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Influence of different sugars on pullulan production and activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase and glucosyltransferase involved in pullulan synthesis in *Aureobasidium pullulans* Y68

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Abstract

Effects of different sugars on pullulan production, UDP-glucose level, and activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase and glucosyltransferase in *Aureobasidium pullulans* Y68 were examined. It was found that more pullulan was produced when the yeast strain was grown in the medium containing glucose than when it was cultivated in the medium supplementing other sugars. Our results demonstrate that when more pullulan was synthesized, 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 activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase and glycosyltransferase. Therefore, high pullulan yield is related to high activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase and glucosyltransferase in *A. pullulans* Y68 grown on different sugars. A pathway of pullulan biosynthesis in *A. pullulan* Y68 was proposed based on the results of this study and those from other researchers. This study will be helpful to metabolism-engineer the yeast strain to further enhance pullulan yield.

Keywords: Pullulan; *Aureobasidium pullulans*; UDP-glucose level; Glucosyltransferase activity

1. Introduction

Pullulan which is a linear α-D-glucan, made mainly of maltotriose repeating units interconnected by α(1 → 6) linkages is the water-soluble homopolysaccharide produced extracellularly by the polymorphic micromycete *Aureobasidium pullulans* (Sutherland, 1998). So far, a number of applications have been reported for pullulan (Shingel, 2004; Sutherland, 1998).

In our previous studies (Chi & Zhao, 2003), it was found that yeast strain Y68 that was isolated from the leaves in China could produce very high level of pullulan. This strain was finally reidentified as a strain of *A. pullulans* according to the results of routine identification of the yeasts and 18S rDNA sequence (Duan, Chi, Li, & Gao, 2007).

Grobben, M.R., Sikkema, and de Bont (1996) showed that the proportions of glucose and fructose as carbohydrate sources influenced both the amount and monomeric composition of the exopolysaccharide (EPS) produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772. Both the amount of EPS produced and the carbohydrate source consumption rates were clearly influenced by carbohydrate sources 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 (Degeest & Vuyst, 2000). The bacterial

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strain produced 25 mg/l exopolysaccharide when it was grown on fructose, while the carbohydrate source was switched to a mixture of fructose and glucose, the exopolysaccharide production increased to 80 mg/l (Degeest & Vuyst, 2000). 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 (Grobben et al., 1996). However, relatively little is understood about the mechanisms of pullulan biosynthesis in *A. pullulans* although it has been applied to different fields in industry and intense investigations of cytological and physiological characteristics of *A. pullulans* have been carried out. So far, no enzymes involved in pullulan biosynthesis have been identified at this time (Shingel, 2004). Pullulan can be synthesized from sucrose by cell-free enzymes of *A. pullulans* when both ATP and UDP-glucose are added to a reaction mixture (Shingel, 2004). It was found that the pullulan chains or pullulan precursors originate from UDP-glucose (Shingel, 2004). Cately and McDowell (1982) have proposed the order of the biochemical events preceding pullulan formation. We thought that UDP-glucose pool and glucosyltransferase activity in the cells of *A. pullulans* may be correlated with high pullulan production. Therefore, the main scope of this present study was to investigate effects of different sugars on pullulan production, UDP-glucose pool and activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase and glucosyltransferase in the cells of *A. pullulans* Y68.

2. Materials and methods

2.1. Yeast strain and cell cultivation

Aureobasidium pullulans Y68 kept in this laboratory was maintained in YPD medium containing (g/l) yeast extract 10.0, polypeptone 20.0, glucose 20.0 and agar 20.0. The seed cultures in 250-ml flasks were prepared by growing the yeast cells from the slant at 28 °C and 180 rpm for 24 h in 50 ml of the medium (pH 7.0) of the following composition (g/l): glucose 20.0, soybean cake hydrolysate 20.0, K_2HPO_4 5.0, NaCl 1.0, $MgSO_4{\cdot}7H_2O$ 0.2, $(NH_4)_2SO_4$ 0.6 (Chi & Zhao, 2003). All the chemicals were purchased from Sigma.

2.2. Preparation of soybean cake hydrolysate

Thirty two grams of soybean cake were mixed with 250 ml of 0.25 N HCl. The mixture was autoclaved at 115 °C for 25 min. After cooling, pH of the mixture was adjusted to 7.0 with 1.0 M NaOH solution and the suspension was filtered. The filtrate was diluted to 800 ml (Chi, Liu, & Zhang, 2001).

2.3. Pullulan production

The pullulan production was carried out in a 5 l stirred tank fermentor (FMG-5 l made in Shanghai Guoqiang Bioengineering Equipment Co., Ltd.) with a working volume of 3 l of the production medium containing (g/l) different sugars (glucose, fructose, xylose, maltose, sucrose and dextrin) 80.0, soybean cake hydrolysate 20.0, K_2HPO_4 5.0, NaCl 1.0, $MgSO_4{\cdot}7H_2O$ 0.2, $(NH_4)_2SO_4$, 0.6, pH 7.0. pH, dissolved oxygen, agitation speed, temperature and aeration rate in the vessel can be monitored and controlled automatically. The fermentor with 2700 ml of the production medium was sterilized at 121 °C for 30 min. After cooling, the medium was inoculated with 300 ml of the seed culture to make $OD_{600\,nm}$ value of the initial culture be 0.5–0.8. The pullulan production was carried out at constant temperature of 28 °C, aeration rate of 6.5 l/min agitation speed of 300 rpm and pH 7.0, respectively. After cell growth started, the culture in the fermentor was collected for determination of content of exopolysaccharide and preparation of cell-free extract at the time interval of 8 h.

2.4. Isolation and purification of extracellular polysaccharide

The culture grown for 3 days in the fermentor as described in Section 2.3 was heated at 100 °C in water bath for 15 min, then was cooled to room temperature. The heated culture was centrifuged at 14,046*g* and 4 °C for 8 min to remove cells and other precipitates. Supernatant (3.0 ml) was transferred into a test tube, then 6.0 ml of cold ethanol (absolute ethanol or 95% ethanol) was added to the test tube and mixed thoroughly and held at 4 °C for 12 h to precipitate the extracellular polysaccharide. After removal of residual ethanol, the precipitate was dissolved in 3.0 ml of deionized water at 80 °C and the solution was dialyzed against deionized water for 48 h to remove small molecules in the solution. After the exopolysaccharide was precipitated again by using 6.0 ml of the cold ethanol, the precipitate was dried at 80 °C to a constant weight (Lee et al., 2001).

2.5. Monosaccharide release and preparation of glucose derivates and GC–MS analysis

In order to analyze compositions of pullulan produced from different sugars, monosaccharide release from the purified pullulan and preparation of glucose derivates were performed as described by Chi, Su, and Lu (2007). Inositol was used as internal standard. GC–MS analysis of monosaccharide was carried out by using GC6890–MS5973N (Agilent Company, USA) (Chi et al., 2007). The chromatography column was a fused silica HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness); injector temperature: 220 °C; carrier gas: Helium, 1.0 ml/min; temperature program: 50 °C, held for 2.0 min, from 50 to 150 °C at 8 °C per min, held for 2.0 min, then to 250 °C at 6 °C per min, held for 3.0 min; injection volume: 1.0 ml; ionization mode: electron impact at 70 eV; temperature of ion source: 230 °C; temperature of quadrupole: 150 °C; acquiring mode: scan, from *m/z* 50 to *m/z* 550; Solvent delay: 4.0 min.

2.6. Preparation of cell-free extract

The cells in 5.0 ml of the yeast culture during pullulan production as described in Section 2.3 were collected by centrifugation at 8000*g* and 4 °C for 5 min and washed three times with ice-cold distilled water. The pellet was suspended in 1.0 ml of ice-cold 1.0 M Tris–HCl (pH 7.6) to make 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 14,006*g* and 4 °C for 30 min and the supernatant obtained was the cell-free extract (also the enzyme preparation). Protein concentration in the cell-free extract was measured by the method of Bradford, and bovine serum albumin served as standard (Bradford, 1976).

2.7. Determination of UDP-glucose

One hundred microliters of 0.1 M Tris–HCl (pH 8.5), 40.0 μl of 26 μM of NAD, 100 μl of UDP-glucose solution (for standard curve) or cell-free extract were added to a quartz microcuvette having a light path of 1 cm and a final volume was adjusted to 1.0 ml using distilled water. After UDP-glucose dehydrogenase (0.05 U) was added, the optical density was read at 340 nm at 1-min intervals immediately, and the reading was continued until no further reaction was detected (Ma & Stöckigt, 2001; Strominger, Elizabeth, & Kalckar, 1957). The mixture without 100 μl of UDP-glucose solution or cell-free extract was used as control.

2.8. Enzymes assays

Glucosyltransferase activity was determined according to methods described by McCleary et al. (1989). A suitably diluted enzyme preparation (0.2 ml) was incubated with pre-equilibrated *p*-nitrophenyl α-D-glucopyranoside (0.2 ml, 10 mM) in 0.1 M sodium acetate buffer (pH 4.0) for 5 min at 40 °C. The reaction was terminated by the addition of an aqueous solution of Trizma base (Sigma; 3.0 ml, 2.0% w/v), and the absorbance at 410 nm was measured. Enzyme activity is expressed in terms of μM of *p*-nitrophenol released/min and calculated by reference to a standard curve (the standard curve was made by measuring $OD_{410\,nm}$ values of the solutions with different concentrations of *p*-nitrophenol). The mixture with the diluted enzyme preparation that had been heated at 100 °C for 5 min was used as the control.

UDPG-pyrophosphorylase activity was measured according to Dutra, Silva, Aattos, and Panek (1996). The reaction mixture contained 1.4 mM $NADP^+$, 4.0 mM $MgCl_2$, 1.0 mM UDP-glucose, 10 μM glucose 1,6-diphosphate, 66 mU of α-phosphoglucomutase, 220 mU of glucose 6-phosphate dehydrogenase, 75 mM Tris–HCl (pH 7.0) and 10 μl of a suitably diluted enzyme preparation in a final volume of 0.5 ml. The assay was developed in a 1 cm path cuvette in a spectrophotometer at 30 °C. Absorbance at 340 nm was measured. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1.0 μM of substrate in 1 min under the assay conditions. The mixture with the diluted enzyme preparation that had been heated at 100 °C for 5 min was used as the control.

Measurement of α-phosphoglucomutase activity was performed according to methods described by Qian, Stanley, Hahn-Hagerdal, and Prådstrom (1994). The reaction mixture contained 50 μM triethanolamine hydrochloride buffer (pH 7.5), 0.5 μM $MgCl_2$, 0.4 μM $NADP^+$, 0.05 μM glucose 1,6-diphosphate, 0.01 ml of glucose 6-phosphate (180 U/ml) dehydrogenase (5.0 U), and the cell-free extract. 1.0 μM glucose 1-phosphate was added to start the reaction. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1.0 μM of substrate in 1 min under the assay conditions. The mixture with the cell-free extract that had been heated at 100 °C for 5 min was used as the control.

2.9. Determination of cell dry weight

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

3. Results

3.1. Effects of different sugars on cell growth and pullulan production

Several studies have shown that different carbon sources had profound influences on EPS yield by bacteria (Grobben et al., 1996; Velasco et al., 2007). However, little is known about effects of different carbon sources on cell growth and pullulan production by *A. pullulans*. The results in Table 1 indicate that pullulan yield was the highest when the cells were grown in the medium containing glucose (8.0%) while the pullulan yield was much lower when carbon sources in the medium were sucrose, fructose, xylose, dextrin and maltose (8.0%), respectively. This means that glucose is the best carbon source for pullulan production by *A. pullulans* Y68. It also can be seen from Table 1 that cell growth was the poorest when xylose was used as the carbon source.

3.2. Change of UDP-glucose level

It has been well known that UDP-glucose plays a pivotal role in the intermediary metabolism of yeast since it serves as a glucosyl donor in numerous biosynthesis pathways including the biosynthesis of reserve carbohydrates, formation of cell wall β-glucans and glucomannoproteins, protein *N*-glycosylation and galactose entry into glycolysis

Table 1
Effects of different sugars on cell growth and pullulan production

Sugars	Glucose	Sucrose	Dextrin	Fructose	Maltose	Xylose
Cell dry weight (g/l)	9.45 ± 0.27	10.52 ± 0.67	10.97 ± 0.90	10.12 ± 0.42	9.19 ± 0.64	6.78 ± 0.58
Pullulan yield (g/l)	52.47 ± 0.09	40.54 ± 1.36	38.83 ± 0.25	25.54 ± 1.79	23.26 ± 0.78	15.72 ± 3.98

The initial sugar concentrations in the media were 8.0% (w/v). Data are given as means ± SD, $n = 3$.

(Daran, Bell, & Francois, 1997). It also has been reported that UDP-glucose cannot be replaced by ADP-glucose during pullulan production by *A. pullulans*, indicating that the pullulan chains or pullulan precursors originate from UDP-glucose (Shingel, 2004). As shown in Fig. 3 below, UDP-glucose is the only precursor for pullulan biosynthesis in *A. pullulans*. Therefore, it must play a very important role in pullulan production. It has been shown that when more EPS is synthesized, more UDP-glucose exists in the cells of lactic bacteria (Grobben et al., 1996). However, it is very interesting to observe from the results in Fig. 1 that less UDP-glucose was detected in the cells of *A. pullulans* Y68 when it was cultivated in the medium with glucose (8.0%) than when it was cultivated in the medium containing other sugars (8.0%). As indicated in Table 1, pullulan yield was the highest when the yeast was grown in the medium containing glucose. Therefore, our results demonstrate that when more pullulan was synthesized, less UDP-glucose was left in the cells of *A. pullulans* Y68. This clearly indicates that our results were not in agreement with those from lactic bacteria (Grobben et al., 1996). It may be suggested that when more pullulan was produced, more UDP-glucose was converted into pullulan so that less UDP-glucose was left in the cells of strain Y68.

It has been confirmed that UDP-glucose is formed from UTP and glucose 1-phosphate in reverse reaction catalyzed by UDP-glucose pyrophosphorylase (Daran et al., 1997; Shingel, 2004):

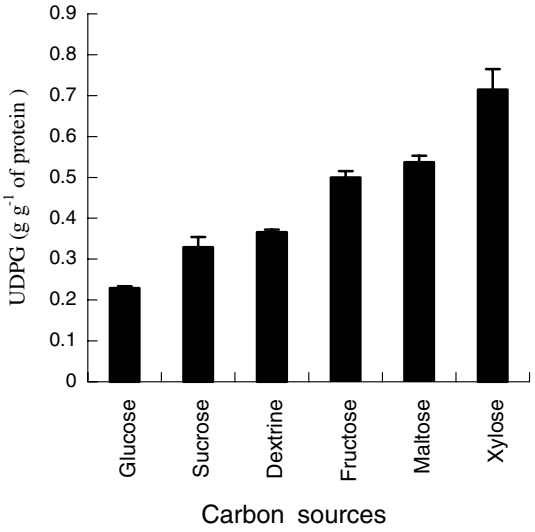

Fig. 1. Effects of different sugars on UDP-glucose level. The initial sugar concentrations in the medium were 8.0% (w/v). Data are given as means ± SD, $n = 3$.

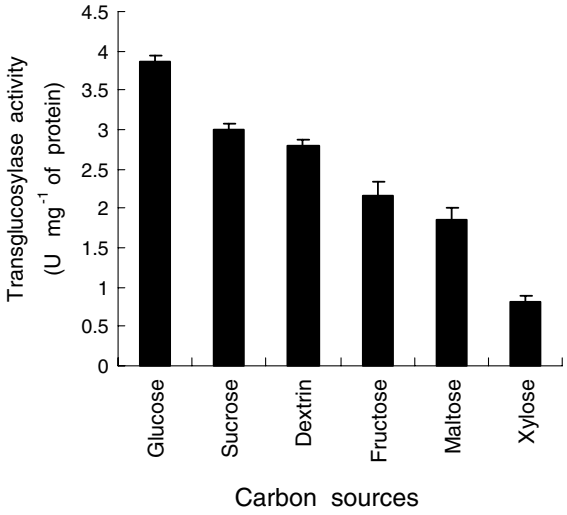

Fig. 2. Effects of different sugars on glucosyltransferase activity. The initial sugar concentrations in the media were 8.0% (w/v). Data are given as means ± SD, $n = 3$.

UTP + glucose-1-phosphate → UDP-glucose + PPi.

This is an essential step for the formation of UDP-glucose, a precursor for pullulan synthesis in *A. pullulans* (Shingel, 2004). Therefore, UDP-glucose pyrophosphorylase, the key enzyme involved in the sugar metabolism and potentially involved in the production of precursors for pullulan biosynthesis in *A. pullulans* Y68 grown on different carbohydrates was analyzed. The results in Table 2 demonstrate that UDP-glucose pyrophosphorylase activity in *A. pullulans* Y68 grown on glucose (8.0%) was higher than that in the cultures grown on other carbohydrates (8.0%). From the results in Fig. 1 and Table 2, it may be concluded that when less UDP-glucose was left in the cells of *A. pullulans* Y68, more UDP-glucose pyrophosphorylase activity was detected. However, the activity of the UDP-glucose pyrophosphorylase was very low in *Pediococcus parvulus* 2.6, irrespective of the carbon source (Velasco et al., 2007).

Glucose 1-phosphate is one of the substrates for biosynthesis of UDP-glucose and is formed from glucose 6-phosphate catalyzed by phosphoglucomutase (Daran et al., 1997; Grobben et al., 1996). Therefore, phosphoglucomutase activity also affects the biosynthesis of UDP-glucose. So, the activity of phosphoglucomutase, the key enzyme involved in the sugar metabolism and potentially involved in the production of precursors for pullulan biosynthesis in *A. pullulans* Y68 grown on different carbohydrates was also

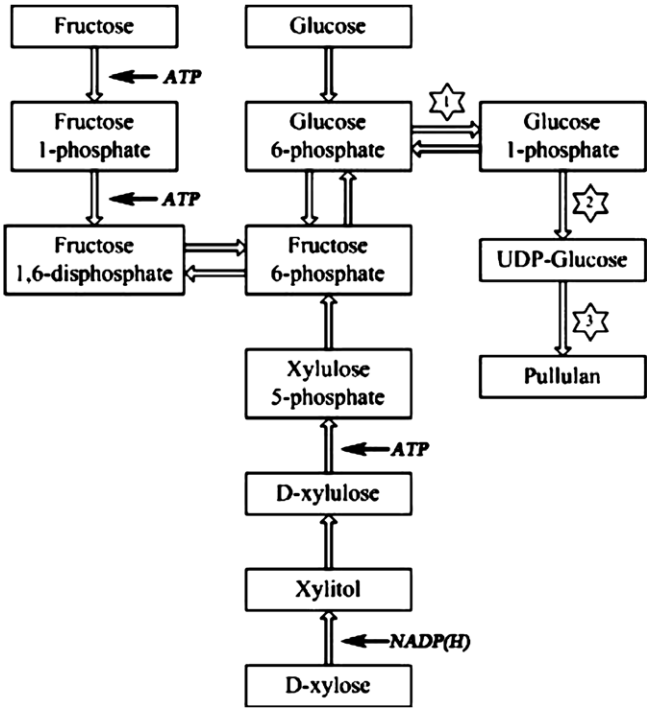

Fig. 3. Proposed pathway of pullulan biosynthesis in *A. pullulans* Y68. 1, α-phosphoglucose mutase; 2, UDPG-pyrophosphorylase; 3, glucosyltransferase.

analyzed. The results in Table 2 demonstrate that phosphoglucomutase activity in *A. pullulans* Y68 grown on glucose (8.0%) was also higher than that in the cultures grown on other carbohydrates (8.0%). Combining the results in Fig. 1 and Table 2, we can make a conclusion that the lower the level of UDP-glucose was left in the cells of *A. pullulans* Y68, the more phosphoglucomutase activity was detected. The results obtained above demonstrate that both activities of UDP-glucose pyrophosphorylase and phosphoglucomutase were enhanced on glucose cultivated cells, showing a high correlation with pullulan production. Velasco et al. (2007) also reported phosphoglucomutase activity in *Pediococcus parvulus* 2.6 grown on glucose was significantly higher than that grown on fructose.

3.3. Changes in glucosyltransferase activity

Certain experimental results indicate that glucose-containing lipid intermediates play a crucial role in pullulan biosynthesis (Shingel, 2004). Cately and McDowell (1982) have proposed the following order of the biochemical events preceding pullulan formation. The first stage is UDPG-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. In the next step, isomaltose participates in the reaction with lipid-linked glucose to yield an isopanosyl residue. Further, isopanosyl residues are polymerized into the pullulan chain (Daran et al., 1997). Therefore, we conclude that glucosyltransferase in *A. pullulans* Y68 may play an important role in biosynthesis of pullulan with high yield. So, the level of glucosyltransferase activity, the key enzyme potentially involved in the production of pullulan in *A. pullulans* Y68 grown on different carbohydrates, was determined. The results in Fig. 2 reveal that glucosyltransferase activity in *A. pullulans* Y68 grown on glucose (8.0%) was higher than that in the cultures grown on other carbohydrates (8.0%). Combining the results in Table 1 and Fig. 2, it may be suggested that the higher the level of glucosyltransferase activity existed, the higher the amount of pullulan was produced by the cells of *A. pullulans* Y68.

4. Discussion

A pathway of pullulan biosynthesis in *A. pullulan* Y68 is proposed in Fig. 3 based on the results of this study and those from other researchers (Daran et al., 1997; Grobben et al., 1996; Jeffries, 2006; Shingel, 2004).

Our results show that the pullulan yield was the highest when *A. pullulans* Y68 is grown in a medium containing glucose (Table 1). In our previous study (Chi & Zhao, 2003), we confirmed that the yeast strain could produce very high level of pullulan within the short period and the purified polysaccharide from the culture was shown to be pullulan by analyzing hydrolysates of the purified pullulan using pullulanase. The GC–MS analysis performed in this study demonstrates that the pullulan produced from different sugars contains only glucose (Fig. 4). However, the yield of pullulan was very low when the yeast strain was grown in a medium containing maltose, fructose or xylose (Table 1). It has been reported that a low exopolymer synthesis was also observed when *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 was grown in the fructose-containing medium (Grobben et al., 1996). In the case of this study, the lower amount of pullulan produced by *A. pullulans* Y68 from fructose and xylose may be caused by the longer biosynthetic pathway leading from fructose and xylose to UDP-glucose as shown in Fig. 3. It is very interesting to note that the glucosyltransferase activity was also the highest when the yeast strain was cultivated

Table 2
Effect of different sugars on UDPG-pyrophosphorylase and phosphoglucomutase activity

	Glucose	Sucrose	Dextrin	Fructose	Maltose	Xylose
UDPG-pyrophosphorylase activity (mU/mg of protein)	94.5 ± 3.3	77.6 ± 1.4	75.1 ± 0.8	39.7 ± 5.8	28.8 ± 2.1	7.2 ± 0.6
Phosphoglucomutase activity (mU/mg of protein)	13.6 ± 0.3	9.2 ± 0.49	8.4 ± 0.05	3.7 ± 0.03	3.4 ± 0.29	1.8 ± 0.02

The initial sugar concentrations in the media were 8.0% (w/v). Data are given as means ± SD, $n = 3$.

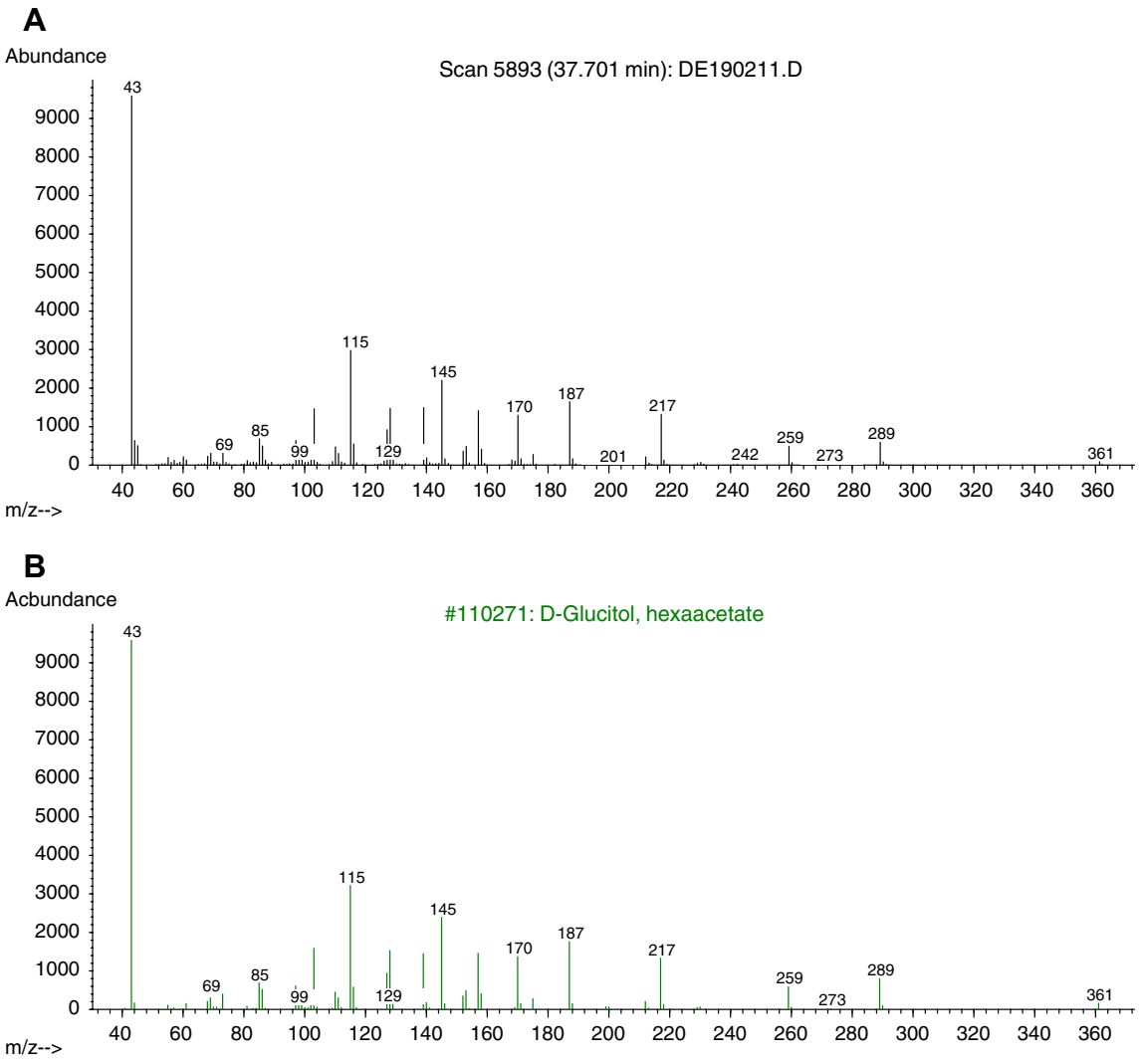

Fig. 4. The MS spectra of the monosaccharide alditol of hydrolysate of the purified pullulan produced from glucose, fructose, xylose, maltose, sucrose and dextrin, respectively (A) and standard D-glucitol (B). Because the MS spectra of the monosaccharide alditol of hydrolysate of the purified pullulan produced from glucose, fructose, xylose, maltose, sucrose and dextrin, respectively, are the same, here only the MS spectra of the monosaccharide alditol of hydrolysate of the purified pullulan produced from glucose are listed.

in the medium containing glucose (Fig. 2). We think that most of UDP-glucose was used to synthesize pullulan when the glucosyltransferase activity was very high, leading to very low UDP-glucose level in the yeast cells (Fig. 1). This may imply that very high glucosyltransferase activity was the unique characteristics of *A. pullulans* Y68 which can produce high yield of pullulan (Chi & Zhao, 2003). Because the phosphoglucomutase and UDPG-pyrophosphorylase activity in the yeast cells grown in the medium containing glucose was also very high (Table 2), UDP-glucose was synthesized continuously to supply the precursors for high pullulan synthesis (Fig. 3) when the very high glucosyltransferase activity occurred in the cells of *A. pullulans* Y68. However, UDP-glucose was left when the yeast cells were grown in the medium containing xylose and fructose, respectively (Fig. 2), due to low glucosyltransferase activity (Fig. 2). In order to metabolism-engineer the yeast strain to further enhance pullulan yield, purification and characterization of glucosyltransferase and the cloning of the gene encoding glucosyltransferase from *A. pullulans* Y68 are being undertaken in this laboratory.

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