3.64± 1.04 [c]	4.75 ± 0.50 [b]	3.49 ± 0.64 [a]	6.14± 0.99 [c]
4	6.5	5.88 ± 1.37 [a]	5.24 ± 0.43 [a]	3.44 ± 0.56 [a]	8.50 ± 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 × 96 h × pH 6.5 and 34° C × 120 h × pH 4, respectively. The highest and lowest amount of exoglucanase was produced at 28 °C × 96 h × pH 4.8 and 34 °C × 120 h × 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 × 120 h × pH 4.8 and 34°C × 48 h × pH 4, respectively. The highest amount of total cellulase was produced at 25 °C × 120 h × pH 6.5, while the lowest amount was produced at 34 °C × 120 h × 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.

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CONFLICT OF INTEREST

All contributing authors declare no conflicts of interest.

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