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Note

Production of β-Glucosidase by a Transformant of *Aspergillus oryzae* RIB40 in a Liquid-Surface Immobilization (LSI) System

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The production of β-glucosidase by *Aspergillus oryzae* RIB40 and a transformant, RIB40-TF harboring the *bglA* gene was assessed in a liquid-surface immobilization (LSI) system that uses particles of a unique polymeric material, a ballooned microsphere. In a submerged cultivation system, RIB40-TF produced significantly more β-glucosidase than RIB40, although the enzyme was not secreted into the medium. However, when using the LSI system, RIB40-TF secreted β-glucosidase into the liquid medium to a level of 1.36 U/ml. Furthermore, we showed that the LSI system was capable of supporting repeated production of β-glucosidase at least 3 cycles over a period of 39 days.

Key words: xylanase, *Aspergillus*, liquid-surface cultivation, immobilization, microsphere

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) has recently attracted considerable attention for use in bioethanol production from cellulose, a linear polymer of D-glucose units linked by 1,4-β-D-glucosidic bonds. This enzyme hydrolyzes cellobiose to D-glucose to counteract the inhibition of *endo*-1,4-β-glucanase (EC 3.2.1.4) and cellobiohydrolase (EC 3.2.1.91) by cellobiose. Thus, a multicomponent enzyme system consisting of β-glucosidase, *endo*-1,4-β-glucanase, and cellobiohydrolase is necessary for the commercial production of bioethanol from cellulose (1, 2).

β-Glucosidase is produced by various fungi, such as *Trichoderma* (3), *Paecilomyces* (4), *Aureobasidium* (5),

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Alternaria (6), and *Aspergillus*. Two species that have been particularly targeted for the production and characterization of β-glucosidase are *Aspergillus niger* (7-10) and *Aspergillus oryzae* (11-13). The production of β-glucosidase by these fungi is commonly examined in submerged cultivation (SmC), although solid-state cultivation (SSC) has also been used (5, 12).

It is well known that particular cultivation methods influence the production and stability of enzymes. Compared to SmC, SSC generally enables high enzyme yield and enhances the stability of produced enzymes (14). For example, the production of tannase, pectinase, and polygalacturonase by *A. niger* in SSC is significantly higher than that in SmC (15, 16). The higher productivity with SSC results from various factors:, higher titers and productivities of enzymes, low levels of catabolite repression and proteolytic digestion, and increased stability of the secreted enzymes (14, 17). However, SSC has some practical disadvantages, such as poor heat dissipation and slow diffusion of nutrients, products, water, and oxygen in a packed bed (14, 18).

Recently, we presented 3 types of unique cultivation and application systems for fungi: liquid-surface immobilization (LSI) (19, 20), a liquid-liquid interface bioreactor (L-L IBR) (19, 21, 22), and the extractive liquid-surface immobilization (Ext-LSI) systems (23). Among these systems, LSI displayed enzyme productivity superior to SmC for lipase and xylanase. In the LSI system (Fig. 1), which consists of a liquid medium overlaid by a mat of fungus-microsphere (MS), fungal differentiation, sufficient nutrient, water and

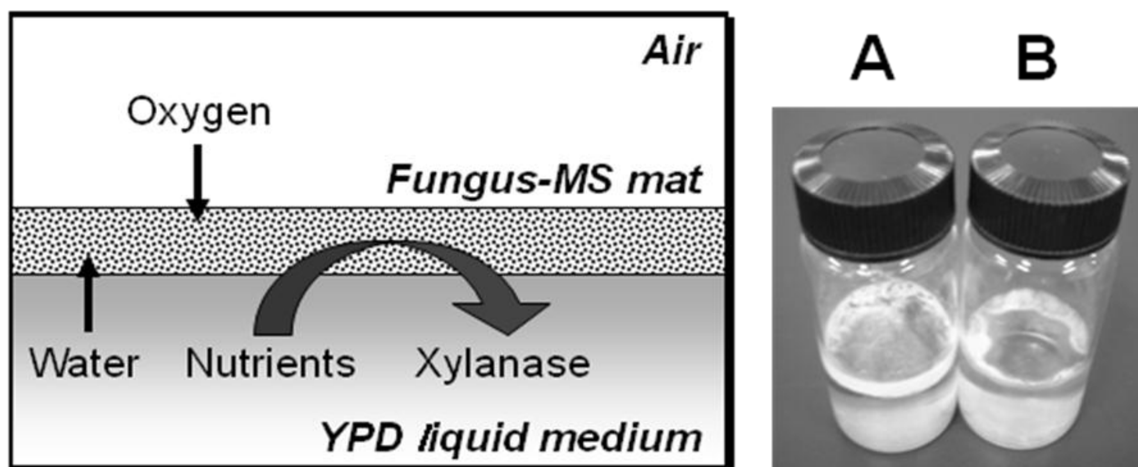

Fig. 1. Production of β-glucosidase in the liquid-surface immobilization (LSI) system. Spores of *A. oryzae* RIB40 (A) or its transformant RIB40-TF (B) were floated on the surface of YPD medium with ballooned microsphere (MS) particles. Cultivation in the thick fungus-MS mat used nutrients and water in the liquid medium, and oxygen in the atmosphere. The fungus-MS mat exhibited high yield of β-glucosidase.

oxygen supply, and higher enzyme production were achieved, despite the stationary conditions (19, 20). We expected that the LSI system would also be suitable for the production of other enzymes, such as other hydrolytic enzymes and peroxidases. Here, we report the application of the LSI system to the production of β-glucosidase an important enzyme in the production of bioethanol from cellulolytic materials with a *bglA* transformant of *A. oryzae* RIB40. The enzyme productivity was compared with that of SmC with *A. oryzae* RIB40; the transformant was shown to have a higher β-glucosidase production capability.

By analyzing an *A. oryzae* DNA microarray, the β-glucosidase gene (*bglA*: AO090009000356) was seen to be more highly expressed in the liquid culture medium of the rice bran (data not shown). An expression vector containing *bglA*, pNEN-BGLA (12.2 kb), was constructed. Plasmid pBGLA, which has a genomic fragment containing *bglA*, was used as a template for PCR with the following primers: primer 1 (5'-ACGCGTCGACGCGTATGAAGCTTGGTTGGATCGAGGTG-3'/-14-24 in *bglA*, 38-mer) which introduces a *Sal*I site (underlined) just upstream from the translational initiation codon of *A. oryzae celA*, and primer 2 (5'-ATAGTTTAGCGGCCGCATTCTTATTTACTGGGCCTTAGGCAG-3'/-2565-2610 in *bglA*, 48-mer) which includes a *Not*I site (underlined) downstream from the termination codon. Amplification was carried out by using LA Taq polymerase (Takara-bio Co., Kyoto) in a thermal cycler (30 s at 95 °C; 40 s at 55 °C; 60 s at 72 °C; 25 cycles). The 2627-bp PCR product was double digested with *Sal*I and *Not*I. Then, the fragment was cloned into the corresponding sites between P-*enoA*142 and T-*agdA*, a fungal high-level expression vector pNEN142 (9.3 kb) (24). P-*enoA*142 is the promoter from the *A. oryzae* enolase gene, improved by introducing 12 copies of "region III" that had previously been discovered to contain *cis*-acting sequence involved in the positive regulation of *A. oryzae* amylases, P-*enoA*, the promoter *A. oryzae* enolase gene.

The resulting plasmid, pNEN-BGLA, was introduced into *A. oryzae* niaD300 (*niaD*) according to a previously method (25). Transformants were selected based on the ability to grow on nitrate resulting from the introduction of the entire *niaD* gene. Recombinant β-glucosidase activity was measured in 50 mM sodium acetate buffer (pH 5.0) at 40 °C for 10 min with 1 mM *p*-nitrophenyl β-D-glucopyranoside. One unit of β-glucosidase activity in the supernatant, isolated by filtration (pore size, 0.45 μm), was defined as the amount of

enzyme that released 1 μmol of *p*-nitrophenol per min.

We then examined the β-glucosidase yield of the transformants. The transformant and the untransformed host were grown for 3 days in YPD medium (1% yeast extract, 2% peptone, 2% dextrose, pH 6.5), after which they were analyzed for β-glucosidase activity. The specific β-glucosidase activity, prepared from a cell extract of transformant RIB40-TF, was 130-fold higher than that of RIB40, and reached 1.03 U/ml in SmC. However, neither strains secreted significant β-glucosidase into the medium, similar to the situation reported for *Volvariella volvacea* (26) and *Trichoderma reesei* (27).

Next, RIB40 and RIB40-TF were applied to the LSI system for the production of β-glucosidase. Spore suspension (final concentration, 1 × 10^6 spores/ml) and 10 ml of ballooned polyacrylnitrile microspheres (MMS-DE-1 [former MFL-80SDE]: mean diameter, 40 μm; density, 0.06; Matsumoto Yushi-Seiyaku, Co., Ltd., Osaka) were mixed with 50 ml of YPD medium and poured into a polypropylene vessel (55 mm i.d. × 70 mm). After precultivation (30 °C, without shaking, 3 days), β-glucosidase accumulation in the YPD medium was determined over an 18-day period. As shown in Fig. 2A, although RIB40 accumulated little β-glucosidase in the medium, RIB40-TF secreted significant amounts of β-glucosidase (maximum 1.36 U/ml) in the medium up to day 11. β-Glucosidase specific activity of RIB40-TF was 667-fold

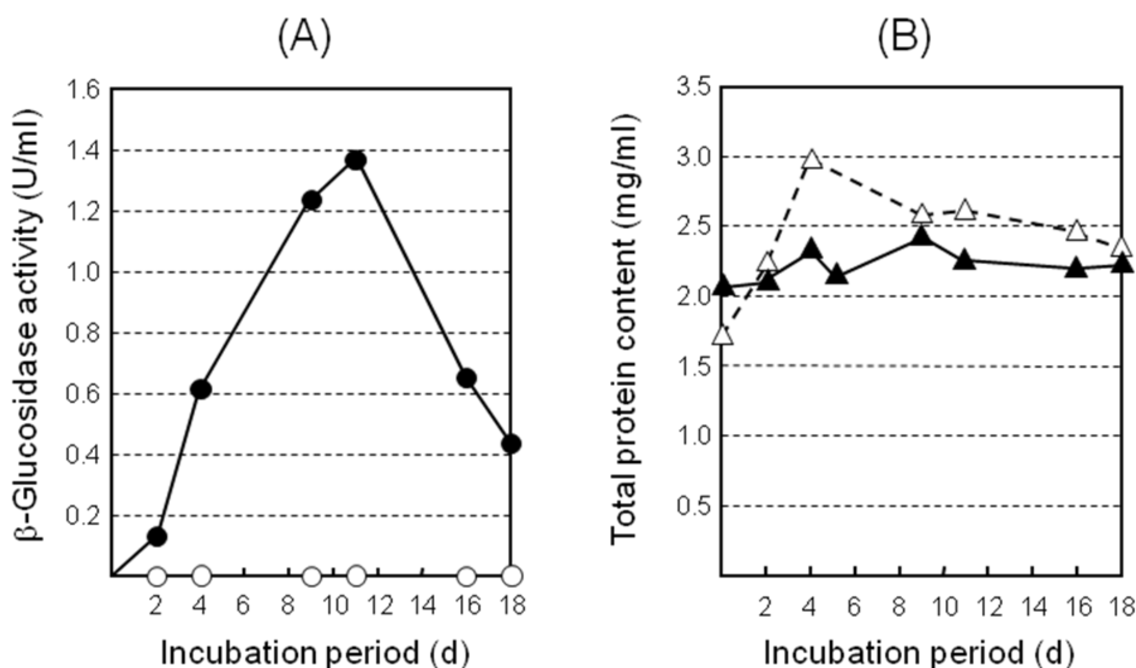

Fig. 2. Time course of β-glucosidase and total protein production by *A. oryzae* RIB40 and its transformant, RIB40-TF, in the LSI system. (A) Production of β-glucosidase; (B) production of total protein. Open circles/triangles: RIB40; closed circles/triangles: RIB40-TF.

higher than that of RIB40. However, β-glucosidase activity gradually reduced after this time. It is assumed that prolonged cultivation leads to digestion of β-glucosidase by endogenous proteases. The levels of other proteins in the medium were higher with RIB40 than with RIB40-TF (Fig. 2B). This might have resulted from titration of common regulatory proteins that interact with Region III (pNEN-BGLA) (28).

Finally, production of β-glucosidase with RIB40-TF was performed in an LSI system that used a 600 ml polypropylene vessel with inlet and outlet ports. A mixture of spore suspension (final concentration, 10^6 spores/ml), 40 ml of MMS-DE-1, and 300 ml of YPD medium was poured to the vessel. A magnet was put to a bottle in the vessel, and a polypropylene net was included to stabilize the fungus-MS mat. After precultivation (30 °C, without shaking, 3 days), β-glucosidase production in the medium was periodically determined. The medium was exchanged with fresh YPD medium (300 ml) at days 15 and 27. As shown in Fig. 3, repeated batch production of β-glucosidase was stably achieved 3 times over 39 days. Thus, it was demonstrated that *A. oryzae* RIB40-TF was stably maintained in the LSI system, and that it secreted active enzyme over this period.

The maximum specific activity of β-glucosidase produced by RIB40-TF was 1.36 U/ml-medium (Fig. 2A). By comparison, the β-glucosidase specific activities reported for *Aureobasidium pullulans* (5), *Alternaria alternata* (6), *Aspergillus terreus* (29), *Aspergillus niger* (30), and *Schizophyllum commune* (31) are 0.46, 2.5, 2.18, 8.5, and 22.2 U/ml-medium, respectively. Therefore, our β-glucosidase titer is currently insufficient for practical production purposes. It will be necessary to modify the media composition, culture condition, and strain to achieve competitive production rates.

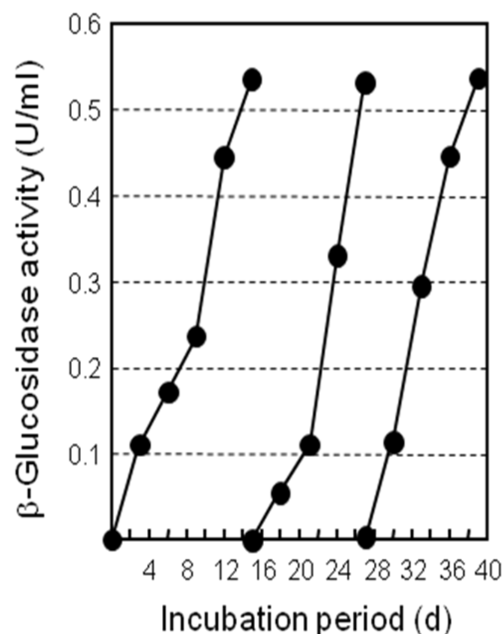

Fig. 3. Repeated batch production of β-glucosidase by *A. oryzae* RIB40-TF in the LSI system. At days 15 and 27, 300 ml of YPD medium was exchanged with fresh medium.

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REFERENCES

1. **Kadam, S. K. and Demain, A. L.**: Addition of cloned β-glucosidase enhances the degradation of crystalline cellulose by the *Clostridium thermocellum* cellulase complex. Biochem. Biophys. Res. Commun., **161**, 706-711 (1989).
2. **Pushalkar, S., Rao, K. K., and Menon, K.**: Production of β-glucosidase by *Aspergillus terreus*. Current Microbiol., **30**, 255-258 (1995).
3. **Herr, D.**: Secretion of cellulase and β-glucosidase by *Trichoderma viride* ITCC-1433 in submerged culture on different substrates. Biotechnol. Bioeng., **21**, 1361-1371 (1979).
4. **Qin, Y., Zhou, X. - W., Zhou, W., Li, X. - W., Fneg, M. - Q., and Zhou, P.**: Purification and properties of a novel β-glucosidase, hydrolyzing ginsenoside Rb1 to CK, from *Paecilomyces bainier*. J. Microbiol. Biotechnol., **18**, 1081-1089 (2008).
5. **Saha, B. C., Freer, S. N., and Bothast, R. J.**: Production, purification, and properties of a thermostable β-glucosidase from a color variant strain of *Aureobasidium pullulans*. Appl. Environ. Microbiol., **60**, 3774-3780 (1994).
6. **Macris, B. J.**: Production and characterization of cellulase and β-glucosidase from a mutant of *Alternaria alternata*. Appl. Environ. Microbiol., **47**, 560-565 (1984).
7. **Watanabe, T., Sato, T., Yoshioka, S., Koshijima, T., and Kuwahara, M.**: Purification and properties of *Aspergillus niger* β-glucosidase. Eur. J. Biochem., **209**, 651-659 (1992).
8. **Rashid, M. H. and Siddiqui, K.S.**: The stability of extracellular β-glucosidase from *Aspergillus niger* is significantly enhanced by non-covalently attached polysaccharides. Folia Microbiol., **41**, 341-346 (1996).
9. **Rashid, M. H. and Siddiqui, K. S.**: Purification and characterization of a β-glucosidase from *Aspergillus niger*. Folia Microbiol., **42**, 544-550 (1997).
10. **Xie, Y., Gao, Y., and Chen, Z.**: Purification and characterization of an extracellular β-glucosidase with high transglycosylation activity and stability from *Aspergillus niger* No. 5.1. Appl. Biochem. Biotechnol., **119**, 229-240 (2004).
11. **Riou, C., Salmon, J. -M., Vallier, M. -J., Günata, Z., and Barre, P.**:

Purification, characterization, and substrate specificity of a novel highly glucose-tolerant β-glucosidase from *Aspergillus oryzae*. Appl. Environ. Microbiol., **64**, 3607-3614 (1998).

12. **Yamane, Y., Fujita, J., Izuwa, S., Fukuchi, K., Shimizu, R., Hiyohsi, A., Fukuda, H., Mikami, S., Kizaki, Y., and Wakabayashi, S.**: Properties of cellulose-degrading enzymes from *Aspergillus oryzae* and their contribution to material utilization and alcohol yield in sake mash fermentation. J. Biosci. Bioeng., **93**, 479-484 (2002).
13. **Langston, J., Sheehy, N., and Xu, F.**: Substrate specificity of *Aspergillus oryzae* family 3 β-glucosidase. Biochim. Biophys. Acta, **1764**, 972-978 (2006).
14. **Hölker, U., Höfer, M., and Lenz, J.**: Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Appl. Microbiol. Biotechnol., **64**, 175-186 (2004).
15. **Aguilar, C. N., Augur, C., Favela-Torres, E., and Viniegra-González, G.**: Induction and repression patterns of fungal tannase in solid-state and submerged cultures. Process Biochem., **36**, 565-570 (2001).
16. **Maldonado, M. C., and Strasser de Saad, A. M.**: Production of pectinesterase and polygalacturonase by *Aspergillus niger* in submerged and solid state systems. J. Ind. Microbiol. Biotechnol., **20**, 34-38 (1998).
17. **Asther, M., Haon, M., Roussos, S., Record, E., Delattre, M., Lesage-Meessen, L., Labat, M., and Asther, M.**: Feruloyl esterase from *Aspergillus niger*: a comparison of the production in solid state and submerged fermentation. Process Biochem., **38**, 685-691 (2002).
18. **Viccini, G., Mitchell, D. A., Boit, S. D., Gern, J. C., de Rosa, A. S., Costa, R. M., Dalsenter, F. D. H., von Meien, O. F., and Krieger, N.**: Analysis of growth kinetic profiles in solid-state fermentation. Food Technol. Biotechnol., **39**, 271-294 (2001).
19. **Oda, S. and Isshiki, K.**: Liquid-surface immobilization system and liquid-liquid interface bioreactor: application to fungal hydrolysis. Process Biochem., **42**, 1553-1560 (2007).
20. **Ozeki, K., Takagi, Y., Oda, S., and Ohashi, S.**: Production of xylanase with a transformant of *Aspergillus oryzae* RIB40 in a liqud-surface immobilization (LSI) system. J. Biosci. Bioeng., **109**, 224-226 (2010).
21. **Oda, S. and Isshiki, K.**: Asymmetric reduction of benzil to (*S*)-benzoin with *Penicillium claviforme* IAM 7294 in a liquid-liquid interface bioreactor (L-L IBR). Biosci. Biotechnol. Biochem., **72**, 1364-1367 (2008).
22. **Oda, S., Isshiki, K., and Ohashi, S.**: Regio- and stereoselective subterminal

hydroxylations of *n*-decane by fungi in a liquid-liquid interface bioreactor (L-L IBR). Bull. Chem. Soc. Jpn., **82**, 105-109 (2009).

23. **Oda, S., Isshiki, K., and Ohashi, S.**: Production of 6-pentyl-α-pyrone with *Trichoderma atroviride* and its mutant in a novel extractive liquid-surface immobilization (Ext-LSI) system. Process Biochem., **44**, 625-630 (2009).
24. **Tsuboi, H., Koda, A., Toda, T., Minetoki, T., Hirotsune, M., and Machida, M.:** Improvement of the *Aspergillus oryzae* enolase promoter (P-*enoA*) by the intoroduction of *cis*-element repeats. Biosci. Biotechnol. Biochem., **69**, 206-208 (2005).
25. **Ozeki, K., Kanda, A., Hamachi, M., and Nunokawa, Y.:** Construction of a promoter probe vector autonomously maintained in *Aspergillus* and characterization of promoter regions derived from *A. niger* and *A. oryzae* genomes. Biosci. Biotechnol. Biochem., **60**, 383-389 (1996).
26. **Cai, Y.J., Chapman, S. J., Buswell, J. A., and Chang, S. -T.**: Production and distribution of endoglucanase, cellobiohydrolase, and β-glucosidase components of the cellulolytic system of *Volvariella volvacea*, the edible straw mushroom. Appl. Environ. Microbiol., **65**, 553-559 (1999).
27. **Messner, R., Hagspiel, K., and Kubicek, C. P.**: Isolation of a β-glucosidase binding and activating polysaccharide from cell walls of *Trichoderma reesei*. Arch. Microbiol., **150**, 150-155 (1990).
28. **Minetoki, T., Tsuboi, H., Koda, A., and Ozeki, K.:** Development of high expression system with the improved promoter using the *cis*-acting element in *Aspergillus* species. J. Biol. Macromol., **3**, 89-96 (2003).
29. **Pushalkar, S., Rao, K. K., and Menon, K.**: Production of β-glucosidase by *Aspergillus terreus*. Curr. Microbiol., **30**, 255-258 (1995).
30. **García-Kirchner, O., Segura-Granados, M., and Rodríguez-Pascual, P.**: Effect of media composition and growth conditions on production of β-glucosidase by *Aspergillus niger* C-6. Appl. Biochem. Biotechnol., **121**, 347-359 (2005).
31. **Desrochers, M., Jurasek, L., and Paice, M. G.**: High production of β-glucosidase in *Schizophyllum commune*: isolation of the enzyme and effect of the culture filtrate on cellulose hydrolysis. Appl. Environ. Microbiol., **41**, 222-228 (1981).

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