Iran Agricultural Research, Vol. 25, No. 2 and Vol. 26, No. 1-2
Printed in the Islamic Republic of Iran, 2007

Cellulase Production by *Trichoderma reesei* using Sugar Beet Pulp

M. Moosavi-Nasab[1] and M. Majdi-Nasab[1*]**

[1]Department of Food Science and Technology, College of Agriculture, Shiraz University, Shiraz, I. R. Iran

ABSTRACT- Cellulase production by the fungus *Trichoderma reesei* was studied using sugar beet pulp (SBP) as a substrate. The subculture medium was a salt solution consisting of KH_2PO_4, $CaCl_2$, etc. Fungal cells were sub-cultured in an orbital shaker (180 rpm) at 30°C for 1-2 generations (two days for each generation) and were then used as an inoculum. Exponential cells were inoculated into a medium containing SBP. Data showed that cellulose in SBP has a potential to be used as a substrate to produce cellulase. The effect of cellulose concentration on cellulase production was investigated. Using the medium composition (the mixture of chemical salts and SBP substrate) and a fermentation time of 4-6 days, a maximum cellulase activity of 0.46 IU/ml of filter paper activity was obtained.

Keywords: Cellulase, Sugar beet pulp, *Trichoderma reesei*

INTRODUCTION

Sugar beet pulp (SBP) is a by-product of the sugar beet industry. SBP has been reported to contain large amounts of cellulose (29). Cellulose might be converted to useful materials, bio-based chemicals and energy. One possible approach to SBP lignocellulose utilization is to hydrolyze the materials into fermentable saccharides, which can then be converted into value-added products or bioenergy (3). To effectively convert lignocellulose into reducing sugars, commercial cellulase enzymes could be used (30). The process, however, is considered non-economical because the cost of commercial cellulase enzymes remains very high (28). If cellulase is produced directly from SBP and then applied to further degrade the SBP cellulose, the cost of cellulase will be significantly reduced.

As a potentially less expensive alternative, cellulolytic enzyme could be produced by a number of bacteria and fungi. The cellulolytic fungi *Trichoderma viride* and *Trichoderma reesei* have been extensively studied for their cellulase production (4, 6, 11, 12, 14, and 26). To enhance the cellulase titer, various mutants of *Trichoderma* have been developed, among which *T. reesei* RUT C30 is of industrial interest because of its high cellulase production level (14) as well as its ability to grow on waste cellulosic material (4, 9 and 19).

Cellulolytic fungi can use cellulose as a primary carbon source. Pure, crystalline cellulose, such as Solka Floc, Avicel, and cotton are good cellulose inducers, but expensive. To keep costs down, it is therefore important to use a less expensive substrate. Many cellulosic materials such as wood (5 and 19), wastepaper (9), bagasse (17 and 18), wheat straw (10 and 21), corncob (32), wheat bran (22), and

* Assistant Professor and Graduate Student, respectively
** Corresponding Author

fruit pomace (8 and 25) have been studied as potential substrates for the production of cellulase. There is, however, a lack of investigation on the cellulase production from SBP cellulosic materials. The aim of the present work was to study the potential of using SBP for cellulolytic enzyme production by the fungus *T. reesei*. This information would be useful for the development of a cost-effective process for cellulase production and subsequent enzymatic hydrolysis of SBP lignocellulose.

MATERIALS AND METHODS

Biomass material

Fresh sugar beet pulp was collected from the Marvdasht sugar factory. This pulp was obtained from extracted cossettes after diffusion and press, and was without formaline. SBP was dried at 60°C for 16 h. Dry pulp was milled and reduced to 60 meshes for uniformity of particle size. Pulp powders were stored at 25°C and kept away from light and moisture. This pulp powders were used for physical and chemical analyses.

Analysis of biomass components

Chemical analysis of biomass (SBP) was performed. Fat content was determined by soxhlet method using chloroform and methanol as solvents (28). Protein content was determined by the microKjeldahl method and with a conversion factor of 6.25 (15). Moisture content (2) and ash (13) were also determined for SBP.

Cellulose measurement of SBP

A biomass of 2 g was transferred into a 250 ml Erlenmeyer flask. One hundred ml of 96% ethyl alcohol and 50 ml of 65% nitric acid were added. The flask was put on a heater equipped with a condenser and heated for 1 h. After hydrolysis, flask contents were filtered. Once more, remaining cellulose on the filter paper was transferred into the flask. This step was repeated similarly to the previous stage. This process was repeated for the third time and then cellulose together with the filter paper were dried at 102°C. The cellulose content was calculated from the following equation (16 and 20):

$$\% \text{ Cellulose} = \frac{\text{Cellulose dry weight}}{\text{Sample dry weight}} \times 100$$

Microorganism, medium and culture conditions

Fungal strain of *T. reesei* (PTCC 5142) was used in this study. The fungi were transferred into Nutrient broth and incubated at 30°C for activation, cultured in PDA (Potato Dextrose Agar) plates and incubated at 30°C to form yellow colonies. Then, the fungi were maintained in the above mentioned medium at 4°C. The salt solution was prepared in a 500 ml Erlenmeyer flask containing 100 ml of medium (30). The subculture medium was a salt solution with 2 ml/L tween-80, 1 g/L peptone, and 10 g/L glucose added (Table 1). The initial pH of the medium was adjusted to 4.8 before being autoclaved at 121°C for 15 min. Fungal cells were sub-cultured in an orbital shaker (180 rpm) at 30°C for 1-2 generations (two days for each generation) and were then used as inoculum.

Fifteen milliliters (15 ml) of exponential cells were inoculated into 100 ml of medium containing SBP as a substrate. Pure crystalline cellulose was also used as a substrate, simultaneously. In this study, both SBP and pure crystalline cellulose were used and both concentrations were adjusted to 10 g/L (dry basis). The medium composition was the same as the subculture medium (Table 1), except that peptone was eliminated and glucose was replaced with SBP or pure crystalline cellulose.

Table 1. Medium composition for subculturing and cellulase production from the fungus *T. reesei*

Components	Unit	Concentration	
		Subculture	Cellulase production
Salt solution			
KH_2PO_4	g/L	2.0	2.0
$CaCl_2.2H_2O$	g/L	0.4	0.4
$MgSO_4.7H_2O$	g/L	0.3	0.3
$(NH_4)_2SO_4$	g/L	1.4	1.4
Urea	g/L	0.3	0.3
Trace elements			
$FeSO_4.7H_2O$	mg/L	5.0	5.0
$MnSO_4.H_2O$	mg/L	1.6	1.6
$ZnSO_4.7H_2O$	mg/L	1.4	1.4
$CoCl_2$	mg/L	2.0	2.0
Tween-80	ml/L	2.0	2.0
Peptone	g/L	2.0	-
Glucose	g/L	1.0	-
Sugarbeet pulp (DM) †	g/L	10	10

†Dry Matter

Enzyme activity

The activity of total cellulase (filter paper activity, FPA) was determined according to the standard IUPAC procedure and expressed as an international unit (IU). Each unit of FPA activity was defined as the amount of enzyme which releases 1 μmol of glucose equivalents from Whatman No. 1 filter paper in 1 min (7).

Acid pretreatment and enzymatic saccharification

In order to inspect the saccharification effect of the produced enzyme on cellulose substrate, SBP was used. Lignocellulosic biomass is composed of carbohydrate polymers (cellulose, hemicelluloses and lignin). The combination of hemicellulose and lignin provides a protective sheath around the cellulose, which must be modified or removed before any efficient hydrolysis of cellulose can occur. Therefore, for cost-effective hydrolysis of cellulose, more advanced pretreatment technologies are required. For this reason, sugar beet pulp was pretreated with 1% H_2SO_4 at 120°C for 1 h. Biomass collected after pretreatment was separated by filtration into a solid residue and liquid. The solid residue was thoroughly washed with water and then dried. Pretreated and non- pretreated SBP were mixed separately with 50 mM sodium citrate buffer (pH 5.5). Cellulase enzyme was then added to the mixtures and they were incubated at 50°C for cellulose hydrolysis.

Determination and quantification of released sugars

In order to detect the released sugars after enzymatic hydrolysis, thin layer chromatography (TLC) was performed. A solvent consisting acetate, pyridine and water (55:25:20 by volume, respectively) was poured into the tank and the lid was replaced. TLC plastic sheets pre-coated with silica gel (sheet dimension: 20×20 cm and layer thickness: 0.2 mm) were used. One drop of each sugar solution (consisting 1% of raffinose, maltose, xylose, arabinose, fructose, galactose and glucose) and one drop of the unknown (pretreated and non-pretreated SBP after enzymatic hydrolysis) were applied to nine separated origins. After drying the spots, the sheet was placed into the tank covered with the lid. When running was finished, the sheet was removed from the tank and dried until it was free from the solvent. In order to detect the spots, freshly-prepared reagent (m-phenylene diamine, stannous chloride, acetic acid and ethanol) was sprayed on the sheet and placed in the oven at 110ºC for 10 min (31). The amount of released sugars from non-treated SBP and pretreated SBP influenced by the cellulase enzyme were determined using AOAC method (2).

Statistical analysis

All data were statistically analyzed by independent and paired T tests and ANOVAs using the SPSS/PC software and the mean comparison was performed using DMRT ($p<0.05$).

RESULTS AND DISCUSSION

Biomass characterization

The chemical characteristics of sugar beet pulp are given in Table 2. SBP contained 97.12% dry matter after drying, 25.55% of which was cellulose, a suitable percentage for microbial cellulase production. The amounts of other ingredients of SBP consisting of fat, protein and ash indicate that in terms of nutrition, SBP is a relatively worthless material and the use of this biomass reduces the production cost.

Table 2. Major composition of SBP[a]

Ingredient	% ± SD
Fat	1.50 ± 0.70
Protein	5.95 ± 0.35
Moisture	2.88 ± 0.10
Ash	4.00 ± 0.50
Cellulose	25.55 ± 1.76

[a]**Data is expressed as mean ± SD of three replicate samples**

Amount and type of sugars released from SBP after enzymatic saccharification

The results of thin layer chromatography (TLC) including the R_f values of unknown samples and the comparison of the R_f of each sugar revealed that the released sugar of pretreated SBP affected by cellulase enzyme was only glucose, but no sugar was detected from the SBP with no cellulose added. The amount of released sugar from pretreated SBP affected by cellulase enzyme was 3% against < 0.1% in SBP without cellulase.

Possibility of using SBP as a substrate for cellulase production

Sugar beet pulp has cellulosic components that can induce the production of cellulase when used as a carbon source for fungus growth. *T. reesei* has been the most extensively studied, with the mutants *T. reesei* RU-T C30 and *T. reesei* QM 9414 having been identified as possessing improved filter paper activity (4, 6, 9, 19, and 22). In this study, *T. reesei* was grown in a medium containing SBP as a substrate. Both SBP and pure crystalline cellulose were used.

It was found that this fungus could produce cellulase in medium containing SBP or pure crystalline cellulose (Fig. 1A). The pure crystalline cellulose resulted in a lower FPA than the SBP indicating that an increase in cellulose concentration causes a decrease in cellulase activity. The pattern of cellulase production indicates that the cellulase activity increased during the first four to five days, reached the maximum on day 5 and then decreased at the end of cultivation (Fig. 1B).

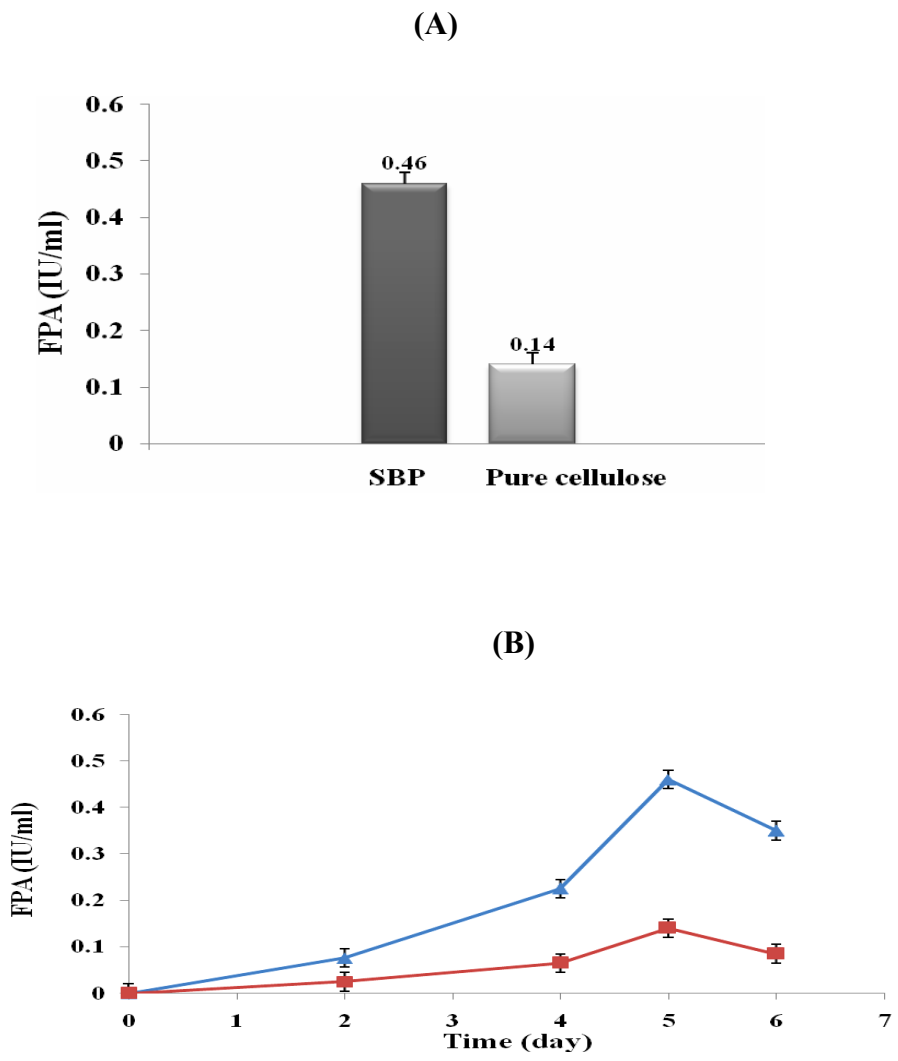

Fig 1. Cellulase production by *T. reesei* using SBP and pure crystalline cellulose as substrates. (A) Filter paper activity after five days of cultivation in medium containing SBP and pure crystalline cellulose. (B) Time course of FPA in medium containing, ▲SBP; ■ pure crystalline cellulose. Data are means of three replicates and error bars show standard deviation.

Mutants of *T. reesei* produce higher cellulase activity. Cellulose production by the fungus *T. reesei* was studied using dairy manure as a substrate by Wen et al. (30). Data showed that the mutant of *T. reesei* RUT- C30 had higher cellulose production than *T. reesei* QM 9414. The maximum cellulose production activity of 1.74 IU/ml of filter paper activity was obtained by *T. reesei* RUT-C30.

Membrillo *et al*. used two strains of *Pleurotus ostreatus* (IE-8 and CP-50) for the production of lignocellulolytic enzymes. Mycelia from these cultures were used as an inoculum for solid fermentation using sugar cane bagasse. Strain IE-8 produced the highest level of cellulase (0.18 IU/g dry wt on smallest particles of substrate) (13).

Ahamed and Vermette investigated production of cellulase by co-culturing *Trichoderma reesei* and *Aspergillus niger* in a bioreactor to convert cellulose substrate into soluble sugars through a synergetic action of enzyme complex simultaneously produced by these two fungi. The results of mixed culture experiments exhibited a highly significant increase in the production of filter paper activity (7.1 U mL^{-1}) (1).

Sun et al. used *Trichoderma reesei* Rut C-30 for production of cellulase. The use of alkaline-treated rice straw sticks and non-pretreated rice straw powder as the inducing substrates in the batch culture could result in cellulase activities of 1.07 and 0.71 FPU/ml, respectively (23).

Thus, the results of cellulase production and its activity in this research are comparable to values reported in the literature on the use of other cellulose sources as inducers (Table 3). Since the produced enzyme from SBP had a relatively high enzyme activity and this substrate is abundant in the waste of sugar factories, sugar beet pulp can be used as a suitable substrate for cellulase enzyme production especially in Iran.

Table 3. Comparison of cellulase production by different fungal species and substrates

Fungal species	Substrate	PFU (IU/ml)	Reference
Lignocellulosic substrate			
***Trichoderma reesei* RUT C30**	**Steam-treated willow**	**0.66**	**Reczey et al. (19)**
***Trichoderma reesei* RUT C30**	**Steam-treated willow**	**1.55**	**Szengyel & Zacchi (24)**
***Trichoderma reesei* RUT C30**	**Wastepaper**	**0.30**	**Ju & Afolabi (9)**
***Trichoderma reesei* RUT C30**	**Dairy manure**	**1.72**	**Wen et al. (29)**
Chaetomium globosum	**Oil palm fruit fiber**	**0.95**	**Umikalsom et al. (25)**
***Neurospora crassa* 4335 (cell-1)**	**Wheat straw**	**1.33**	**Romero et al. (21)**
***Scytalidium thermophilum* 3-A**	**Apple pomace**	**0.39**	**Ogel et al. (17)**
***Scytalidium thermophilum* 3-A**	**Lentil bran**	**0.23**	**Ogel et al. (17)**
***Scytalidium thermophilum* 3-A**	**Bagasse**	**0.21**	**Ogel et al. (17)**
Trichoderma reesei	**Sugar beet pulp**	**0.46**	**This Study**
Pure cellulose or reducing sugar			
***Trichoderma reesei* QM 9414**	**Acid-swollen cellulose**	**0.54**	**Gadgil et al. (6)**
***Trichoderma reesei* RUT NG14**	**Acid-swollen cellulose**	**15**	**Montenecourt & Eveleigh (14)**
***Trichoderma reesei* RUT C30**	**Acid-swollen cellulose**	**15**	**Montenecourt & Eveleigh (14)**
***Trichoderma reesei* RUT C30**	**Solka floc (cellulose)**	**4.65**	**Velkovska et al. (26)**
***Trichoderma reesei* RUT C30**	**Solka floc (cellulose)**	**2.10**	**Domingues et al. (4)**
***Trichoderma reesei* RUT C30**	**Lactose**	**1.30**	**Domingues et al. (4)**
***Trichoderma viride* QM 6a**	**Solka floc (cellulose)** †	**3.3**	**Mandels & Weber (11)**

†**0.2% peptone was added to the culture medium**

CONCLUSIONS

The present work showed that sugar beet pulp (SBP) was a suitable substrate for cellulase production by *T. reesei*. With the optimal culture condition and SBP as a suitable source of cellulose content, filter paper activity achived 0.46 IU/ml, which is much higher than the results, obtained using pure crystalline cellulose and other lignocellulosics residue, although wild type of *T. reesei* with low cellulase activity was used. Further work is needed to find out how to enhance cellulase activity.

ACKNOWLEDGEMENT

The authors would like to thank the Shiraz University Entrepreneurship Centre for the financial support of this project.

REFERENCES

1. Ahamed, A., and P. Vermette. 2008. Enhanced enzyme production from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* LMA grown as fed batch in a stirred tank bioreactor. Biochem.Eng. 42: 41-46.
2. AOAC. 2002. Official Methods of Analysis. 17th edition. Association of Official Agricultural Chemists, Incoporated. Washington, D.C. USA.
3. Colberg, P.J. 1988. Anaerobic microbial degradation of cellulose, lignin, oligonols, and monoaromatic lignin derivatives. ***In***: Zehnder, A.J.B. **(*ed.*)**, Biology of Anaerobic Microorganisms. John Wiley and Sons, New York, 333-372.
4. Domigues, F. C., J. A. Queiroz, J. M. C. Cabral and L. P. Fonseca. 2000. The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30. Enzyme Microb. Tnolech. 26: 394-401.
5. Duff, S. J. B., and W. D. Murray. 1996. Bioconversion of forest products industry waste cellulosics of fuel ethanol: a review. Bioresour. Technol. 55: 1-33.
6. Gadgil, N. J., H. F. Daginawala, T. Chakrabarti and P. Khanna. 1995. Enhanced cellulase production by a mutant of *Trichoderma reesei.* Enzyme Microb. Technol. 17: 942-946.
7. Ghose, T. K. 1987. Measurement of cellulase activities. Pure Appl. Chem. 59: 257-268.
8. Haddadin, M. S. Y., I. M. Abu-Reesh, F. A. S. Haddadin and R. K. Robinson. 2001. Utilisation of tomato pomace as a substrate for the production of vitamin B12- a preliminary appraisal. Bioresour. Technol. 78: 225-230.
9. Ju, L. K., and O. A. Afolabi. 1999. Wastepaper hydrolysate as soluble inducing substrate for cellulase production in continuous culture of *Trichoderma reesei*. Biotechnol. Prog. 15: 91-97.
10. Kalogerist, E., P. Christakopoulos, P. Katapodis, A. Alexiou, S. Vlachou, D. Kekos and B. J. Macris. 2003. Production and characterization of cellulolytic enzymes from the thermophilic fungus *Thermoascus*

aurantiacus under solid state cultivation of agricultural wastes. Process Biochem. 38: 1099-1104.

11. Mandels, M., and J. Weber. 1969. The production of cellulases. Adv. Chem. Ser. 95: 391-414.

12. Mandels, M., J. Weber and R. Parizek. 1971. Enhanced cellulase production by a mutant of *Trichoderma viride*. Appl. Microbiol. 21: 152-154.

13. Membrillo, I., C. Sánchez, M. Meneses, E. Favela and O. Loera. 2008. Effect of substrate particle size and additional nitrogen source on production of lignocellulolytic enzymes by *Pleurotus ostreatus* strains. Biorec. Technol. 99: 7842-7847.

14. Montenecourt, B. S., and D. E. Eveleigh. 1979. Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei*. Adv. Chem. Ser. 181: 289-301.

15. Morris, B., and S. Jacob. 1965. Chemical Analysis of Food and Food Products. 3rd ed. D-van nostrand Company Inc. New Jersey. 30-31.

16. Oakley, E. T. 1984. Determination of cellulose index of tobacco. Am. Chem. Soc. 32: 1192-1194.

17. Ogel, Z. B., K. Yarangumeli, H. Dundar and I. Ifrij. 2001. Submerged cultivation of *Schatalidium thermophilium* on complex lignocellulosic biomass for endoglucanase production. Enzyme Microb. Technol. 28: 689-695.

18. Rajoka, M. I., and K. A. Malik. 1997. Cellulase production by Cellulomonas bioazotea cultured in medium containing different cellulosic substrate. Bioresour. Technol. 59: 21-27.

19. Reczey, K., Z. Szengyel, R. Eklund and G. Zacchi. 1996. Cellulase production by *T. reesei*. Bioresour. Technol. 57: 25-30.

20. Ritter, H., and L. C. Fleck. 1924. Determination of cellulose in wood. Ind. Eng. Chem. 16: 147-148.

21. Romero, M. D., J. Aguado, L. Gonzalez and M. Ladero. 1999. Cellulase production by *Neurospora crassa* on wheat straw. Enzyme Microb. Technol. 25: 244-250.

22. Smits, J. P., A. Rinzema, J. Tramper, H. M. van Sonsbeek and W. Knol. 1996. Solid-state fermentation of wheat bran by *Trichoderma reesei* QM9414: substrate composition changes, C balance enzyme production, growth and kinetics. Appl. Microbiol. Biotechnol. 46: 489-496.

23. Sun, W. C., C. H. Cheng and W. C. Lee. 2008. Protein expression and enzymatic activity of cellulases produced by *Trichoderma reesei* Rut C-30 on rice straw. Metabolic. Eng. 43: 1083-1087.

24. Szengyel, Z., and G. Zacchi. 2000. Effect of acetic acid and furfural on cellulose production of *Trichoderma reesei* RUT C30. Appl. Biochem. Biotechnol. 89: 31-42.

25. Umikalsom, M. S., A. B. Arrif, Z. H. Shamasuddin, C. C. Tong, M. A. Hassan and M. I. A. Karim. 1997. Production of cellulase by a wild strain of Chaetomium globosum using delignified oil palm empty fruit-bunch fiber as substrate. Appl. Microbiol. Biotechnol. 47: 590-595.

26. Velkovska, S., M. R. Marten and D. F. Ollis. 1997. Kinetic model for batch cellulase production by *Trichoderma reesei* RUT C30. J. Biotechnol. 54: 83-94.

27. Von Sivers, M., and G. Zacchi. 1995. A techno-economical comparison of three processes for the production of ethanol. Biorec. Technol. 51: 43-52.

28. Wen, L. F., K. C. Chang and D. D. Gallaher. 1998. Isolation and characterization of hemicellulose and cellulose from sugar beet pulp. J. Food Sci. 53: 826-829.

29. Wen, Z., W. Liao and S. Chen. 2004. Hydrolysis of animal manure lignocellulosic for reducing sugar production. Bioresour. Technol. 91: 31-39.

30. Wen, Z., W. Liao and Sh. Chen. 2005. Production of cellulase by *Trichoderma reesei* from dairy manure. Bioresour. Technol. 96: 491-499.

31. White, B. J., and J. F. Robyt. 1988. Simultaneous determination of pentoses and hexoses from a hydrolysed gum (polysaccharide). Chem. Ed. 65: 164-166.

32. Xia, L., and P. Cen. 1999. Cellulase production by solid state fermentation on lignocellulosic waste from the xylose industry. Process Biochem. 34: 909-912.

تولید آنزیم سلولاز توسط قارچ تریکودرما ریزیی با استفاده از تفاله چغندر قند

مرضیه موسوی نسب[۱] و مرجان مجدی نسب[۱]*

[۱] بخش علوم و صنایع غذایی، دانشکده کشاورزی، دانشگاه شیراز، شیراز، جمهوری اسلامی ایران

چکیده- در این پژوهش تولید سلولاز توسط قارچ تریکودرما ریزیی با استفاده از تفاله چغندر قند به عنوان سوبسترا، مورد بررسی قرار گرفت. محیط کشت یک محلول نمکی شامل KH_2PO_4 ، $CaCl_2$ و ترکیبات دیگر بود. سلول های قارچ در شیکر چرخشی (۱۸۰ دور در دقیقه) با دمای 30^oC به مدت ۱ تا ۲ دوره (هر دوره دو روز) کشت داده شده و سپس به عنوان مایه تلقیح استفاده شدند. سلول های رشد یافته به محیطی که محتوی سوبسترای تفاله چغندر قند بود تلقیح گردیدند. نتایج نشان داد که تفاله چغندر قند می تواند به عنوان سوبسترا برای تولید آنزیم سلولاز استفاده شود. تاثیر غلظت سلولز بر تولید سلولاز بررسی شد. با استفاده از ترکیبات محیط (مخلوطی از نمک های شیمیایی و سوبسترای تفاله چغندر قند) و مدت زمان تخمیر ٦-٤ روز، بیشترین فعالیت آنزیمی سلولاز ۰/٤٦ IU/ml بود که با استفاده از روش فعالیت کاغذ صافی بدست آمد.

واژه های کلیدی: سلولاز، تفاله چغندر قند، تریکودرما ریزیی

* به ترتیب استادیار و دانشجوی کارشناسی ارشد

** مکاتبه کننده