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High pullulan yield is related to low UDP-glucose level and high pullulan-related synthases activity in *Aureobasidium pullulans* Y68

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Abstract - Effects of different pH and carbon sources on pullulan production, UDP-glucose level and pullulan-related synthases activity in *Aureobasidium pullulans* Y68 were examined. It was found that more pullulan was produced when the yeast strain was grown in the medium with initial pH 7.0 than when it was grown in the same medium with constant pH 6.0. The results also show that higher pullulan yield was obtained when the cells were grown in the medium containing glucose than when they were cultivated in the medium supplementing other carbon sources. Our results demonstrate that the more pullulan was synthesized, the less UDP-glucose was left in the cells of *A. pullulans* Y68. However, it was observed that more pullulan was synthesized; the cells had higher pullulan-related synthase activity. Therefore, high pullulan yield was related to low UDP-glucose level and high pullulan-related synthases activity in *Aureobasidium pullulans* Y68.

Key words: pullulan, *Aureobasidium pullulans*, UDP-glucose level, pullulan-related synthases activity.

INTRODUCTION

In recent years, extra-cellular polysaccharides (EPS) from microorganisms have received increasing attention since they have many potential applications in food, cosmetic, pharmaceutical and chemical industries (Yuen, 1974; Deshpande *et al*., 1992; Lee *et al*., 2001; Lazaridon *et al*., 2002). Due to high yield, easy purification, diverse chemical structure, unique properties and functions, EPS produced by microorganisms has many advantages. For example, pullulan which is a linear a-D-glucan, made mainly of maltotriose repeating units interconnected by a(1→6) linkages is the water-soluble homopolysaccharide produced extracellularly by the polymorphic micromycete *Aureobasidium pullulans* (Sutherland, 1998). Due to high yield, easy purification, unique properties and functions, pullulan produced by this microorganism has many advantages over by other organisms (Chi and Zhao, 2003). A number of potential applications have been reported for pullulan as a result of its good film-forming properties; pullulan can form thin films that are transparent, oil resistant and impermeable to oxygen. Pullulan may be used as coating and packing material; as a sizing agent for paper; as a starch replacer in food formulations, in cosmetic emulsions, and in other industrial and medical applications (Lazaridon *et al*., 2002). Pullulan can be chemically modified to produce medicines that have an anticoagulant, antithrombotic and antiviral activities and important materials that can be used in chemical industries (Alban *et al*., 2002).

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It has been noted that initial pH in the fermentation medium without pH adjustment has profound effects on both the rate of production and the synthesis of extracellular polysaccharide. In a previous study, the highest pullulan production in shaken cultures of *A. pullulans* Y68 was obtained when the initial pH of growth medium was 7.0 (Chi and Zhao, 2003). By contrast, optimal pullulan productions in liquid cultures of other *A. pullulans* strains were observed at distinct initial pH values (Auer and Seviour, 1990; Roukas and Liakopoulou-Kyriakides, 1999; Vijayendra *et al.*, 2001). This implies that the optimal initial pH values for pullulan production depend on different yeast strains, composition of the fermentation medium and growth conditions.

Grobben *et al*. (1996) showed that the proportions of glucose and fructose as carbon sources influenced both the amount and monomeric composition of the EPS produced by *L. delbrueckii* subsp. *bulgaricus* NCFB 2772. Both the amount of EPS production and the carbon source consumption rates were clearly influenced by the type of energy and carbon source used. A combination of lactose and glucose resulted in the largest amounts of EPS. It has been reported that the levels of activity of α-phosphoglucomutase, UDP-galactose 4-epimerase, and UDP-glucose pyrophosphorylase are highly correlated with the amount of EPS produced by *Streptococcus thermophilus* LY03 (Degeest and Vuyst, 2000). A three-fold increase in EPS production was attained when the same strain was grown on mixture of both glucose and fructose with respect to that observed on fructose as the sole carbon source. It was found that in cell-free extracts of glucose-grown cells the activity of UDP-glucose pyrophosphorylase was higher than

that in cell-free extracts of fructose-grown cells in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 (Grobben *et al*., 1996).

Relatively little is understood about the mechanisms of pullulan biosynthesis in *A. pullulans* although it has been applied to different fields in industry. So far, no enzymes involved in pullulan biosynthesis have been identified in *A. pullulans*. However, it has been reported that pullulan can be synthesized from sucrose by cell-free enzymes of *A. pullulans* in the presence of both ATP and UDP-glucose which suggested possible participation of glucosyltransferase action in the process (Taguchi *et al*., 1973). Furthermore, it was found that the pullulan chains or pullulan precursors originate from UDP-glucose. Catley and McDowell (1982) have proposed the following order of the biochemical events preceding pullulan formation. The first stage is the UDP-glucose-mediated attachment of a D-glucose residue to the lipid molecule (LPh) with a phosphoester bridge. A further transfer of the D-glucose residue from UDP-glucose gives lipid-linked isomaltose by glucosyltransferase.

We think that UDP-glucose pool and pullulan-related synthases (including glucosyltransferase) activity in the cells may be correlated with high pullulan production by *A. pullulans* Y68. Therefore, the main scope of this present study was to investigate effects of different pH, different carbon sources on pullulan production, UDP-glucose pool and pullulan-related synthases (including glucosyltransferase) activity in the cells of *A. pullulans* Y68.

MATERIALS AND METHODS

Yeast strain and cell cultivation. Yeast strain Y68 kept in this laboratory was maintained in YPD medium containing 10 g/l yeast extract, 20 g/l polypeptone, 20 g/l glucose and 20 g/l agar. The inoculum cultures were prepared by growing the yeast cells at 28 °C and 180 rpm for 24 h in the medium (pH 7.0) of the following composition: 20 g/l glucose, 20 g/l soybean cake hydrolysate, 5 g/l K_2HPO_4, 1 g/l NaCl, 2 g/l $MgSO_4{\cdot}7H_2O$, 0.6 g/l $(NH_4)_2SO_4$ (Chi and Zhao, 2003).

Preparation of soybean cake hydrolysate. Thirty two grams of soybean cake were mixed with 250 ml of tap water containing 0.25 N HCl. The mixture was autoclaved at 121 °C for 25 min. After cooling, pH of the mixture was adjusted to 7.0 with 1 N NaOH solution and the suspension was filtered. The filtrate was diluted to 800 ml (Chi *et al*., 2001).

DNA extraction and PCR. DNA extraction and PCR techniques for amplification of 18S rDNA in the yeast were performed according to the methods described by Chi *et al*. (2007). The 18S rDNA fragment inserted on the vector was sequenced by Shanghai Sangon Company.

Phylogenetic analysis. The sequence obtained above was aligned by using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST). For comparison with currently available sequences, we retrieved 20 sequences with over 97% similarity belonging to 20 different genera from NCBI server (http://www.ncbi.nlm.nih.gov) and performed multiple alignment by using Bioedit 7.0

Routine identification of the yeast. The routine methods for identification of the yeasts were performed by using the methods as described by Kurtzman and Fell (1998).

Fermentation. The fermentation was carried out in a 5-litre stirred tank fermentor (FMG-5l made in Shanghai Guoqiang Bioengineering Equipment CO., LTD) with a working volume of 3 l of the production medium containing 80 g/l glucose, 20 g/l soybean cake hydrolysate, 5 g/l K_2HPO_4, 1 g/l NaCl, 0.2 g/l $MgSO_4.7H_2O$, 0.6 g/l $(NH_4)_2SO_4$, pH7.0. The changes in pH, dissolved oxygen and temperature during the fermentation could be automatically recorded by the computer. The fermentor with 2.7 l of the production medium was sterilized at 121 °C for 30 min. After cooling, the medium was inoculated with 300 ml of inoculum to reach OD_{600nm} values ranging from 0.5 to 0.8. The fermentation was carried out at constant temperature of 28 °C, aeration rate of 6.5 l/min in the fermentor, agitation speed of 300 rpm and different constant pH 7.0, 6.0 and 5.0, respectively. After the fermentation started, the culture in the fermentor was collected for determination of content of EPS and cell dry weight at the time interval of 8 h.

Isolation and purification of extracellular polysaccharide. Aliquots of liquid cultures were incubated in a boiling water bath for 15 min, cooled at room temperature and then centrifuged at 12000 rpm, 4 °C for 8 min. Absolute ethanol (6 ml) was added to 3 ml of culture supernatant and the mixture was allowed to stand at 4 °C for 12 h to precipitate EPS. After removal of residual ethanol, the precipitate was dissolved in 3 ml of deionised water at 80 °C and the solution was dialyzed against deionised water for 48 h to remove small molecules in the solution. Then, the exopolysaccharide was precipitated again by using 6 ml of the cold ethanol; the precipitate was dried at 80 °C to a constant weight (Lee *et al*., 2001).

Preparation of cell free extract. In order to recover cells, aliquots of strain Y68 liquid cultures (15 ml), grown at 28 °C in the production medium, were centrifuged at 8000 rpm, 4 °C for 5 min. The pellet was washed three times with ice-cold distilled water and then suspended in 1 ml of ice-cold 0.1 M Tris-HCl buffer at pH 7.0 to yield a thick paste. The thick paste was homogenized in a DY89-I Type Electric Glass Homogenizer (Xinzhi, Zhejiang, China) and homogenization proceeded for 1 h on the ice. The cell debris was removed by centrifugation at 12000 rpm and 4 °C for 30 min and the supernatant obtained was used as the cell free extract. The amount of protein in the cell-free extract was determined by the method of Bradford, and bovine serum albumin served as standard (Bradford, 1976).

Determination of pullulan-related synthases activity. The pullulan-related synthases activity was measured according to the methods described by Taguchi *et al*. (1973). The reaction mixture contained 20 µmol of UDP-glucose, 40 µmol of ATP, 50 µl of 0.1 M Tris-HCl (pH 7.0), 50 µl of 0.1 M $MgCl_2$, and 50 µl of cell-free extract in total volume 250 µl. The mixture was incubated at 30 °C for 90 min, and boiled to stop the reaction. An aliquot of the reaction mixture was treated with 0.1 ml pullulanase (SIGMA, EC No. 2.32-983-9) at 40 ºC for 2 h, and then heated in

0.05 N HCl at 100 °C for 10 min to decompose UDP-Glucose left in the solution. The solution was neutralised, and analysed for reducing sugar by using the Nelson-Somogyi method (Spiro, 1966). Another aliquot was taken as blank from the boiled reaction mixture treated in 0.05 N HCl at 100 °C for 10 min, neutralised, and its reducing sugar content was determined. The value of reducing sugar in the blank was subtracted from that in the pullulanase-treated sample and reducing sugar was calculated as maltotriose. One unit of pullulan-related synthase is defined as the amount of the enzyme required to produce one μmol of reducing sugar per minute under the conditions described above. The specific pullulan-related synthases activity was defined as units per mg of protein in the cell-free extract.

Determination of UDP-glucose. One hundred microlitres of 0.1 M Tris-HCl buffer at pH 8.5 were added to a quartz microcuvette containing 40 μl of 26 μM of NAD^+, 100 μl of UDP-glucose solution (for standard curve) or cell-free extract and the final volume was adjusted to 1.0 ml by adding distilled water. Then UDP-glucose dehydrogenase (0.05 U) was added, the optical density was read at 340 nm at 1-minute intervals immediately, and the reading was continued until no further reaction was detected (Strominger *et al*., 1957; Ma and Joachim, 2001).

Determination of cell dry weight. The yeast cells from 3.0 ml of culture were harvested and washed three times with distilled water by centrifugation at 4000 rpm for 5 min. Then, cells in the tube were dried at 100 °C until the cell dry weight was constant (Chi *et al*., 2001).

RESULTS AND DISCUSSION

Phylogenetic analysis

18S rDNA sequence of yeast strain Y68 was deposited in NCBI server and the accession number is DQ278883. Phylogenetic analysis of twenty 18S rDNA sequences with over 97% similarity belonging to twenty different genera from NCBI server shows that the similarities between 18S rDNA sequences of yeast strain Y68 and *Aureobasidium pullulans* were 100% while results of routine identification of yeasts also show that yeast strain Y68 was closely related to *Aureobasidium pullulans* (data not shown). Therefore, the yeast strain Y68, that was previously assigned to the species *Rhodotorula bacarum* on the basis of BIOLOG analysis (Chi and Zhao, 2003), was unequivocally identified as *Aureobasidium pullulans*. Several studies have shown that EPS produced by distinct *A. pullulans* strains are pullulan or pullulan-like polymers (Sutherland, 1998; Lazaridon *et al*., 2002).

Effect of different constant pHs on pullulan production

In our previous study (Chi and Zhao, 2003), we found that initial pH in the fermentation had great influence on pullulan production by *A. pullulans* Y68. The optimal initial pH for pullulan production by *A. pullulans* Y68 liquid culture was 7.0 in both shaken flasks and stirred tank fermentor. This may imply that it is crucial to keep constant pH around 7.0 in the fermentation medium in order to produce higher yield of pullulan. Therefore, the effects of constant pHs of 5.0, 6.0, 7.0 on pullulan production by *A. pullulans* Y68 were tested, respectively, in a 5-litre stirred tank fermentor in which pH value was kept constant. Fig. 1 shows that pullulan production by *A. pullulans* Y68 was negatively affected when pH of growth medium was kept constant at both 5.0 and 7.0. In particular, at pH 7.0 pullulan production was 2.43 g/g of cell dry weight within 88 h of fermentation, whereas 12.6 g pullulan/g of cell dry weight within the same time when pH was kept constant at 6.0. Furthermore, from the results in Fig. 1, it is very interesting to observe that more pullulan production (14.7 g/g of cell dry weight) was attained when initial pH of the fermentation medium without pH adjustment was 7.0 than when constant pH of the fermentation medium was 6.0. These results show that pullulan production was highly affected by medium pH and point out that the best pullulan production by the strain under the study did not require pH maintenance at the set initial value of 7.0.

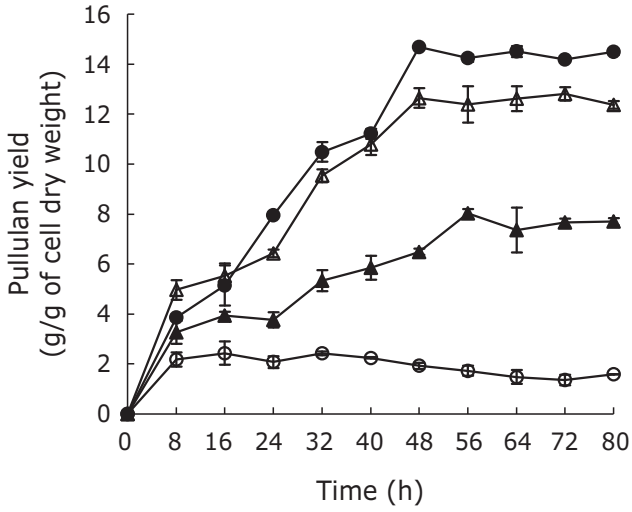

FIG. 1 - Effects of different pHs on pullulan yield. Constant pH 7.0 (○), constant pH 6.0 (△) , constant pH 5.0 (▲) and initial pH 7.0 (●). Data are given as means ± SD, n = 3.

Effects of different carbon sources on pullulan production

Several studies have shown that different carbon sources had profound influences on exopolysaccharide yield by lactic bacteria (Grobben *et al*., 1996; Vijayendra *et al*., 2001). However, little is known about effects of different carbon sources on pullulan production by *A. pullulans*. The results in Fig. 2 indicate that the highest pullulan yield was obtained when the cells were grown in the medium containing glucose. Grobben *et al*. (1996) suggested that the lower production of exopolysaccharides in cultures grown on fructose might be caused by the more complex pathway involved in the synthesis of sugar nucleotides. In *A. pullulans*, glucose can be converted into UDP-glucose directly for biosynthesis of pullulan (Catley and McDowell, 1982). However, other carbon sources, such as xylose must be converted into glucose, and then glucose is converted into UDP-glucose for biosynthesis of pullulan. Therefore, the lower production of pullulan in cultures grown on other car-

bon sources may also be caused by the more complex pathway involved in the synthesis of UDP-glucose.

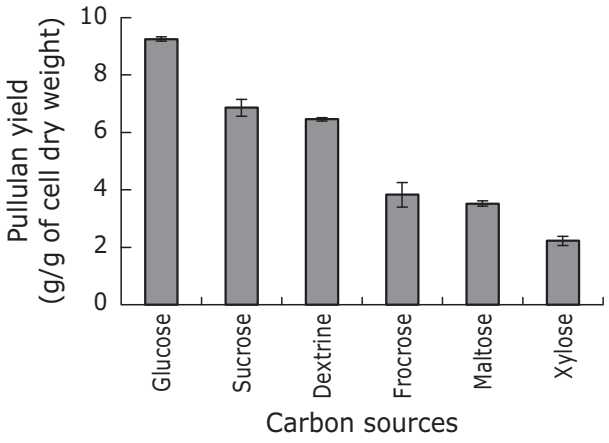

FIG. 2 - Effect of different carbon sources on pullulan yield by strain Y68. Data are given as means ± SD, n = 3.

Determination of UDP-glucose

UDP-glucose is the only precursor for pullulan biosynthesis in *A. pullulans* (Shingel, 2004). In yeasts, UDP-glucose is the glucosyl donor for the synthesis of cell-wall glucan, glycogen, trehalose and is also involved in N-glycosylation of proteins (Daran *et al*., 1995). Therefore, it must play a very important role in pullulan production. It has been reported that the more exopolysaccharide is synthesized, the more UDP-glucose exists in the cells of lactic bacteria (Degeest and Vuyst, 2000). However, it is very interesting to note that less UDP-glucose was detected in the cells of *A. pullulans* Y68 when they grew in the medium with initial pH 7.0 than when they grew in the medium with constant pH 6.0 (Fig. 3). It has been shown in Fig. 1 that the pullulan yield was much higher when strain Y68 was grown in the medium with initial pH 7.0 than when it was grown in the medium with constant pH 6.0. For example, after the cells were grown in the production medium with initial pH 7.0 within 64 h of fermentation, the pullulan yield was 14.5 g/g of cell dry weight and UDP-glucose level was 0.48 mg per mg of protein while the pullulan yield was 12.61 g/g of cell dry weight and UDP-glucose level was 2.38 mg/mg of protein after the cells were grown in the production medium with constant pH 6.0 within the same period of the fermentation. The results in Fig. 1 and Fig. 4 indicate that more pullulan was produced and less UDP-glucose level was detected when the yeast strain was grown in the medium containing glucose than when grown in the same medium containing other carbon sources.

Analysis of the intracellular level of sugar nucleotides in glucose-grown cultures of *L. delbrueckii* subsp. *bulgaricus* showed the presence of UDP-glucose and UDP-galactose in a ratio of 3.3:1, respectively, a similar ratio and slightly lower concentrations were found in fructose-grown cultures (Degeest and Vuyst, 2000). In contrast, our results show that the more pullulan was synthesized, the less UDP-glucose was left in the cells of *A. pullulans* Y68 (Fig. 1-4). Therefore, the results were not in agreement with those from lactic bacteria (Degeest and Vuyst, 2000). Consequently, it might be suggested that the more pullulan was produced, the more UDP-glucose was converted into pullulan and therefore the less UDP-glucose was left in the cells of strain Y68.

Pullulan-related synthases activity

Taguchi *et al*. (1973) reported that pullulan was synthesized from UDP-glucose by cell-free extract prepared from *A. pullulans*. Therefore, pullulan-related synthase activity in the presence of UDP-glucose, ATP and the cell-free extract from *A. pullulans* Y68 was determined according to the procedures described by Taguchi *et al*. (1973). The results in Fig. 5 indicate that higher pullulan-related synthases activ-

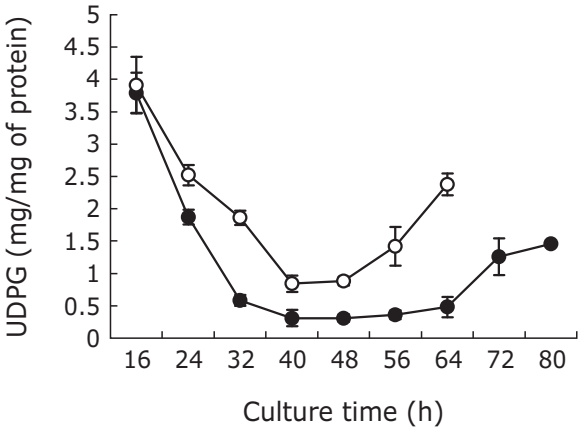

FIG. 3 - Effects of different pH in the medium on UDPG levels. Initial pH 7.0 (●) and constant pH 6.0 (○). Data are given as means ± SD, n = 3.

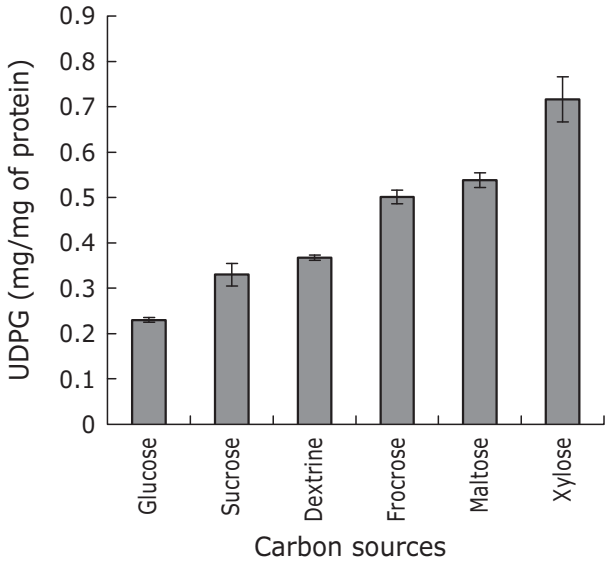

FIG. 4 - Effects of different carbon sources on UDP-glucose levels. Data are given as means ± SD, n = 3.

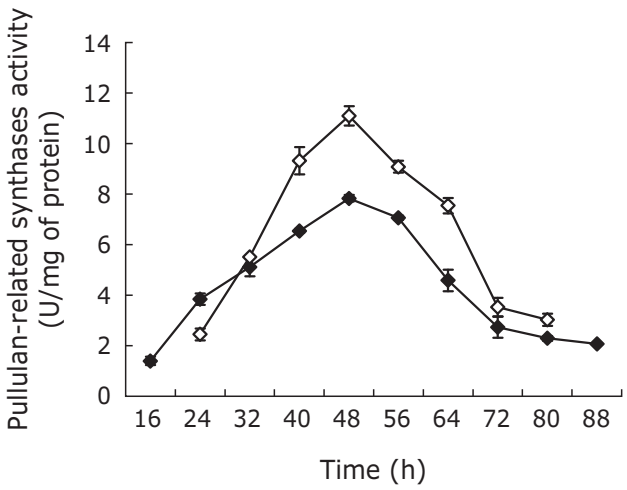

FIG. 5 - Effects of different pHs on pullulan-related synthases activity. Initial pH 7.0 (◇); constant pH 6.0 (◆). Data are given as means ± SD, n = 3.

ity was detected in the cells of *A. pullulans* Y68 when they grew in the production medium with initial pH 7.0 than when they grew in the medium with constant pH 6.0. It has been shown in Fig. 1 that the pullulan yield was much higher when strain Y68 was grown in the medium with initial pH 7.0 than when it was grown in the medium with constant pH 6.0. For example, after the cells were grown in the medium with initial pH 7.0 within 64 h of the fermentation, the pullulan yield was 9.25 g/g of cell dry weight and pullulan-related synthase activity was 7.54 U/mg of protein while the pullulan yield was 6.86 g/g of cell dry weight and pullulan-related synthase activity was 4.58 U/mg of protein after the cells were grown in the production medium with constant pH 6.0 within the same period of the fermentation. Meanwhile, the pullulan-related synthases activity in the cell-free extract was 5.26 U/mg of protein when the cells were cultivated in the production medium with glucose while the pullulan-related synthases activity in the cell-free extract was less than 4.33 U/mg of protein when it was cultivated in the medium containing other carbon sources (Fig. 6). Therefore, our results demonstrate that more pullulan was synthesized when the cells had higher pullulan-related synthases activity. Therefore, it is very important how to stimulate pullulan-related synthases activity in strain Y68 in order to enhance pullulan yields.

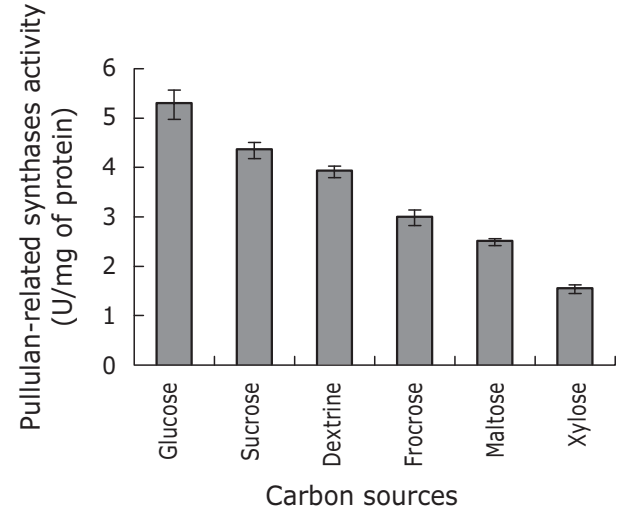

FIG. 6 - Effects of different carbon sources on pullulan-related synthases activity. Data are given as means ± SD, n = 3.

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