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Production of β-Glucosidase in Mixed Culture of *Aspergillus niger* BKMF 1305 and *Trichoderma reesei* RUT C30

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Summary

The aim of the present study was to investigate a new approach to β-glucosidase production of an *Aspergillus* strain using cheap lignocellulosic material *i.e.* waste paper in order to substitute glucose, a generally used carbon source, and thereby reduce the production cost. The enzyme production was performed and optimized for the highest β-glucosidase yield in cofermentation with a *Trichoderma* strain to support the degradation of cellulose and to provide the non-cellulolytic *Aspergillus* with water soluble carbon source. Batch fermentation experiments of *Aspergillus niger* BKMF 1305 and *Trichoderma reesei* RUT C30 were carried out in shake flask cultures. The factors influencing the enzyme production, such as the concentrations of nutrients and carbon source, the inoculum ratio of the two species, and the delay in *A. niger* inoculation were investigated using a 2^3 full factorial design. The results were analyzed with the response surface methodology using commercially available software, Statistica for Windows. All three examined factors were found significant. The highest β-glucosidase activity of 3.07 IU/mL was obtained after 7 days of incubation, if 3.3 % *Aspergillus* and 6.7 % *Trichoderma* inoculum were added at the same time to modified Mandels' medium, in which the concentration of nutrients was doubled compared to normal Mandels' medium and the carbon source concentration was set to 20 g/L waste paper.

Key words: Aspergillus niger, *Trichoderma reesei*, β-glucosidase production, cofermentation

Introduction

For the complete hydrolysis of cellulose, cellulolytic microorganisms produce a whole set of enzymes *i.e.* cellulases, which act synergistically in the degradation process. There are three enzyme groups involved in the breakdown of cellulose to glucose. These are the endoglucanases, exoglucanases or cellobiohydrolases (CBH) and β-glucosidase. Strictly speaking, β-glucosidase enzyme is not a cellulase since this enzyme does not act on water insoluble cellulose, however it shares a common feature with the endo- and exoglucanases, namely the specificity towards β-1,4-glucosidic bonds.

In most of the cellulases produced by cellulolytic fungi, such as in *Trichoderma* cellulases, the ammount of β-glucosidase is lower than needed for the efficient cellulose to glucose hydrolysis, therefore the main product of the hydrolysis is cellobiose (*1*). Cellobiose is a strong inhibitor of endo- and exoglucanases, and the accumulation of cellobiose will slow down the hydrolysis process significantly (*2*). By addition of β-glucosidase to cellulases from external sources, the problem can be solved *i.e.* the inhibitory effect of cellobiose is ceased by the conversion to glucose (*3*). Furthermore, glucose is better car-

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bon source than cellobiose for other microorganisms, for instance for ethanol fermenting baker's yeast.

One of the potential applications of cellulases and β-glucosidase in the future will be the production of fuel ethanol from lignocellulosic biomass (*4*), which is a good substitute for gasoline in internal combustion engines. The most promising technology for conversion of lignocellulosic biomass to fuel ethanol is based on the enzymatic breakdown of cellulose utilizing cellulase enzymes. After enzymatic hydrolysis, the water soluble sugars are converted to ethanol using yeasts. β-Glucosidase has several applications in food technology as well. It can degrade the most common pigments *i.e.* anthocyanins into sugars and anthocyanidins. Thus the enzyme is appropriate for decolorization of different drinks and can be used in the processing of orange juices, rosé and white wines (*5*).

Aspergillus strains are known for their ability to produce β-glucosidase with significantly higher yields than *Trichoderma* species. Cofermentations of *Aspergillus* and *Trichoderma* species were examined by Duff *et al*. on starch (*6*) and starch-cellulose mixture (*7*). The highest β-glucosidase activity obtained was 2.9 IU/mL while the enzyme mixture had a cellulolytic activity of 3.0 FPU/mL. Maheshwari *et al*. (*8*) investigated the cofermentation of the two species on cellulosic waste. The enzyme complex derived from the cofermentational broth had two or three times higher cellulose hydrolysis potential than the enzyme derived from monocultures. Madamwar *et al*. (*9*) performed semi-solid state cofermentations of the two fungal species and obtained 20–40 % higher enzyme activities with mixed cultures than with monocultures. The interactions between two different species can be exploited in other applications as well, such as protecting plants from plant pathogenic fungi (*10–12*).

In the present study β-glucosidase production of *Aspergillus niger* BKMF 1305 was examined in mixed culture together with *Trichoderma reesei* RUT C30, with great emphasis on the β-glucosidase production, in order to substitute glucose, a typically used carbon source for *Aspergillus niger*, with cheap lignocellulosic carbon source *i.e.* waste paper. The experiments were designed in such a way that the cellulase production of *Trichoderma reesei* RUT C30 was kept at a low level, just enough for the breakdown of the cellulosic carbon source, thereby supporting the β-glucosidase production of *A. niger*. A 2^3 full factorial design was employed to optimize the process in shake flasks. The results were analyzed using a commercially available software, Statistica for Windows.

Materials and Methods

Strains and inoculum preparation

Aspergillus niger BKMF 1305 and *Trichoderma reesei* RUT C30 were obtained from the strain collection of Department of Agricultural Chemical Technology, Budapest University of Technology and Economics. Stock cultures were maintained on agar slants containing: 20 g/L malt extract, 5 g/L glucose, 1 g/L peptone and 20 g/L agar. After 14 days at 30 °C the conidia were suspended in 5 mL of sterile water and 2 mL of this spore suspension were transferred aseptically to 750 mL Erlenmeyer flask containing 150 mL of the sterile and pH adjusted (pH=5.5) medium prepared according to Mandels (*13*), in which the mass concentrations of nutrients were: urea 0.3 g/L, $(NH_4)_2SO_4$ 1.4 g/L, KH_2PO_4 2.0 g/L, $CaCl_2$ 0.3 g/L, $MgSO_4$ 0.3 g/L, yeast extract 0.25 g/L, proteose peptone 0.75 g/L together with either 7.5 g/L Solka Floc cellulose powder for *Trichoderma reesei* RUT C30 cultivation or 10 g/L glucose for *Aspergillus niger* BKMF 1305. The medium was also supplemented with the following trace elements: $FeSO_4 \cdot 7H_2O$ 5 mg/L, $CoCl_2$ 20 mg/L, $MnSO_4$ 1.6 mg/L, $ZnSO_4$ 1.4 mg/L. After 3–4 days at 30 °C and 350 rpm the inoculum was ready.

Enzyme production in shake flasks

A total volume of 15 mL mycelium suspension, obtained from the inoculum cultures, was used to initiate growth in a 750 mL Erlenmeyer flask containing 150 mL of modified Mandels' medium at pH=5.5, in which the carbon source was 10 g/L waste paper instead of Solka Floc. Each time *Trichoderma reesei* inoculum was added in a volume of 5, 7.5 or 10 mL, depending on the designed inoculum ratio. Then the inoculum of *Aspergillus niger* was added after certain inoculation times in different volumes in order to set the volumetric inoculum ratio (R, volume of *Aspergillus* inoculum / volume of *Trichoderma* inoculum) to 1:2, 1:1, or 2:1. After the inoculation E. flasks were incubated in an orbitary shaker at 30 °C and 350 rpm for 7 days. Samples were withdrawn every day and at the same time pH was adjusted to 6.0 by addition of sterile NaOH or H_2SO_4 solutions. The samples were centrifuged at 8000 rpm for 5 min and the supernatants were collected for the analysis of β-glucosidase activities. In a set of experiments the concentrations of nutrients, as well as the carbon source, were doubled and the same experimental setup was performed as described above.

Enzyme assay

The enzyme activity of samples was determined as β-glucosidase activity using Berghem's method (*14*). One milliliter of 5 mM *p*-nitrophenyl-β-D-glucopyranoside substrate was dissolved in citrate buffer (0.05 M, pH=4.8) and 0.1 mL sample was incubated at 50 °C for 10 min. The reaction was terminated by addition of 2 mL of 1 M sodium carbonate solution. After cooling down to room temperature, 10 mL of distilled water was added. The absorbance was measured at 400 nm. Standard curve was obtained using *p*-nitrophenol. The activity was calculated as IU/mL.

Experimental design

The effects of three factors on the β-glucosidase production were investigated in shake flask cultures using a 2^3 full factorial design (*15*). These factors were the concentration of nutrients and carbon source in the culture medium (M), the inoculum ratio of the two fungal species (R) and the delay in *A. niger* inoculation (D). The experimental setup is outlined in details in Table 1. Each experimental point was performed in triplicate and the average and standard deviations were calculated. The β-glucosidase production was also investigated in the

center point, where 5 parallel cultivation runs were done. Statistical analysis of the results was performed by fitting Equation 1 to the response data (Y), *i.e.* β-glucosidase activity. Multilinear regression was performed using Statistica for Windows.

$$Y = a_0 + (a_1 \cdot M) + (a_2 \cdot D) + (a_3 \cdot R) + (a_{12} \cdot M \cdot D) + (a_{13} \cdot M \cdot R) + (a_{23} \cdot D \cdot R) \qquad /1/$$

Results and Discussion

According to the experimental design (see Table 1) nine different conditions were run. Two additional experiments were performed with monocultures of the two examined fungal species as references (Table 1). As described above, for each condition the average values and standard deviations were calculated for the β-glucosidase activity obtained. Statistical analysis of the standard deviations calculated for each condition showed significant differences between the standard deviations *i.e.* the standard deviation was not constant. Therefore Box-Cox transformation (*16*) of the response data (Y), *i.e.* β-glucosidase activity was carried out according to Equation 2.

$$Y' = Y^{-1.9} \qquad /2/$$

The analysis of the effects of various factors on the β-glucosidase activity was performed with the transformed (Y′) response data. Since the Y′ is expressed with a reciprocal function, the lowest Y′ value means the highest actually reached β-glucosidase activity. To the transformed enzyme activity values (Y′) the surface described by Equation 1 was fitted. On the Pareto chart of standardized effects (see Fig. 1) it can be seen that all three factors had a significant influence on the transformed enzyme activity, therefore on the β-glucosidase activity as well. The analysis also showed that two of the interactions, *i.e.* delay in *A. niger* inoculation with inoculum ratio and the concentration of nutrients and carbon source together with the delay in *A. niger* inoculation, had significant effect on the amount of produced enzyme. Curvature check was also performed and as it can be seen in Fig. 1, the curvature was below 95 % confidence level, thus the model fitted was adequate.

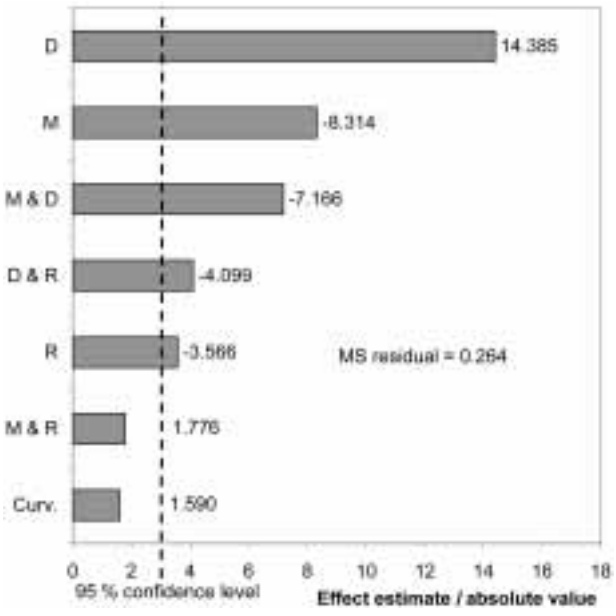

Fig. 1. Pareto chart of standardized effects of 2^3 full factorial design (Box-Cox transformated values)

In order to check the effect of inoculum ratio, experiments were done with varying inoculum ratios (data not shown). However, the data obtained did not confirm the results obtained in the factorial design. A possible explanation could be that the inoculum preparation is not reproducible enough for this kind of statistical analysis. In order to clear up this problem, the inoculum ratio has to be set by microbial biomass determination, which would be more reproducible than the volumetric inoculum ratio.

Table 1. The conditions and results of 2^3 full factorial design and monocultures (*) M: Relative ratios of nutritients and carbon source (1: normal Mandels' medium with 10 g/L waste paper, 2: doubled amounts of nutritients of Mandels' medium with 20 g/L waste paper) D: delay in *A. niger* inoculation, R: volumetric inoculum ratio (*Aspergillus / Trichoderma)*

M	D/h	R	E. activity IU/mL	E. activity IU/mL	Yield IU/g
			4th day	7th day	7th day
1	0	1:2	1.07	1.57	78.5
2	0	1:2	2.17	3.07	153.5
1	24	1:2	0.18	0.29	14.5
2	24	1:2	0.35	0.52	26.0
1	0	2:1	0.71	1.18	59.0
2	0	2:1	1.61	2.32	116.0
1	24	2:1	0.27	0.38	19.0
2	24	2:1	0.42	0.68	34.0
1.5	12	1:1	0.34	0.52	26.0
* 1	–	1:0	0.55	0.75	37.5
* 1	–	0:1	0.18	0.38	18.0

As it is shown in Table 1, the concentration of nutrients and carbon source had a positive significant effect on the β-glucosidase titer, which seems to be rather obvious. Pairwise comparison of the activities obtained at various conditions, where every factor but the concentration of nutrients and carbon source was the same, showed that although the higher enzyme activity was obtained at the higher concentration of carbon source, the enzyme yields based on the amount of carbon source were nearly the same.

Statistical analysis of the data showed that the delay in *A. niger* inoculation affects the β-glucosidase activity obtained in the cofermentation. As it is demonstrated in Fig. 1, this factor has a significant effect, which means that the highest enzyme activity can be reached when the inocula were added at the same time. In order to examine this effect, more experiments were carried out, where the volumetric inoculum ratio was set to 1:1, while the concentrations of nutrients and carbon source were doubled compared to the standard Mandels' medium.

The time of *A. niger* inoculum addition varied between 0 and 32 h. As a reference, the enzyme production was also examined in monocultures of the two fungal species. The results are summarized in Fig. 2. The highest β-glucosidase activity, obtained after 7 days of incubation, was reached when the inoculums were added together at the beginning of the fermentation. A slight difference in enzyme activity was observed when the inoculum of *A. niger* was added after 8 h of incubation. However, β-glucosidase activity was not significantly lower compared to the first case. Further increase of the time of *A. niger* addition significantly decreased the β-glucosidase activity obtained in fermentation.

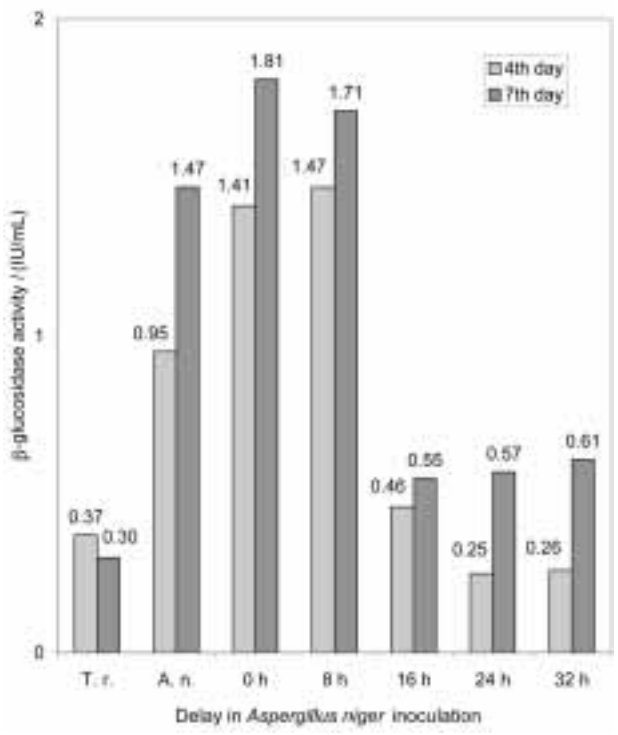

Fig. 2. The enzyme activities of monocultures and cocultures at different values of delay in *Aspergillus* inoculation, after 4 and 7 days of culturing (*T. r.*: monoculture of *Trichoderma reesei*, *A. n.* monoculture of *Aspergillus niger*)

Conclusions

A 2^3 full factorial design was carried out to investigate the effects of concentrations of nutrients, the inoculum ratio of the two species and the delay in *A. niger* inoculation on the β-glucosidase production in shake flask cultivations using *Trichoderma reesei* and *Aspergillus niger*. Statistical analysis showed that all three factors examined had a significant effect on the enzyme production. The concentrations of nutrients and carbon source had a positive effect on the β-glucosidase activity obtained after 7 days of incubation. However, after a closer analysis the comparison of enzyme yields based on added carbon source showed that there were no significant differences between those conditions, where only this factor was changed. The delay in *A. niger* inoculation also proved to be an important factor. Better results were obtained when the inocula of the two species were added together at the beginning of the fermentation. The effect of volumetric inoculum ratio seemed to be important, as well. However, further experiments (data not shown) could not confirm our findings, and more experiments are needed to increase the reproducibility of the inoculum preparation and clear up the effect of inoculum ratio on the enzyme production.

The highest β-glucosidase activity (3.07 IU/mL) was obtained using 20 g/L waste paper and resulted in a yield of 153.5 IU/g substrate, which was 25 % lower than that obtained with the same *Aspergillus* strain on 10 g/L glucose medium (*17*). However, this technique still has a potential for further improvement and by optimizing the process possibly better yields will be reached, using an economically more advantageous carbon source *i.e.* waste paper.

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References

1. B. C. Stockton, D. J. Mitchell, K. Grohmann, M. E. Himmel, *Biotechnol. Lett. 13* (1991) 57–62.
2. J. A. Howell, G. Stuck, *Biotechnol. Bioeng. 17* (1975) 873–893.
3. A. W. Khan, E. Meek, J. R. Henschel, *Enzyme Microb. Technol. 7* (1985) 465–467.
4. S. J. B. Duff, V. D. Murray, *Bioresource Technol. 55* (1996) 1–33.
5. A. Martino, P. G. Pifferi, G. Spagna, *J. Chem. Technol. Biotechnol. 60* (1994) 247–252.
6. S. J. B. Duff, *Enzyme Microb. Technol. 9* (1987) 47–51.
7. S. J. B. Duff, *Biotechnol. Lett. 7* (1985) 185–190.
8. D. K. Maheshwari, S. Gohade, J. Paul, A. Varma, *Carbohydr. Polym. 23* (1994) 161–163.
9. D. Madamwar, S. Patel, *World J. Microbiol. Biotechnol. 8* (1992) 183–186.
10. L. Kredics, Z. Antal, L. Manczinger, E. Nagy, *Lett. Appl. Microbiol. 33* (2001) 112–116.
11. L. Kredics, I. Dóczy, Z. Antal, L. Manczinger, *Bull. Environ. Contam. Toxicol. 66* (2001) 249–254.
12. L. Kredics, Z. Antal, L. Manczinger, A. Szekeres, F. Kevei, E. Nagy, *Food Technol. Biotechnol. 41* (2003) 37–42.
13. M. Mandels, J. Weber, *Adv. Chem. Ser. 95* (1969) 391–414.
14. L. E. R. Berghem, L. G. Petterson, *Eur. J. Biochem. 46* (1974) 295–305.
15. G. E. P. Box, J. S. Hunter, *Ann. Math. Stat. 28* (1957) 195–241.
16. G. E. P. Box, D. R. Cox, *J. Roy. Stat. Soc. B 26* (1964) 211–235.
17. A. Brumbauer, K. Réczey, *Acta Aliment. 28* (1999) 361–370.

Proizvodnja β-glukozidaze u mješovitoj kulturi *Aspergillus niger* BKMF 1305 i *Trichoderma reesei* RUT C30

Sažetak

Svrha je rada bila ispitati novi način proizvodnje β-glukozidaze u soju *Aspergillus* koristeći jeftini lignocelulozni materijal, tj. otpadni papir da bi se zamijenila glukoza, kao općeniti izvor ugljika i stoga smanjili troškovi proizvodnje. Proizvodnja enzima provedena je i optimirana na najveće iskorištenje glukozidaze kofermentacijom sa sojem *Trichoderma* kako bi se pojačala degradacija celuloze i omogućilo vrsti *Aspergillus*, koja nema celulolitičko svojstvo, da koristi u vodi topljivi izvor ugljika. Pokusi šaržne fermentacije *Aspergillus niger* BKMF 1305 i *Trichoderma reesei* RUT C30 provedeni su u posudama na tresilici. Faktori koji utječu na proizvodnju enzima, kao što su koncentracija hranjive podloge i izvora ugljika, odnos inokuluma upotrijebljenih dviju vrsta, a i vrijeme inokulacije (zajedno ili u različito vrijeme) ispitani su koristeći 2^3 puni faktorijalni plan. Dobiveni su rezultati analizirani metodologijom odzivne plohe koristeći računalnu podršku (Statistica for Windows). Sva tri ispitivana faktora bila su signifikantna. Najviša vrijednost aktivnosti β-glukozidaze od 3,07 IU/mL dobivena je nakon 7 dana inkubacije ako se istodobno dodao inokulum od 3,3 % vrste *Aspergillus* i 6,7 % vrste *Trichoderma* u modificiranu Mandelsovu podlogu, u kojoj je koncentracija hranjivih tvari udvostručena u usporedbi s normalnom Mandelsovom podlogom, a koncentracija izvora ugljika bila 20 g/L otpadnog papira.