Enhanced Glucosamine Production with *Actinomucor elegans* Based on Stimulating Factor of Methanol

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Abstract Glucosamine (GlcN) is a major and valuable component in the cell wall of fungi. In this study, the cell wall was treated via a two-stage alkali and acid process, and chitin and chitosan were fully deacetylated, partially depolymerized, and converted to GlcN oligosaccharides. Then, the oligosaccharides were analyzed by high performance liquid chromatography. The influences of *Actinomucor elegans* on GlcN production in a flask culture were investigated to achieve an optimum yield of GlcN. The experimental result showed that cultivation in condition of pH 6.0, 100 mL working volume (500 mL flask), 10 % (v/v) inoculum concentration, at 28 °C and 200 rpm for 6 days yielded highest dry cell weight (DCW) which was 23.43 g L⁻¹, with a GlcN concentration of 5.12 g L⁻¹. Methanol as stimulating factor was found to exert the best effect in concentration of 1.5 % (v/v). With addition of methanol into medium, the DCW increased from 23.69 to 32.42 g L⁻¹, leading to maximum GlcN concentration of 6.85 g L⁻¹ obtained. Here, the methanol addition may be useful for industrial production of GlcN, and may also be meaningful for the production of other fine chemicals by filamentous fungi.

Keywords *Actinomucor elegans* · Glucosamine production · Stimulating factor · Methanol

Introduction

As a component of chitin and chitosan, glucosamine (2-amino-2-deoxy-D-glucose, GlcN) is an important hexosamine, which is widespread in fungal cell wall, shrimp and crab shells, as well as animals [1]. GlcN is also a major component of glycoproteins and proteoglycans playing an important role in human articular cartilage [2]. Since the 1960s, GlcN has been widely applied in treatment of osteoarthritis [3]. Currently, shrimp and crab shells are the main raw materials for GlcN extraction. Chitin and chitosan are extracted from shrimp and crab shells, which are decomposed by acid hydrolysis [4]. However, extensive use of concentrated hydrochloric acid may cause serious environmental problems, so the method will be gradually limited by environmental policy. A further concern with GlcN derived from shellfish is that a significant portion of the human population have shellfish allergies that are unsuitable to use products containing ingredients derived from shellfish [5]. Moreover, it is not economically practical that GlcN products derived from shellfish source are completely free of all traces of shellfish allergens [6]. In comparison with the extraction method, the microbial production of GlcN has some advantages such as the lack of allergens and less environmental pollution [7].

The present work shows microbial fermentation to produce GlcN with fungi, because the fungal cell wall contains chitin and chitosan as structural materials, as above mentioned. Fungi, *Aspergillus* sp. BCRC31742 (7.48 g L⁻¹), *Monascus pilosus* BCRC31527 (0.72 g L⁻¹) and *Rhizopus oligosporus* BCRC31996 (0.39 g L⁻¹) have been used to produce GlcN [8, 9]. However, production of GlcN from fungi is a difficult task, because of the low levels at which it typically presents. Nevertheless, due to the higher crystallinity of chitin, this polymer may exhibit a

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higher resistance toward hydrolysis compared to chitosan. Therefore, chitosan may be a preferred substrate for GlcN production. Chitin and chitosan make up around 25–27 % of the cell wall of *Actinomucor elegans*, while chitosan usually occurs at higher portions [10]. Therefore, in this work, *A. elegans* CGMCC3539 was cultivated and influences on GlcN production were investigated. Furthermore, different methanol concentration were employed to enhance GlcN production in a flask culture system.

Materials and Methods

Materials

Actinomucor elegans CGMCC3539 was purchased from China General Microbiological Culture Collection Center (CGMCC) in Beijing, which was cultured on potato dextrose agar (PDA) slants with natural pH. The fermentation medium consisted of: glucose 25 g L⁻¹, peptone (Ao-BoXing, Beijing, China) 25 g L⁻¹, KH₂PO₄ 0.5 g L⁻¹, MgSO₄ 0.5 g L⁻¹ and CaCl₂ 0.1 g L⁻¹. The fermentation medium was sterilized at 121 °C for 15 min to avoid potential Maillard reaction.

Fungal Cultivation

The PDA plate was incubated at 28 °C for 3–5 days for production of *A. elegans* CGMCC3539 spores. After a large number of spores appeared on slants, spores were eluted with sterilized normal saline of 10 mL, and two layers of sterile gauze were used to filter mycelium. The filtrate was added into a flask containing glass beads, fully oscillated. The spores were counted with a hemocytometer. After adjusted with distilled water, the spore suspension reached final cfu of 10⁶ mL⁻¹. Then the spore suspension of 10 % (v/v) was added into a 500 mL flask with fermentation medium (150 mL working volume, pH 7.0), which was cultured at condition of 28 °C and 200 rpm rotary shaker for 8 days. In further studies, various pH, working volumes and inoculum concentrations would be employed.

Treatment of Fungal Mycelium

The mycelium was collected from fermentation broth by filtration of Buchner funnel, which was washed with distilled water, and dried to constant weight in 60–80 °C oven. 30 mL of 0.5 M NaOH was added into dry mycelium per gram, which was heated in 121 °C steam for 10–60 min. The solution was removed by centrifugation (10 min, 4,000×g), and the precipitate was washed with distilled water until neutral pH was reached. Then, the

precipitate was dried to constant weight in 45 °C to obtain mycelium alkali insoluble matter (AIM). It was weighed and stored at room temperature. Then 500 mg AIM was added into a test tube, and then 5 mL 6 M HCl, which was placed into oven, heating at 100 °C for 5–40 h. After the reaction, the test tube was cooled to room temperature. 10 mL of distilled water was added into the test tube, and the pH was adjusted to 7.0 with 5 M NaOH. The mixture was filtered with 0.45 µm membrane, and 1 mL of the solution was added into microcentrifuge tube of 1.5 mL for high performance liquid chromatography (HPLC) and colorimetric method analysis.

Determination of Fungal GlcN

The analysis was performed on instrument of Shimadzu LC Prominence-20A, using Inertsil HPLC Column (NH₂ 5 µm; 4.6 × 250 mm) with phosphate buffer (Taking 0.8 g KH₂PO₄ into a 1 L distilled water adjusted to neutral pH with 5 M KOH)/acetonitrile (35:65, v/v) as mobile phase, at a flow rate of 1 mL min⁻¹ with column temperature of 30 °C. The variable wavelength detector was 195 nm. And the *N*-acetylglucosamine (GlcNAc) concentration was determined by Morgan–Elson [11].

Data and Statistical Analysis

Fermentation profiles were evaluated specifically in terms of dry cell weight (DCW) (g L⁻¹), GlcN concentration (g L⁻¹) as well as residual glucose (g L⁻¹), content (w_{GlcN}/w_{DCW}) and productivity (mg L⁻¹ h⁻¹). Residual glucose in the fermentation broth was measured by glucose-glutamate analyzer SBA-40C (Biology Institute of Shandong Academy of Sciences, Jinan, China). All the values (mean ± standard error) were from three independent experiments.

Results and Discussion

Treatment and Determination

The times of alkali treatment and acid degradation are vital to obtain maximum amount of GlcN. Figure 1a shows that the amount of AIM increased along with the extension of alkali treatment. When the time was 40 min, the maximum AIM concentration reached 14.45 g L⁻¹, and remained unchanged thereafter. And it is clearly shown in Fig. 1b that the best time of acid degradation was 30 h, after that GlcN and GlcNAc concentration maintained substantially constant, and maximum concentration of GlcNAc was just 0.26 g L⁻¹. So under this condition, mycelium could be effectively degraded and converted to GlcN oligosaccharides. The test sample and standard sample of GlcN were

Fig. 1 The determination of treatment conditions of fungal mycelium. **a** The alkali treatment. **b** The acid degradation. (—●—) AIM, (—■—) GlcN, (—□—) GlcNAc

Fig. 2 Chromatograms of standard GlcN and test sample. The Cr^{+1} peak of test sample derived from HCl

studied and results could be seen in Fig. 2. It indicates that the developed method is applicable to determination of GlcN derived from fungi.

Effects of Fermentation pH

Different pH was studied and results could be seen in Fig. 3, which demonstrates that cell growth and the quantity of GlcN production are closely related to pH variations and pH 6.0 is the suitable operating factor. Figure 2d shows that the maximum GlcN concentration was 5.08 g L^{-1} (residual glucose 5.32 g L^{-1} , DCW 23.57 g L^{-1} , AIM 6.12 g L^{-1}) in condition of pH 6.0 and cultivation time 144 h. Moreover, the increase of DCW was generally accompanied with higher concentrations of GlcN. Hence, the GlcN concentration in *A. elegans* CGMCC3539 was inferred as growth-associated product formation. When pH was changed from 3.0 to 5.0, the residual glucose concentration increased, and the fungal growth rate was

slower, leading to a lower GlcN concentration. When pH was 7.0 or 8.0, the fungal growth rate was rapid within pre-inoculation, but later slowed down. It might indicate that pH changed the charge of membrane, leading to abnormal variations of membrane osmotic pressure [12, 13].

Effect of Working Volume and Inoculum Concentration

Working volume and agitation rate affect cell growth and accumulation of metabolites in aerobic fermentation. Especially in mold fermentation, the pellet of mycelium could increase the viscosity of fermentation broth to reduce oxygen transfer efficiency, leading to the decrease of fermentation products [14, 15]. In flask fermentation process, optimal working volume could not only improve oxygen transfer efficiency, but also help get maximum amount of desired products [16]. Different working volumes (50, 100, 150, 200, 250 and 300 mL) were studied. The fermentation occurred in the condition of pH 7.0, 10 % (v/v) inoculum concentration, 28°C , and 200 rpm rotary shaker for 8 days. The results indicated that optimal condition of this flask culture system was 100 mL working volume, and the GlcN concentration reached a maximum of 5.12 g L^{-1} . With the increase of working volume, DCW and GlcN concentration decreased because of the reduced oxygen transfer efficiency. When the fungus was under low oxygen supply, the oxygen demand was not satisfied, resulting in a lowered yield. However, excessive oxygen supply might also cause a decrease in productivity because of the losses of substrates in direct oxidation.

Generally, inoculum concentration is 5–10 % (v/v) in fermentation processes, which is determined by growth rate. To understand the effects of inoculum concentration in GlcN production, the fermentation was investigated with different inoculum concentrations (5, 10, 15, 20 %, v/v).

Fig. 3 Plot of residual glucose concentration, DCW, AIM and GlcN concentration in fermentation medium at different pH. pH: **a** 3, **b** 4, **c** 5, **d** 6, **e** 7, **f** 8. (■) Residual glucose, (-Δ-) DCW, (-□-) AIM, (-▲-) GlcN. Fermentation medium (150 mL) was adjusted to specific pH and maintained at pH 3.0–8.0 in flask culture system with 5 M

NaOH. The fermentation was in the condition of 150 mL working volume, 10 % (v/v) inoculum concentration, at 28 °C and 200 rpm rotary shaker for 8 days. The residual glucose, DCW, AIM and GlcN concentration were obtained

Figure 4 illustrates the profiles of residual glucose, DCW, AIM and GlcN titer in flask culture system. The maximum DCW reached 16.62, 22.53, 19.12 and 18.61 g L⁻¹ respectively when inoculum concentration was maintained at 5, 10, 15 and 20 % (v/v). It indicates that 10 % (v/v) was the optimal inoculum concentration for cell growth. The GlcN titer reached 3.82, 4.87, 3.93 and 3.19 g L⁻¹ respectively when inoculum concentration maintained at 5,

10, 15 and 20 % (v/v), which demonstrates that the maximum GlcN titer was 4.87 g L⁻¹ with an inoculum concentration of 10 % (v/v). As known, a larger inoculum concentration may shorten the growth time, so that products are synthesized in advance. However, if inoculum concentration is excessive, the fungal growth may be fast so the medium viscosity increase, leading to the decrease of dissolved oxygen.

Fig. 4 Plot of residual glucose concentration, DCW, AIM and GlcN concentration in fermentation medium at different inoculum concentration. Inoculum concentration: **a** 5 %, **b** 10 %, **c** 15 %, **d** 20 %. (-■-) Residual glucose, (-▲-) GlcN, (-□-) AIM, (-△-) DCW. The

fermentation was in the condition of pH 7.0, 150 mL working volume, at 28 °C and 200 rpm rotary shaker for 8 days. The residual glucose, DCW, AIM and GlcN concentration were obtained

Stimulating Factor of Methanol to Improve GlcN Concentration

Different alcohols, especially methanol and ethanol have stimulation effects on synthesis of certain substances derived from fungi [17]. Haq et al. [18] studied the alcohol stimulations in the production of citric acid using *Aspergillus niger* GCB-47. They suggested that the addition of methanol could increase the permeability of cell membrane, leading to the transfer of citric acid from intracellular to fermentation broth. Moreover, increased permeability of cell membrane could make more promoting factors permeate into the microbial cells from fermentation broth, leading to an increase of product synthesis.

In this study, the growth of fungal mycelium was partly attributed to the increase in chitin and chitosan contents, which could be degraded to GlcN by alkali and acid treatment as above mentioned. Table 1 shows that the optimal dosage of methanol was 1.5 % (v/v) in this flask culture system in condition of pH 6.0, 100 mL working volume and 10 % (v/v) inoculation volume, under which the maximum GlcN concentration reached 6.85 g L⁻¹. Furthermore, Table 1 illustrates that the addition of

methanol increased DCW and GlcN significantly from 8.29 to 32.54 g L⁻¹ and from 1.37 to 6.85 g L⁻¹, respectively. It indicates that methanol could promote the growth of fungal mycelium. Methanol appeared to be an effective stimulating factor in the fermentation of *A. elegans* CGMCC3539. Table 1 shows that methanol volume of 2 % (v/v) could promote mycelium growth, however, the concentration of AIM and GlcN were 7.81¹ and 6.73 g L⁻¹, respectively, which was lower than 1.5 % (v/v) (AIM 8.42 g L⁻¹ and GlcN 6.85 g L⁻¹). It could be explained that the increase of methanol could promote mycelium growth and accumulation of chitin and chitosan, but the addition of high amount of methanol might reduce the content of mycelium (chitin and chitosan) and hinder GlcN synthesis. Moreover, GlcN productivity reached 0.048 (g L⁻¹ h⁻¹) and 0.036 (g L⁻¹ h⁻¹), when addition of methanol volume were 1.5 % (v/v) and 2 % (v/v), respectively, which further indicates that excessive methanol was not conducive to GlcN accumulation in mycelium. On the other hand, intracellular metabolites are regulated by intake of appropriate substrates. Nutrients transfer at the liquid–solid interface between cell surface and medium is important for microorganisms. Since larger transport surface is available for small pellets, nutrient

Table 1 Comparison of maximum GlcN production at different methanol levels

	Fermentation parameters	Methanol level (% v/v)			
		0.5	1	1.5	2
The fermentation was in condition of pH 6.0, 100 mL working volume, 10 % (v/v) inoculum concentration, at 28 °C and 200 rpm rotary shaker for 8 days DCW dry cell weight, AIM alkali insoluble matter	Glucose consumption (g L ⁻¹)	19.81 ± 0.13	19.95 ± 0.22	24.68 ± 0.24	24.42 ± 0.21
	Maximum DCW (g L ⁻¹)	23.69 ± 0.17	23.82 ± 0.21	32.42 ± 0.23	32.54 ± 0.15
	Maximum AIM (g L ⁻¹)	6.24 ± 0.15	6.37 ± 0.11	8.42 ± 0.13	7.81 ± 0.15
	Maximum GlcN (g L ⁻¹)	5.19 ± 0.14	5.32 ± 0.11	6.85 ± 0.14	6.73 ± 0.09
	AIM yield on DCW (g g ⁻¹)	0.263 ± 0.002	0.267 ± 0.003	0.260 ± 0.015	0.240 ± 0.008
	GlcN yield on glucose (g g ⁻¹)	0.262 ± 0.004	0.267 ± 0.011	0.278 ± 0.009	0.276 ± 0.011
	GlcN yield on AIM (g g ⁻¹)	0.832 ± 0.003	0.835 ± 0.002	0.814 ± 0.011	0.862 ± 0.007
	Mycelium productivity (g L ⁻¹ h ⁻¹)	0.165 ± 0.007	0.165 ± 0.008	0.225 ± 0.003	0.271 ± 0.002
	GlcN productivity (g L ⁻¹ h ⁻¹)	0.036 ± 0.005	0.037 ± 0.011	0.048 ± 0.009	0.036 ± 0.004

Table 2 Different wild-type fungi used to produce GlcN

Strains	GlcN (g L ⁻¹)	Content (mg g ⁻¹ DCW)	Productivity (mg L ⁻¹ h ⁻¹)	GlcN yield (mg g ⁻¹ carbon)	Reference
<i>R. oligosporus</i> NRRL2710	–	0.11	–	–	Sparringa and Owens [20]
<i>Aspergillus</i> sp.	–	24.10	–	–	Carter et al. [21]
<i>M. pilosus</i>	0.26	–	–	13.20	Yu et al. [22]
<i>Aspergillus</i> sp. BCRC31742	3.43	185.00	20.40	137.00	Hsieh et al. [8]
<i>M. pilosus</i> BCRC31527	0.72	40.40	4.28	35.90	Hsieh et al. [8]
<i>R. oligosporus</i> BCRC31996	0.39	188.00	2.34	13.20	Hsieh et al. [8]
<i>Rhizopus oryzae</i> ATCC20344	–	160.00	–	–	Liao et al. [23]
<i>Aspergillus</i> sp. BCRC31742	7.05	210.00	58.73	210.00	Sitanggang et al. [9]
<i>Aspergillus</i> sp. BCRC31742	7.48	260.00	62.33	220.00	Sitanggang et al. [9]
<i>A. elegans</i> CGMCC3539	6.85	211.29	48	278	This study

transportation will be easier as compared to larger pellets. Generally, substrate limitation at the core of pellet will happen in fungi with big pellet morphology which affects fungi growth [9]. However, the fungus was mycelia pellet morphology of yeast-like with a 1.5 % (v/v) methanol addition into medium and GlcN concentration reached a maximum of 6.85 g L⁻¹. It could be explained that methanol has a significant effect on fungi growth in terms of GlcN production regarding to limitations of nutrient transport and oxygen uptake rate.

The use of wild-type fungi seemed to be an efficient way to produce GlcN. Some studies have reported on production of GlcN with wild-type fungi (Table 2). Most of those studies used wild-type fungi that belong to *Ascomycotina* (*Aspergillus* sp.) and *Zygomycotina* (*Rhizopus* sp., *Mucor* sp.) subdivisions [8]. In every production of native products, productivity is the most important parameter being looked at, especially in maintaining scale-up and monitoring process as it relates to cost expenditure [19]. This study originally used *A. elegans* to produce GlcN and its production could be up to least twofold as compared to other studies, besides *Aspergillus* sp. BCRC31742 studied by Sitanggang et al. The selection of suitable carbon source

and nitrogen source for a fermentation process is a critical factor for microbial growth and biopolymer formation. In the present work, glucose and peptone were used for carbon source and nitrogen source, respectively, but it will be necessary to screen optimal carbon source and nitrogen source for mycelial growth and biopolymer production. Therefore, we think that the GlcN concentration will reach more than 10 g L⁻¹ by the wild-type *A. elegans* when the fermentation medium is further optimized.

Conclusions

Actinomucor elegans CGMCC3539 was used to produce GlcN cultured in flask system. The mycelial pellet morphology, pH, working volume, inoculum concentration and stimulating factor were found to affect fermentation performances. In this study, pH variations are closely related to the quantity of GlcN production, pH 6.0 is the suitable operating factor. With increase of working volume and inoculum concentration, the reduced oxygen transfer efficiency decreased DCW and GlcN concentration. The optimal condition of pH 6.0, 100 mL working volume and

10 % (v/v) inoculum concentration at 30 °C and 200 rpm yielded highest DCW of 23.43 g L⁻¹, with GlcN concentration of 5.12 g L⁻¹. For the fungus, 1.5 % (v/v) methanol was found to be an effective stimulating factor, which could increase GlcN concentration up to 6.85 g L⁻¹. It could be explained that methanol has a significant effect on fungi growth in terms of GlcN production regarding to limitations of nutrient transport and oxygen uptake rate. Methanol addition may be useful for the industrial GlcN production by fungi.

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References

- Uhde A, Youn JW, Maeda T, Clermont L, Matano C, Kramer R, Marin K (2013) Glucosamine as carbon source for amino acid-producing *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 97:1679–1687
- Mennini T (2012) Controversy on glucosamine. Nutrafoods 11:37–41
- Nakanura H (2011) Application of glucosamine on human disease—osteoarthritis. Carbohydr Polym 84:835–839
- Chen X, Liu L, Li JH, Du GC, Chen J (2012) Improved glucosamine and *N*-acetylglucosamine production by an engineered *Escherichia coli* via step-wise regulation of dissolved oxygen level. Bioresour Technol 110:534–538
- Zhang JX, Liu L, Li JH, Du GC, Chen J (2012) Enhanced glucosamine production by *Aspergillus* sp. BCRC 31742 based on the time-variant kinetics analysis of dissolved oxygen level. Bioresour Technol 111:507–511
- Chen X, Liu L, Li JH, Liu J, Du G, Chen J (2012) Optimization of glucose feeding approaches for enhanced glucosamine and *N*-acetylglucosamine production by an engineered *Escherichia coli*. J Ind Microbiol Biotechnol 39:359–365
- Liu L, Liu Y, Shin HD, Chen R, Li J, Du G, Chen J (2013) Microbial production of glucosamine and *N*-acetylglucosamine: advances and perspectives. Appl Microbiol Biotechnol 97:6149–6158
- Hsieh JW, Wu HS, Wei YH, Wang SS (2007) Determination and kinetics of producing glucosamine using fungi. Biotechnol Prog 23:1009–1016
- Sitanggang AB, Wu HS, Wang SS, Ho YC (2010) Effect of pellet size and stimulating factor on the glucosamine production using *Aspergillus* sp. BCRC31742. Bioresour Technol 10:3595–3601
- Bowman SM, Free SJ (2006) The structure and synthesis of the fungal cell wall. BioEssays 28:799–808
- Deng MD, Severson DK, Grund AD, Wassink SL, Burlingame RP, Berry A, Running JA (2005) Metabolic engineering of *Escherichia coli* for industrial production of glucosamine and *N*-acetylglucosamine. Metab Eng 7:201–214
- Kennedy MJ, Reader SL, Davies RJ, Rhoades DA, Silby HW (1994) The scale up of mycelial shake flask fermentations: a case study of gamma-linolenic acid production by *Mucor hiemalis* IRL 51. J Ind Microbiol 13:212–216
- Siedenberg D, Gerlach SR, Schugerl K, Giuseppin MLF, Hunik J (1998) Production of xylanase by *Aspergillus awamori* on synthetic medium in shake flask cultures. Process Biochem 33:429–433
- Wang ZJ, Wang HY, Li YL, Chu J, Huang MZ, Zhuang YP, Zhang SL (2010) Improved vitamin B₁₂ production by step-wise reduction of oxygen uptake rate under dissolved oxygen limiting level during fermentation process. Bioresour Technol 101:2845–2852
- Abdul-Aziz S, Fernandez CC, Salleh MM, Illias RM, Hassan MA (2008) Effect of agitation and aeration rates on chitinase production using *Trichoderma virens* UKM1 in 2-l stirred tank reactor. Appl Biochem Biotechnol 150:193–204
- Bodizs L, Titica M, Faria N, Srinivasan B, Dochain D, Bonvin D (2007) Oxygen control for an industrial pilot-scale fed-batch filamentous fungal fermentation. J Process Control 17:595–606
- Moyer AJ (1953) Effect of methanol on the mycological production of citric acid in surface and submerged culture. Appl Microbiol 1:1–7
- Haq IU, Ali S, Qadeer MA, Iqbal J (2003) Stimulatory effect of alcohols (methanol and ethanol) on citric acid productivity by a 2-deoxy-D-glucose resistant culture of *Aspergillus niger* GCB-47. Bioresour Technol 86:227–233
- Chmielowski RA, Wu HS, Wang SS (2007) Scale-up of upstream and downstream operations of the production of glucosamine using microbial fermentation. Biotechnol J 2:996–1006
- Sparringa RA, Owens JD (1999) Short communication: glucosamine content of tempe mould, *Rhizopus oligosporus*. Int J Microbiol 47:153–157
- Carter SB, Nokes SE, Crofcheck CL (2004) The influence of environmental temperature and substrate initial moisture content on *Aspergillus niger* growth and phytase production in solid state cultivation. Trans ASAE 47:945–949
- Yu KW, Kim YS, Shin KS, Kim JM, Suh HJ (2005) Macrophage stimulating activity of exo-biopolymer from cultured rice bran with *Monascus pilosus*. Appl Biochem Biotechnol 126:35–48
- Liao W, Liu Y, Frear C, Chen S (2008) Co-production of fumaric acid and chitin from a nitrogen-rich lignocellulosic material-dairy manure using a pelletized filamentous fungus *Rhizopus oryzae* ATCC 2034. Bioresour Technol 99:5859–5866