Effects of Morphology and Rheology on Neo-fructosyltransferase Production by *Penicillium citrinum*

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Abstract In this study, we investigated the relationship between the morphology and the rheological properties of *Penicillium citrinum* to improve the production of neo-fructosyltransferase (neo-FTase). In a 2.5 L bioreactor culture of *P. citrinum*, it was observed that agitation speed and aeration rate had significant effects on the production of neo-FTase and that maximum cell mass and neo-FTase production obtained at 500 rpm and 1.5 vvm were 8.14 g/L and 53.2×10^{-3} U/mL, respectively. Cell mass and neo-FTase production increased to 91.53 and 25.17%, respectively. In the morphology and rheology studies, *P. citrinum* showed a typical pellet morphology that was explained by a shaving mechanism; this phenomenon was significantly affected by carbon sources. The rheology of neo-FTase fermentation by *P. citrinum* was dependent on cell growth and fungal morphology.

Keywords: neo-fructosyltransferase, neo-fructooligosaccharide, pellet morphology, *Penicillium citrinum*, rheology

INTRODUCTION

The production of fructooligosaccharides has received special attention in recent years because of the excellent biological and functional properties of the fructooligosaccharides; these properties have created a demand for the development of efficient enzymatic systems [1].

Fructooligosaccharides are fructose oligomers that are mainly composed of 1-ketose, nystose and 1-β-fructofuranosyl nystose in which one to three fructosyl units are bound at the β-2,1 position of sucrose.

The production of fructosyltransferase (FOS) is achieved by aerobic submerged fermentation with some fungal strains [2-5]. Although the fermentation parameters of aeration, agitation, pH, and temperature should be established for each microorganism, the general conditions for producing FOS by culturing of organisms have been well demonstrated [6-8]. For example, sucrose is the best carbon source for cell growth and enzyme activity. Maintenance of a pH above 5.0 is important and the optimum temperature for growth ranges from 25 to 30°C [3-7]. The effects of medium composition on the production of FOS from *Aureobasidium* sp. have been well described [2-5]. Other nutritional requirements and the time courses of enzyme production were similar in intracellular enzyme that was excreted into the culture broth after the static phase of growth. Although studies on the fermentation parameters of *Aspergillus* sp. and *Aureobasidium* sp. have been comprehensive, it was expected that the fermentative characteristics of *Penicillium citrinum* would differ from those of other microorganisms.

The purpose of this study was to provide basic information on the fermentative production of neo-FTase by *P. citrinum* and to investigate the relationship between the morphology and rheological properties of *P. citrinum* to improve neo-FTase production.

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MATERIALS AND METHODS

Microorganism and Culture Conditions

P. citrinum KCTC 18080P was grown on a medium consisting of 20% (w/v) sucrose, 0.2% $NaNO_3$, 0.5% K_2HPO_4, 2% yeast extract, 0.1% $MgSO_4 \cdot 7H_20$, 0.1% KCl, and 0.002% $FeSO_4 \cdot 7H_2O$. The pH was adjusted to 6.0 before the sterilization of the medium. The inoculum was grown for 72 h at 28°C with shaking at 200 rpm, and was then transferred to 500 mL Erlenmeyer flasks containing 100 mL of medium at 5% (v/v) and grown on the same medium for 90 h at 28°C, 200 rpm. Fermentation was carried out in a stirred-tank fermentor (2.5 L) at 28°C. The operating volume was 1.5 L.

Enzyme Assay

The enzyme activity was determined as follows. The reaction mixture consisted of 60% sucrose (2 mL) as the substrate, 0.1 M citrate buffer (3 mL, pH 6.0) and enzyme solution (1 mL). The enzyme reaction was carried

out at 50°C, 100 rpm in a water bath for 6 h. The FOS activity was determined from the amount of neo-kestose. The enzyme activity was defined as the amount of enzyme required to produce 1 μmol of neo-kestose [9].

Morphological Characteristics of *P. citrinum*

Pellet morphologies were studied using photomicrographs taken on an optical microscope (Samwon Scientific Ind. Co. Ltd., Korea) using Image Pro 3.0 software (Media Cybernetics, Silver Spring, MD, USA) and [10,11]. Morphological factors such as mean area and mean diameter were automatically measured after sorting and classification of images. Circularity or shape factors were defined as the ratio of the Feret's minimum diameter to the Feret's maximum diameter of the pellets. Hairiness was defined as the ratio of the outer areas to interior pellet areas. All data were calculated from approximately 20 observations.

Rheological Properties of the Culture Broths of *P. citrinum*

The rheological properties of culture broths of *P. citrinum* were determined using an ARES (Rheometric Co. Ltd., USA). The shear stress of the fermentation broth was characterized using the Power law model:

$$\tau = K \cdot \gamma^n \quad (1)$$

where, τ is the shear stress, γ is the shear rate, K is consistency index, and n is the flow behavior index.

RESULTS AND DISCUSSION

Effect of Carbon Sources on Cell Growth and neo-FTase Production

The effect of various carbon sources on cell growth and neo-FTase production was investigated (Table 1). Sucrose was the most effective substrate among the carbon sources; the highest neo-FTase production obtained with sucrose was 19.8 × 10^{-3} U/mL. This value was approximately two times higher than those of glucose and fructose. Consequently, sucrose was determined as a carbon source for further study.

Fermentation of *P. citrinum* in a 2.5 L Bioreactor

Higher cell mass and levels of neo-FTase production were obtained in a 2.5 L bioreactor culture (Fig. 1). Time courses of cell mass, pH, and neo-FTase production showed similar patterns to those of flask culture. Maximum cell mass and neo-FTase production were 7.88 g/L and 50.8 × 10^{-3} U/mL, respectively. To investigate the effects of agitation speed and aeration rate on the production of neo-FTase, cultures were carried out at different agitation speeds (200~600 rpm) and aeration rates (0.5~2.5 vvm). As agitation speed increased, cell mass and neo-FTase production increased (Fig. 2). Maximum cell mass and neo-FTase production were obtained at 500 rpm and were 7.95 g/L and 51.2 × 10^{-3} U/mL, respectively. Compared to the effect of agitation speed, the effect of aeration rate on neo-FTase production was small (Fig. 3). However, it was evident that a higher aeration rate could improve neo-FTase production. Maximum cell mass and neo-FTase production were obtained at 1.5 vvm, and were 8.14 g/L and 53.2 × 10^{-3} U/mL, respectively.

Table 1. Effect of carbon sources on cell growth and neo-fructosyltransferase production

Carbon source	Final pH	D.C.W (g/L)	Total protein (mg/mL)	Neo-Ftase production (× 10^{-3} U/mL)
Glucose	5.54	3.85	0.31	10.5
Fructose	5.64	3.72	0.29	11.8
Sucrose	6.0	4.23	0.59	19.8
Maltose	5.73	2.73	0.36	3.2

Cultures were carried out at 28°C, pH 6, and 500 rpm for 90 h in the medium containing 5% (w/v) carbon sources and 2% (w/v) yeast extract.

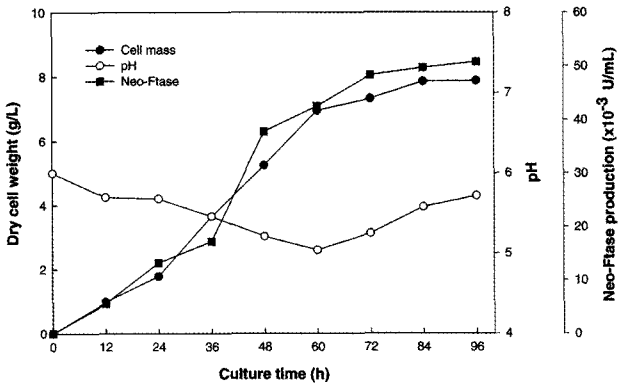

Fig. 1. Time course of neo-FTase production by *P. citrinum* in a 2.5 L bioreactor. Cultures were carried out at 28°C, pH 6, and 400 rpm for 96 h.

Morphological Characteristics of *P. citrinum*

Pellet morphology has been reported as one of the key factors that determines fermentation productivity [12,13]. To investigate the relationship between morphological changes of *P. citrinum* and neo-FTase production, the pellet morphologies of *P. citrinum* were characterized in a 2.5 L bioreactor culture. Fig. 4 shows the morphological changes of *P. citrinum* in a 2.5 L bioreactor. Pellets were the predominant form observed during the early period of fermentation and pellet morphology varied during the progression of fermentation. Rapid pellet formation was observed from the beginning of fermentation, and then pellet growth continued in an irregular manner for up to 36 h of fermentation. After 36 h, pellets lost their rigidity

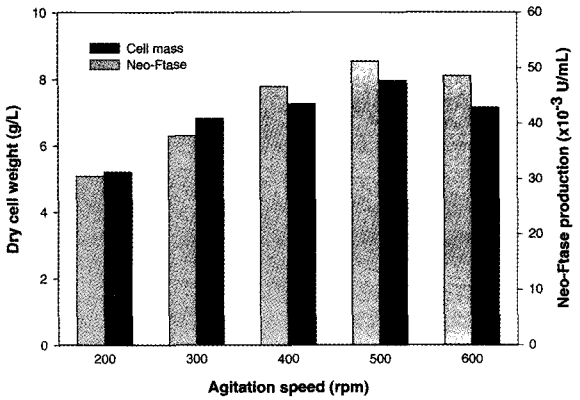

Fig. 2. Effect of agitation speed on neo-FTase production. Cultures were carried out at 28°C and pH 6 for 90 h.

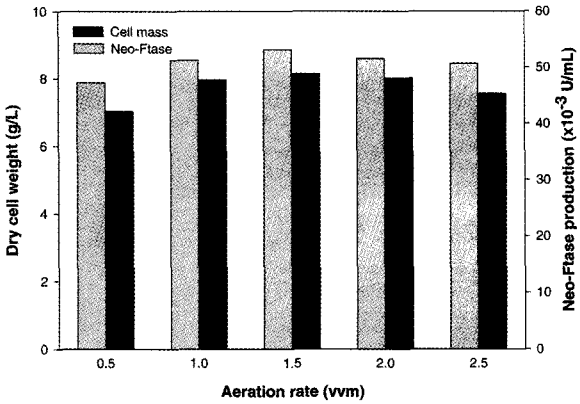

Fig. 3. Effect of aeration rate on neo-FTase production. Cultures were carried out at 28°C, pH 6, and 500 rpm for 90 h.

and broke up. It was observed that the pellet diameter and the pellet surface area increased rapidly over 36 h of fermentation and then reduced again in size after 48 h of fermentation (Fig. 5). Pellet hairiness increased from the beginning of fermentation until 36 h, and then subsequently decreased (Fig. 6). In terms of the circularity factor, it decreased rapidly until the end of fermentation (Fig. 7). Overall, observed morphological changes of *P. citrinum* included changes in its pellet-like morphology, which could be explained by a shaving mechanism [14]. As hairs from pellets were shaved by fermentative parameters (such as nutritional effect, mechanical agitation, and aeration effect), pellets lost their rigidity and broke up. These changes in pellet morphology might facilitate enzyme secretion. In fact, it was observed that neo-FTase production increased as pellets disintegrated.

Morphological changes of *P. citrinum* were affected by carbon source but were not affected by nitrogen source. Table 2 shows the effect of different carbon sources on the morphology of *P. citrinum* in shaking flask culture. It was thought that the effect of carbon source on the morphology of *P. citrinum* would be related to the production of neo-FTase. In the early stage of fermentation, the largest mean area (0.42 mm^2) was observed when maltose

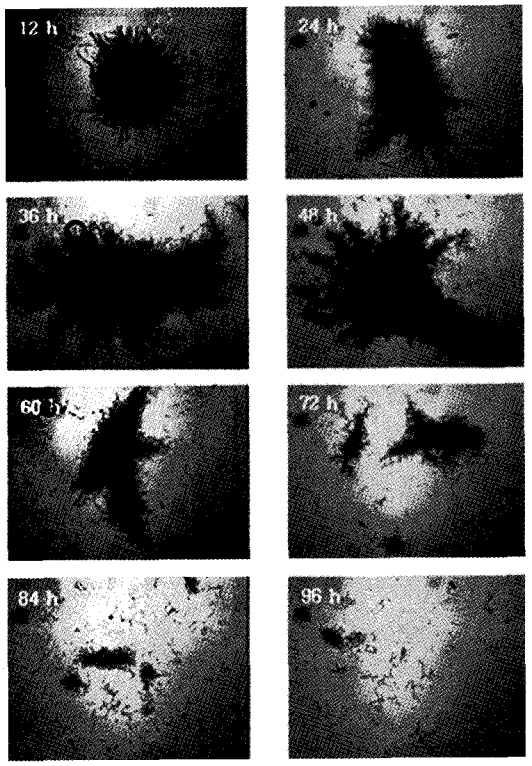

Fig. 4. Typical morphological changes of *P. citrinum* in a 2.5 L bioreactor. Cultures were carried out at 28°C, pH 6, and 500 rpm for 90 h. Morphological changes were measured using Image Pro 3.0.

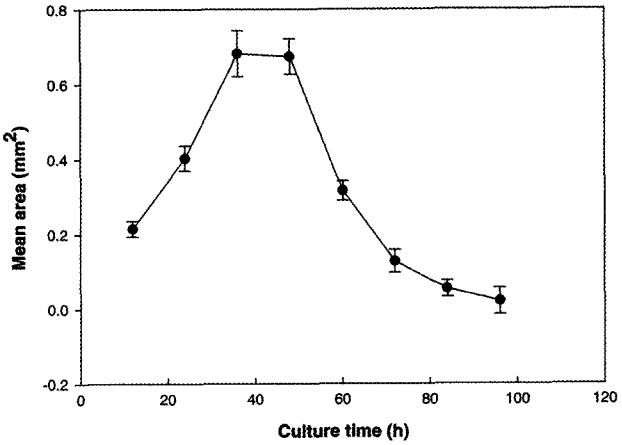

Fig. 5. Variations in mean pellet area of *P. citrinum* in a 2.5 L bioreactor. Cultures were carried out at 28°C, pH 6, and 500 rpm for 90 h. Mean pellet area was measured automatically after sorting and classifying images and calculated from approximately 20 observations using Image Pro 3.0.

was used as a carbon source. Under this condition, pellets had longer hair and a higher circularity factor than in other cases. In particular, when glucose and maltose were used as carbon sources, pellet size increased as fermenta-

Table 2. Effect of carbon sources on the morphology of *P. citrinum* in shaking flask culture

Carbon source	Early stage of fermentation			Late stage of fermentation		
	Mean area (mm^2)	Hairiness	Circularity factor	Mean area (mm^2)	Hairiness	Circularity factor
Glucose	0.24	0.65	0.75	0.48	0.38	0.64
Fructose	0.35	0.49	0.82	0.12	0.12	0.08
Sucrose	0.36	0.52	0.74	0.04	0.11	0.12
Maltose	0.42	0.67	0.89	0.98	0.58	0.76

0~30 h: early stage of fermentation, and 60~90 h: late stage of fermentation.
Cultures were carried out at 28°C, pH 6, and 500 rpm for 90 h.

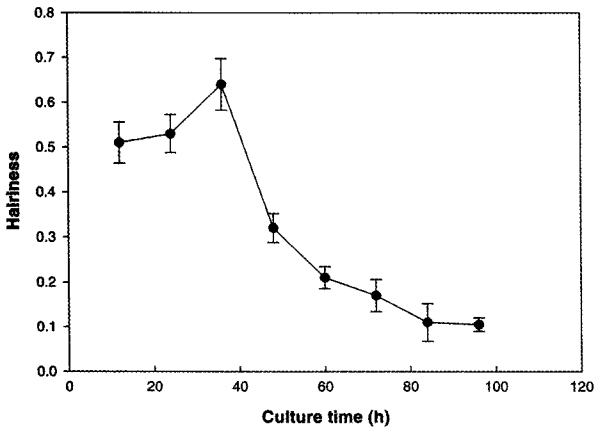

Fig. 6. Variations in pellet hairiness of *P. citrinum* in a 2.5 L bioreactor culture. Cultures were carried out at 28°C, pH 6, and 500 rpm for 90 h. Pellet hairiness was measured automatically after sorting and classifying images and calculated from approximately 20 observations using Image Pro 3.0.

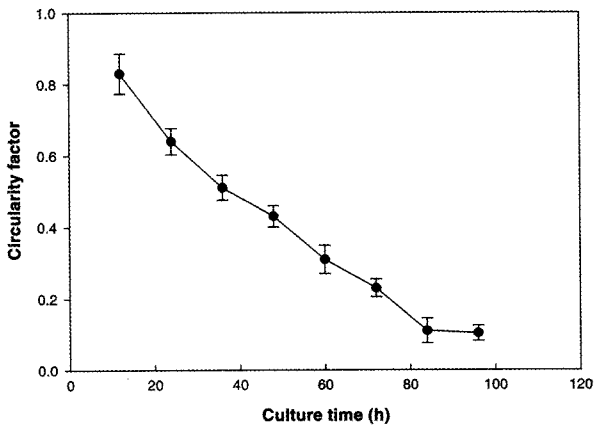

Fig. 7. Variations in pellet circularity factors of *P. citrinum* in a 2.5 L bioreactor culture. Cultures were carried out at 28°C, pH 6, and 500 rpm for 90 h. Circularity factors were defined as the ratio of the Feret's minimum diameter to the Feret's maximum diameter of the pellets and calculated from approximately 20 observations using Image Pro 3.0.

tion proceeded. However, when fructose and sucrose were used as carbon sources, pellet size significantly decreased during fermentation, and neo-FTase production increased. Both the hairiness and the circularity factor decreased from the beginning of fermentation in all cases. From the above results, it was concluded that the production of neo-FTase from *P. citrinum* is facilitated when pellets differentiated into free mycelia, and that this phenomena can be controlled by fermentative parameters like the type of carbon source.

Fermentation Broth Rheology of *P. citrinum*

Broth rheology determines transport phenomena in bioreactors and is a key means of improving the yield of the desired product [15-17]. Therefore, in this study, the fermentation broth rheology of *P. citrinum* and its relationship to morphology was investigated. After the first 12 h of fermentation, the viscosity of the fermentation broth increased rapidly as *P. citrinum* grew exponentially along with the formation of pellets (Fig. 8); this continued until the conclusion of fermentation. The flow behavior of the fermentation broth was essentially pseudoplastic at all stages of fermentation. The rheology of neo-

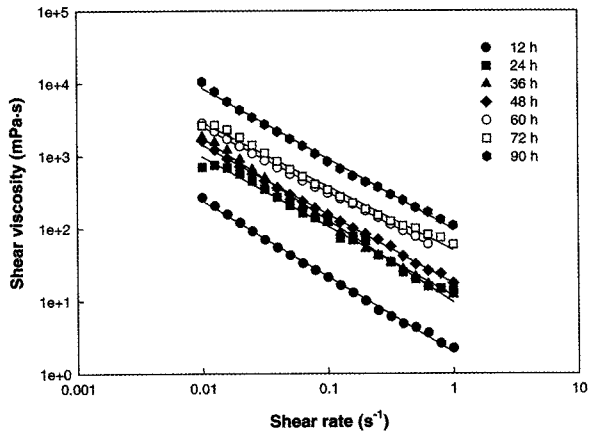

Fig. 8. Variation in shear viscosities of the fermentation broth of *P. citrinum* in a 2.5 L bioreactor. Shear viscosities of fermentation broth were determined using an ARES.

FTase fermentation by *P. citrinum* was dependent on cell growth and fungal morphology, which is similar to those of other fungal fermentations [12,16,17]. Fig. 9 shows the variations in the consistency index (K) and flow be-

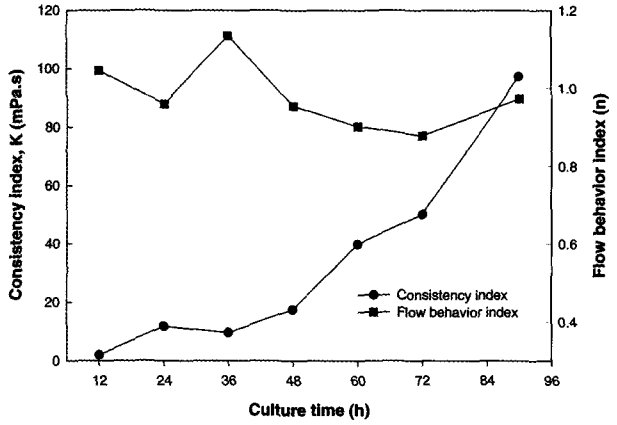

Fig. 9. Variations in flow behavior index and consistency index of fermentation broth of *P. citrinum* in a 2.5 L bioreactor. Behavior and consistency index of fermentation broth were determined using an ARES.

havior index (n) of the fermentation broth. The consistency index increased gradually over 48 h during pellet growth. It then increased rapidly to 97.3 mPa·s at 90 h when most of the pellets had lost rigidity and were in the process of breaking down. Moreover, changes in the flow behavior index were inversely related to those of the consistency index. An increase in the flow behavior index indicated that the pseudoplastic nature of the broth had become more Newtonian in behavior; this phenomenon was observed after 36 h of fermentation. Increased fluid viscosity reduced oxygen transfer to pellets, which may be the reason for the breakdown of pellets after 48 h of fermentation. This process, in combination with cells entering the stationary phase, subsequently increased free mycelia from pellets and increased neo-FTase production during the intermediate and late stages of fermentation.

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REFERENCES

[1] Seo, E. S., J. H. Lee, J. Y. Cho, M. Y. Seo, H. S. Lee, S. S. Chang, H. J. Lee, J. S. Choi, and D. M. Kim (2004) Synthesis and characterization of fructooligosaccharides using levansucrase with a high concentration of sucrose. *Biotechnol. Bioprocess Eng.* 9: 339-344.

[2] Hayashi, S., M. Nonokuchi, K. Imada, and H. Ueno (1990) Production of fructosyl-transferring enzyme by *Aureobasidium* sp. ATCC 20524. *J. Ind. Microbiol.* 5: 395-400.

[3] Yun, J. W. (1996) Fructooligosaccharides-Occurrence, preparation, and application. *Enzyme Microb. Technol.* 19: 107-117.

[4] Jung, K. H., J. Y. Lim, S. J. Yoo, J. H. Lee, and M. Y. Yoo (1987) Production of fructosyltransferase from *Aureobosidium pullulans*. *Biotechnol. Lett.* 9: 703-708.

[5] Hayashi, S., Y. Shimokawa, M. Nogoguchi, Y. Tasasaki, H. Ueno, and K. Imada (1993) Nutritional status of *Aureobasidium* sp. ATCC 20524 for the production of β-fructofuranosidase. *World J. Microbiol. Biotechnol.* 9: 216-220.

[6] Choi, D. B., K. A. Cho, W. S. Cha, and S. R. Ryu (2004) Effect of Triton X-100 on compactin production from *Penicillium citrinum*. *Biotechnol. Bioprocess Eng.* 9: 171-178.

[7] Jung, K. H., J. W. Yun, K. R. Kang, J. Y. Lim, and J. H. Lee (1989) Mathematical model for enzymatic production of fructooligosaccharides from sucrose. *Enzyme Microb. Technol.* 11: 491-494.

[8] Hang, Y., E. Woodams, and K. Jang (1995) Enzymatic conversion of sucrose to kestose by fungal extracellular fructosyltransferase. *Biotechnol. Lett.* 17: 295-298.

[9] Hirayama, M., N. Sumi, and H. Hidaka (1989) Purification and properties of a fructooligosaccharide-producing β-fructofuranosidase from *Aspergillus niger* ATCC 20611. *Agric. Biol. Chem.* 53:667-673.

[10] Lee, M. S., J. S. Lim, C. H. Kim, K. K. Oh, S. I. Hong, and S. W. Kim (2001) Effects of nutrients and culture conditions on morphology in the seed culture of *Cephalosporium acremonium* ATCC 20339. *Biotechnol. Bioprocess Eng.* 6: 156-160.

[11] Lim, J. S., J. H. Kim, C. Y. Kim, and S. W. Kim (2002) Morphological and rheological properties of culture broth of *Cephalosporium acremonium* M25. *Korea-Australia Rheology J.* 14: 11-16.

[12] Sinha, J., J. T. Bae, J. P. Park, C. H. Song, and J. W. Yun (2001) Effect of substrate concentration on broth rheology and fungal morphology during exo-biopolymer production by *Paecilomyces japonica* in a batch bioreactor. *Enzyme Microb. Technol.* 29: 392-399.

[13] Kim, J. H., J. S. Lim, and S. W. Kim (2004) The improvement of cephalosporin C production by fed-batch culture of *Cephalosporium acremonium* M25 using rice oil. *Biotechnol. Bioprocess Eng.* 9: 459-464.

[14] Jimenez-Tobon, G. A., M. J. Penninckx, and R. Lejeune (1997) The relationship between pellet size and production of Mn(II) peroxidase by *Phanerochaete chrysosporium* in submerged culture. *Enzyme Microb. Technol.* 21: 537-542.

[15] Yim, J. H., S. J. Kim, S. H. Aan, and H. K. Lee (2004) Physicochemical and rheological properties of a novel emulsifier, EPS-R, produced by the marine bacterium *Hahella chejuensis*. *Biotechnol. Bioprocess Eng.* 9: 405-413.

[16] Riley, G. L., K. G. Tucker, G. C. Paul, and C. R. Thomas (2000) Effect of biomass concentration and mycelial morphology on fermentation broth rheology. *Biotechnol. Bioeng.* 68: 160-172.

[17] Tucker, K. G. and C. R. Thomas (1993) Effect of biomass concentration and morphology on the rheological parameters of *Penicillium chrysogenum* fermentation broths. *Trans. IchemE. Part C* 71: 111-117.

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