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Effect of cultivating conditions on α-galactosidase production by a novel *Aspergillus foetidus* ZU-G1 strain in solid-state fermentation

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Abstract: The work is intended to achieve optimum culture conditions of α-galactosidase production by a mutant strain *Aspergillus foetidus* ZU-G1 in solid-state fermentation (SSF). Certain fermentation parameters involving moisture content, incubation temperature, cultivation period of seed, inoculum volume, initial pH value, layers of pledget, load size of medium and period of cultivation were investigated separately. The optimal cultivating conditions of α-galactosidase production in SSF were 60% initial moisture of medium, 28 °C incubation temperature, 18 h cultivation period of seed, 10% inoculum volume, 5.0~6.0 initial pH of medium, 6 layers of pledget and 10 g dry matter loadage. Under the optimized cultivation conditions, the maximum α-galactosidase production was 2037.51 U/g dry matter near the 144th hour of fermentation.

Key words: α-Galactosidase, Culture condition, *Aspergillus foetidus* ZU-G1, Solid-state fermentation
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INTRODUCTION

Soybean meal (SBM) is the protein supplement most frequently used in animal feed worldwide. However, SBM is not without limitations because of oligosaccharides such as raffinose and stachyose that are not digested in the intestines of monogastric animals (Gitzelmann and Auricchio, 1965). They cannot synthesize sufficient α-galactosidase (E.C.3.2.1.22) in their intestinal systems to hydrolyze the α-galactosides present in soybeans and other legumes. These disturbances reduce feed efficiency in monogastric animals (LeBlanc *et al*., 2005; Prashanth and Mulimani, 2005). To improve the nutritional quality of SBM for monogastric animals, α-galactosidase is generally used to reduce the level of raffinose series oligosaccharides. α-Galactosidase is known to occur widely in microorganisms, plants (Kang and Lee, 2001) and humans (Gitzelmann and Auricchio, 1965). Various microorganisms produced α-galactosidase, such as fungi (Hin *et al*., 1986; Somiari and Balogh, 1995; Wang *et al*., 2004), yeasts (Oda and Tonomura, 1996) and bacteria (Jin *et al*., 2001; Leenhouts *et al*., 1995).

Up to now, *Aspergillus foetidus* was identified and applied to produce several enzymes. Pariza and Johnson (2001) reported approval of hemicellulase preparations from *A. foetidus* for use in food processing. Shah *et al*.(2006) reported using xylanase of *A. foetidus* to improve the quality of whole wheat bread. Wang and Rakshit (1999) reported producing transferase enzymes used for iso-oligosaccharides production by *Aspergillus foetidus*. Banerjee *et al*.(2005) and Purohit *et al*.(2006) reported using a coculture of the filamentous fungi, *Rhizopus oryzae* (RO IIT RB-13, NRRL 21498) and *Aspergillus foetidus* (GMRB013 MTCC3557) to yield tannase and gallic acid. It was reported that *Aspergillus foetidus* NRRL

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337 produced β-fructofuranosidase (Hang and Woodams, 1995). The isolate *Aspergillus foetidus* does not produce ochratoxin A as reported by Schuster *et al*.(2002). *Aspergillus foetidus* is a member of *Aspergillus niger* group which is considered as GRAS (generally regarded as safe) organism.

Solid-state fermentation (SSF) holds tremendous potential for the production of enzymes due to availabilities of equipment and technique, low capital investment, superior productivity, less energy requirements and effluent generation (Nigam and Singh, 1994). In spite of these promising features, comparatively few applications of SSF are currently running on industrial scale mainly due to unsolved technological and scale-up problems (Lekanda and Pérez-Correa, 2004). There has been considerable interest to produce α-galactosidase in SSF processes. Cruz and Park (1982), Kotwal *et al*.(1998), Li *et al*.(2001) and Wang *et al*.(2004) reported α-galactosidase production in SSF. However, little is known concerning the culture conditions by SSF for production of this enzyme by *Aspergillus foetidus*.

In this work, we investigate the influence of cultivating conditions such as moisture content, incubation temperature, cultivation period of seed, inoculum volume, initial pH of medium, layers of pledget, loadage of media and period of cultivation on the α-galactosidase biosynthesis by *Aspergillus foetidus* ZU-G1 in SSF using agricultural residues as substrates without enrichment of the medium. The culture conditions on α-galactosidase production in laboratory scale can potentially be scaled-up.

MATERIALS AND METHODS

Microorganism and culture medium

Aspergillus foetidus ZU-G1 was isolated from the Chinese traditional soy sauce mash and identified as *Aspergillus foetidus* according to *Manual of Fungi Identification* (Wei, 1979) and *Flora Fungorum Sinicorum (Vol. 5)-Aspergillus et Teleomorphi Cognati Chinese Edition with Latin Name Index* (Qi, 1997). ^{60}Co γ-ray and low energy N^+ ion beam implantation are used to mutate the strain, and obtain a positive mutant strain with high α-galactosidase activity. It is preserved at the China General Microbiological Culture Collection Center (CGMCC No. 1628).

The seed medium was potato dextrose agar (PDA). The fermentation medium in α-galactosidase production was composed as follows (w/w, on dry basis): wheat bran (WB), 83.21%; soybean meal (SBM), 16.64%; $FeSO_4 \cdot 7H_2O$, 0.05%; KH_2PO_4, 0.1%. Distilled water was added. All media were sterilized at 121 °C for 20 min and cooled to room temperature prior to use. Cultivation in SSF was carried out in an incubator where temperature could be controlled automatically. The initial culture condition was 28 °C incubation temperature for 96 h, 12 h cultivation period of seed, 5% inoculum volume, 5.5 initial pH of medium, 8 layers of pledget and 5 g dry matter loadage. Unless otherwise stated, the following tests were conducted in 250 ml flasks under the above conditions.

Crude enzyme extraction

After an appropriate time of incubation, the fermented medium was stirred. One gram of the fermented medium ground sample was soaked with 50 ml of 100 mmol/L McIlvaine buffer (pH 5.0), and disrupted by a vibrator in water bath at 40 °C for 30 min. Then it was filtered by filter paper and the clear filtrate was used for α-galactosidase activity measurement.

Enzyme assays

α-Galactosidase assay was carried out in test tubes by the modified version of the method of Garro *et al*.(2004). The reaction mixture contained: 10 mmol/L pNPG 50 μl, 100 mmol/L McIlvaine buffer pH 5.0 50 μl, cell-free extract 100 μl; final volume: 200 μl. The mixture was incubated at 50 °C for 10 min, and the reaction was stopped by adding 3 ml of sodium carbonate (0.25 mmol/L). One enzyme unit (U) was defined as the amount of enzyme that released 1.0 μmol of pNP from its substrate pNPG per min under the given assay conditions. The results are expressed as U/g dry matter.

Moisture content

The percentage moisture in the solid substrate was determined gravimetrically after drying the samples in an oven at 130 °C for 1.5 h.

RESULTS AND DISCUSSION

Effect of moisture content on α-galactosidase production

In general, the moisture level of the medium is regarded as a fundamental parameter for microbial growth and metabolite formation. Lower moisture level leads to sub-optimal growth, a lower degree of substrate swelling and high surface tension, whereas higher moisture level decrease porosity, which would cause lower oxygen transfer and heat dissipation and enhanced formation of aerial mycelium (Lonsane *et al*., 1985). This test studied the effects of moisture content on the enzyme production. The initial moisture contents (weight ratio of water to gross substrate) were adjusted to the range of 45%~75% (Fig.1). When the initial moisture content of the medium was 60%, α-galactosidase activity reached its peak value (1 677.1 U/g), after which it fell sharply. However, the range of 50%~65% initial moisture had no significant effect on α-galactosidase production. This result differs from that of Kotwal *et al*.(1998), who reported that maximum α-galactosidase activity was obtained when the initial moisture content was 86%. This may be due to the different culture temperatures.

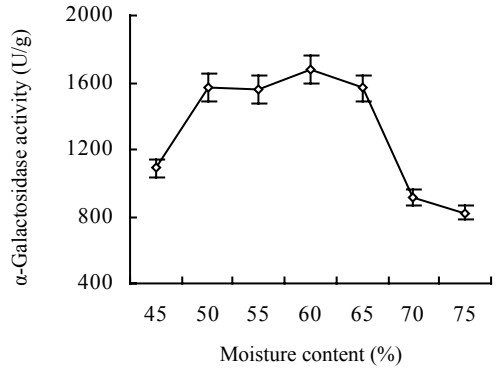

Fig.1 Effect of initial moisture content of medium on α-galactosidase production

Effect of incubation temperature on α-galactosidase production

Temperature is a factor that strongly influences culture growth, as in aerobic fermentations, a large amount of heat is produced during microbial growth. The effect of incubation temperature on α-galactosidase biosynthesis was investigated by incubating the inoculated standard basal media at different temperatures including 25 °C, 28 °C and 30 °C. Since 28 °C produced better enzyme yields as compared to 25 °C and 30 °C (Fig.2), the subsequent optimization studies were carried out at this incubation temperature. It was also found that the spores germinated very slowly when the incubation temperature was 25 °C.

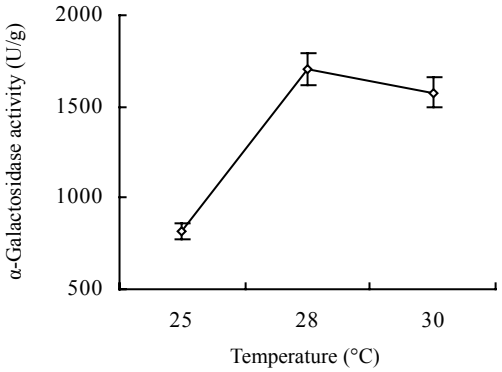

Fig.2 Effect of incubation temperature on α-galactosidase production

Effect of cultivation period of seed on α-galactosidase production

Short time cultivation period of seed will delay fungal growth, and long time will induce microorganism aging. Different incubation time of seed (1×10^6 spores/ml) was employed to study the effect on α-galactosidase production. The result is depicted in Fig.3. The maximum α-galactosidase production was obtained when seeds were cultivated at 18 h. With longer cultivation time the yield of α-galactosidase had decreasing trend. This further confirmed that the highest activity of enzyme would not arise until a sufficient biomass was formed. Further extending incubation time had no positive effect on increasing the enzyme yield.

Effect of inoculum volume on α-galactosidase production

Adequate inoculum can initiate fast mycelium growth and product formation, thereby reducing other organism contamination. The effect of inoculum volume on α-galactosidase production is shown in Fig.4. There was good α-galactosidase production when the inoculum size was 5%~20%. The maximum production occurred at 10% inoculum volume. A small quantity of inoculum obviously postponed

mycelia growth and reduced enzyme productivity. The higher the inoculum volume the more difficult the heat transfer is, although water-holding capacity of media is better.

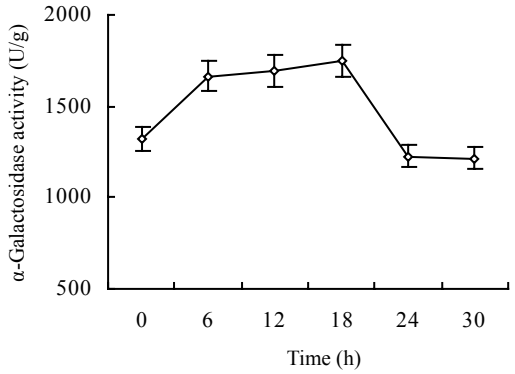

Fig.3 Effect of cultivation period of seed on α-galactosidase production

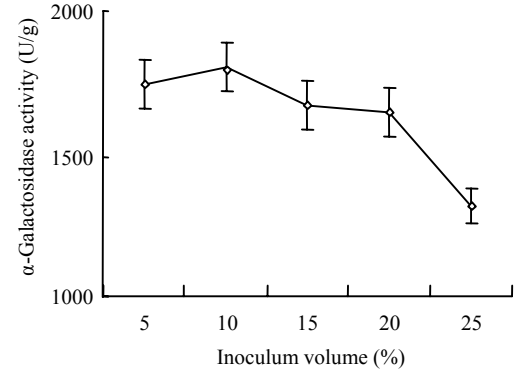

Fig.4 Effect of inoculum volume on α-galactosidase production

Effect of initial medium pH on α-galactosidase production

To investigate the effect of culture pH on α-galactosidase production, culture media was adjusted to different pH (4.5~6.5) with 100 mmol/L McIlvaine buffer. The results revealed that the active pH of the medium for enzyme production was 5.0~6.0 (Fig.5). It could be speculated that solid substrate contributed to a better buffering capacity. This optimum pH was a little lower than that reported by Wang *et al*.(2004) for his strain whose pH was 5.5~6.5.

Effect of layers of pledget on α-galactosidase production

In SSF, aeration has essential functions: oxygen supply for aerobic metabolism, and removal of CO_2, heat, water vapor, and volatile components produced during the metabolism. Aeration also has very important effects on hydration properties in SSF (Gervais and Molin, 2003). For good ventilation, different layer of pledget on α-galactosidase production was studied. The result revealed that 6 layers of pledget were better than 8 layers for enzyme production (Fig.6). This revealed good ventilation contributed to higher enzyme activity.

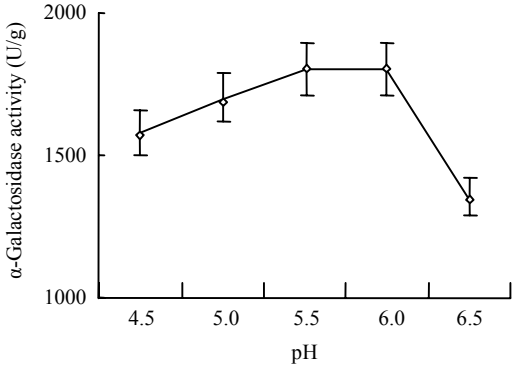

Fig.5 Effect of initial medium pH on α-galactosidase production

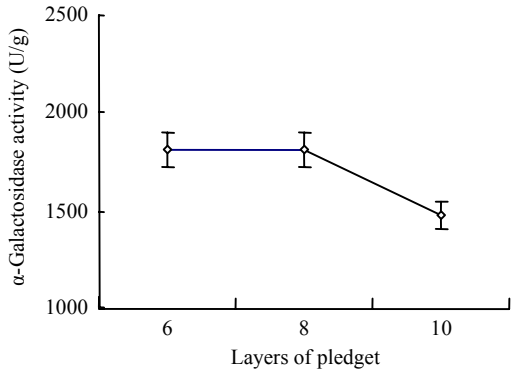

Fig.6 Effect of layers of pledget on α-galactosidase production

Effect of the amount of medium on α-galactosidase production

Lower loadage makes heat dissipation easy, but the loss of water would be fast. Thus, it is necessary to maintain an appropriate ratio of media weight to fermenter volume. The effect of the amount (5~15 g dry matter) of medium on enzyme production in 250 ml flasks is showed in Fig.7. The results revealed that 10 g dry matter of medium contributed to higher enzyme activity than other dry matter weight of medium, and that the maximum reached 1842.17 U/g dry matter.

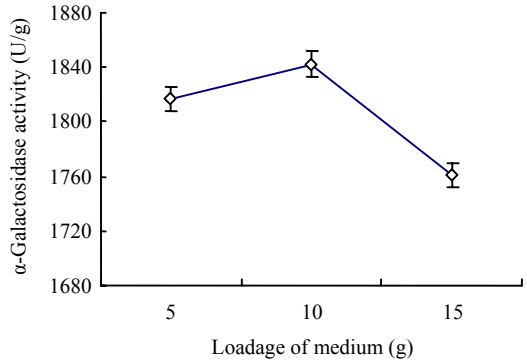

Fig.7 Effect of loadage of medium on α-galactosidase production

Effect of incubation time on α-galactosidase production

Different incubation time (0~192 h) was employed to study the effect on α-galactosidase production. The fermentation was carried out keeping all other conditions at their above optimum levels. Sampling per 12 h was prepared for assay. It is depicted in Fig.8. The maximum enzyme activity was found on the 144th hour of fermentation. The result was the same as that for submerged fermentation (data not publish). With extending cultivation time the yield of α-galactosidase had decreasing trend. This result confirmed that highest activity of enzyme would not occur until a sufficient biomass was formed, and that further extending incubation time had no effect on improving the enzyme yield.

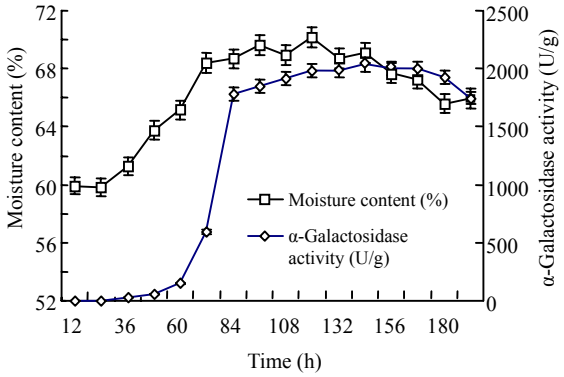

Fig.8 Effect of incubation time on α-galactosidase production

Analysis of the average moisture content at the end of the fermentation runs revealed that medium moisture content increased with α-galactosidase formation at early stages, but decreased at higher fermentation. The moisture content changes during the course of fermentation due to formation of metabolic water during fungi growth (von Meien and Mitchell, 2002), and condensate droplets that might be caused by small differences in air currents and evaporation which is required to remove heat generated by metabolism and utilization by fungi (Lonsane *et al*., 1985; Nagel *et al*., 2001a). The water activity a_w fluctuated near 0.983~0.988 during the fermentation, and optimum a_w for α-galactosidase biosynthesis was 0.987. As a whole, the moisture content and water activity were above the critical levels for growth, i.e. x_w>0.5 kg H_2O/(kg insoluble dry matter) and a_w>0.96 (Nagel *et al*., 2001b). This result indicates that the medium moisture content did not hamper fungal growth.

CONCLUSION

Laboratory-scale experiments may provide a basis for scaling-up of α-galactosidase production in SSF. Cultivating conditions for α-galactosidase biosynthesis by *Aspergillus foetidus* ZU-G1 in SSF was investigated. In our experiment, the substrate was broke up after inoculation. Mixing is needed to avoid aggregation of substrate, but frequent mixing did not benefit α-galactosidase production because of injury to the microbial cells. The optimal cultivation conditions of α-galactosidase in SSF was 60% moisture level of the medium, 28 °C incubation temperature, 18 h cultivation period of seed, 10% inoculum volume, 5.0~6.0 initial pH of medium, 6 layers of pledget and 10 g dry matter loadage. Under the above optimized cultivation conditions, the maximum α-galactosidase production was 2037.51 U/g dry matter near the 144th hour of fermentation. Future research will be focused on optimizing the key factors of culture conditions and nutrient to obtain higher α-galactosidase production at low cost, which is desirable for the industrial development.

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