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Diversity of sugar alcohols produced by *Aureobasidium* spp. and the influence of selected nutrients on mannitol synthesis

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ABSTRACT

Sugar alcohols are a group of diverse polyols derived from monosaccharides that have been used in many commercial products. Mannitol, a six-carbon sugar alcohol, is a natural sweetener produced by many plants such as pumpkin, celery and olive, and microorganisms including yeast, bacteria, fungi, lichen and algae. Mannitol has been widely used as a sugar substitute in food and pharmaceutical industries, especially the products for diabetic patients, since it does not affect blood sugar and insulin levels. Mannitol can be produced via fermentation by yeasts such as *Candida* spp. and *Aureobasidium* spp. In nature, most microorganisms produce mannitol from glucose, fructose, sucrose and glycerol with different efficacies. Thus, this study was aimed to (i) study the diversity of polyols produced by seven *Aureobasidium* strains including *A. pullulans* (NRRL Y-2311-1, CBS 135684, PBUAP55 and PBUAP102) *A. thailandense* (NRRL58543, PBUAP17 and PBUAP70) (ii) select the best strain for mannitol production and (iii) investigate the effect of carbon and nitrogen sources on the mannitol production. It was found that these *Aureobasidium* strains were able to produce a variety of sugar alcohols including mannitol, arabitol, xylitol and glycerol in the strain-dependent manner. *Aureobasidium thailandense* PBUAP70 produced the highest yield of mannitol at 14% conversion comparing with the rest by using glucose as substrate. Glucose and ammonium sulphate were found

to be enhanced the mannitol production of this strain up to 16% conversion which was the highest of the carbon and nitrogen sources tested.

Keywords: *Aureobasidium* ; Sugar alcohol ; Fermentation

INTRODUCTION

Sugar alcohols known as polyhydric alcohols or polyols are hydrogenated carbohydrates. They can be acquired when carbonyl oxygens of the sugars are reduced to polyhydroxyl alcohol. Sugar alcohols are commonly used in food and pharmaceutical industries due to their excellent properties such as sweet taste with low calories, cool mouthfeel and non-cariogenic effect (Park et al., 2016).

Mannitol is a six-carbon polyol which is the most abundant polyol in nature. It is commonly found in fruits, vegetables, and microbial fermentation products. In plant, mannitol serves as a carbon stockpiling compound for reducing power. In fungi, mannitol is an important storage-carbon for spore germination under starvation conditions (Upadhyay et al., 2015). In *Agaricus* mushroom, mannitol also involves in regulating the intracellular NADP/NADPH balance (Meng et al., 2017). Mannitol can be produced by two routes, chemical and biological processes. Industrial mannitol is commercially produced by a chemical process via the catalytic dehydrogenation of a hexose at high temperature and high pressure (Zada et al., 2017). However, this process has disadvantages including low mannitol yield and the formation of by-products such as sorbitol. Therefore, biological processes via microbial fermentation are of great attention since they are free of those problems, give a higher yield and are environmental friendly (Savergave et al., 2013). It has been reported that *Lactobacillus* bacteria and yeasts such as *Candida mannitofaciens*, *Candida magnoliae*, *Yarowwia lipolytica* and *Aureobasidium pullulans* can produce mannitol and other sugar alcohols (Chalfan et al., 1975; Saha and Nakamura, 2003; Onishi and Suzuki, 1970; Song et al., 2002; Rywińska et al., 2015). *Aureobasidium* is a yeast-like fungus commonly found worldwide. Several strains of genetically diverse *Aureobasidium* spp. have been isolated in Thailand, and they were reported to produce many biotechnologically valuable products (Punnapayak et al., 2003; Lotrakul et al., 2009; Prasongsuk et al., 2018). Therefore, it is of interest to investigate the ability to produce sugar alcohols, including mannitol, of Thai *Aureobasidium* yeasts.

In this study, seven chosen strains of *Aureobasidium* spp. were screened for their sugar alcohol production profile and the best mannitol producing strain was selected. The effects of carbon and nitrogen sources on its mannitol production were studied. The results gave a useful data for the development of future commercial mannitol production.

MATERIAL AND METHODS

Microorganisms and culture maintenance

Aureobasidium pullulans PBUAP55, *A. pullulans* PBUAP102, *A. pullulans* CBS 135684, *A. thailandense* NRRL58543, *A. thailandense* PBUAP17 and *A. thailandense* PBUAP70 from Plant Biomass Utilization Research Unit and *A. pullulans* NRRL Y-2311-1 from Microbial Genomics and Bioprocessing Research Unit, the United States Department of Agriculture, were used in this study. For long term storage, the yeasts were cultured in Yeast Malt broth (YMB, (all w/v) 2% glucose, 0.5% yeast extract, 0.5% malt extract and 0.5% peptone) at room temperature (28±2°C), 150 rpm for 3 days before they were transferred into eppendorf tubes containing 10% (w/v) skim milk and lyophilized. The lyophilized cells were kept at 4°C. For short term storage, all strains were maintained on YM agar (YMA) and kept at 4°C.

Screening of sugar alcohol production

The seed culture of each strain was prepared by growing in 20 mL of YMB at room temperature (28 ± 2 ºC), 150 rpm for 3 days. The seed culture was adjusted to 1×10^8 cells/mL before it was inoculated (200 µL) into 20 mL of the standard production medium ((all w/v) 5% glucose, 0.2% $(NH_4)_2SO_4$, 0.2% yeast extract, 0.5% KH_2PO_4, and 0.04% $MgSO_4$(Ping et al., 2013). The cultures were grown under the same condition for another 6 days before sugar alcohol determination.

Determination of sugar alcohols

Supernatants were harvested by centrifugation at 6,000 × g for 10 min and filtered through a 0.45 µm nylon membrane before the quantitative determination of sugar alcohols via High Performance Liquid Chromatography (HPLC) (Waters, alliance 2690, USA) using the Agilent MetaCarb 87H organic acids column (Varian, USA) at 40°C. The mobile phase was 5.0 mM H_2SO_4 with a flow rate of 0.6 mL/min (Guo et al., 2016). The refractive index detector was used to detect the sugar alcohols.

Determination of nutritional factors on sugar alcohol production

Effects of three different carbon (glucose, glycerol and molasses, each at 3% (w/v)) and four different nitrogen sources (peptone, $NaNO_3$, $(NH_4)_2SO_4$ and $(NH_4)_2C_2O_4$, each at 0.2% (w/v)) on sugar alcohol production of the selected *Aureobasidium* strain were determined using one-factor-at-a-time approach. The seed culture preparation and cultivation conditions were carried out as described above. Supernatants were harvested on day 6 and determined for sugar alcohols as described above.

Statistical analysis

All experiments were conducted in triplicate. Data were presented as mean ± one standard deviation and subjected to analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) using SPSS Statistics version 23 (International Business Machines Corporation, USA). Significant differences were considered at $p \leq 0.05$.

RESULTS

Diversity of sugar alcohols produced by *Aureobasidium* spp.

Seven strains of *Aureobasidium* spp. were screened for diversity of sugar alcohols produced. The results were shown in Table 1. Mannitol, arabitol and glycerol were produced by *Aureobasidium* spp. when 5% (w/v) glucose was used as substrate. Three strains, *A. thailandense* PBUAP70 *A. pullulans* NRRL Y-2311-1 and *A. thailandense* PBUAP17, were able to produce all of the aforementioned sugar alcohols whereas *A. pullulans* PBUAP102 produced mannitol and arabitol, but not glycerol. *Aureobasidium thailandense* NRRL58543 and *A. pullulans* CBS 135684 both only produced mannitol. No sugar alcohol was detected in *A. pullulans* PBUAP55 culture. The highest percent conversion of sugar alcohols was observed in *A. thailandense* PBUAP70 culture at 14%, 2.3% and 1.6% for mannitol, arabitol and glycerol, respectively (Table 1). Therefore, *A. thailandense* PBUAP70 was selected for further production study.

Table 1. Sugar alcohol production by *Aureobasidium* spp.

Microorganism	Mannitol (g/g glucose)	Arabitol (g/g glucose)	Glycerol (g/g glucose)
A. pullulans NRRL Y-2311-1	0.112 ± 0.428	0.021 ± 0.055	0.007 ± 0.027
A. pullulans CBS 135684	0.048 ± 0.367	ND	ND
A. pullulans PBUAP102	0.056 ± 0.291	0.008 ± 0.023	ND
A. pullulans PBUAP55	ND**	ND	ND
A. thailandense PBUAP70	0.141 ± 0.191	0.023 ± 0.059	0.016 ± 0.039
A. thailandense NRRL58543	0.016 ± 0.263	ND	ND
A. thailandense PBUAP17	0.043 ± 0.463	0.010 ± 0.060	0.004 ± 0.021

*Data were shown as mean ± one standard deviation derived from three replications.
**ND: not detectable

Effect of carbon and nitrogen sources on mannitol production.

In a preliminary study, a higher percent conversion was obtained when glucose was added to the medium at 3 % (w/v) than at 5 % (w/v) (data not shown). Therefore, the concentration of carbon source used in this experiment was reduced accordingly. When each carbon source was added to the standard production medium containing 0.2 % (w/v) $(NH_4)_2SO_4$ as nitrogen source, it was found that glucose (0.168 ± 0.011) was the best carbon source for mannitol production by *A. thailandense* PBUAP70 comparing with glycerol (0.054 ± 0.004) and molasses (not detectable) (Figure 1). Therefore, glucose was selected as the carbon source for the next experiment.

To further determine the effect of nitrogen source on mannitol production, the standard production medium containing 3 % (w/v) glucose as carbon source was combined with each of the following nitrogen sources: peptone, $NaNO_3$, $(NH_4)_2SO_4$, $(NH_4)_2C_2O_4$. It was found that $(NH_4)_2SO_4$ was the best nitrogen source for mannitol production at 0.115 ± 0.008 (Figure 2). A much lower conversion was found when peptone (0.040 ± 0.008), $NaNO_3$ (0.024 ± 0.009), and $(NH_4)_2C_2O_4$ (0.011 ± 0.005) were used. Thus, $(NH_4)_2SO_4$ was chosen as nitrogen source for mannitol production.

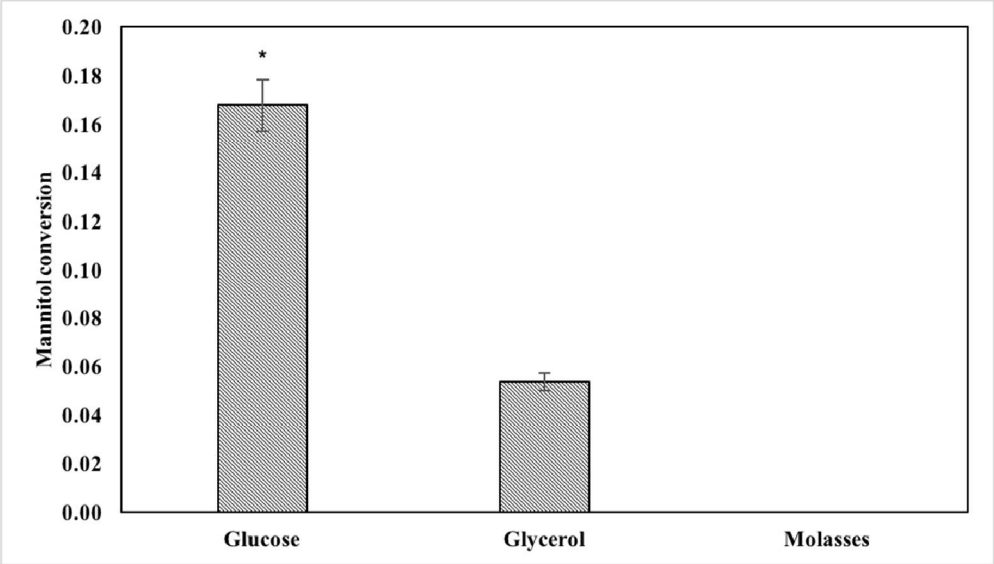

Figure 1. Effects of carbon sources on mannitol production by *A. thailandense* PBUAP70. Data were shown as average values. Bars represented one standard deviation derived from three replications. Asterisk indicated significantly different values ($p \leq 0.05$).

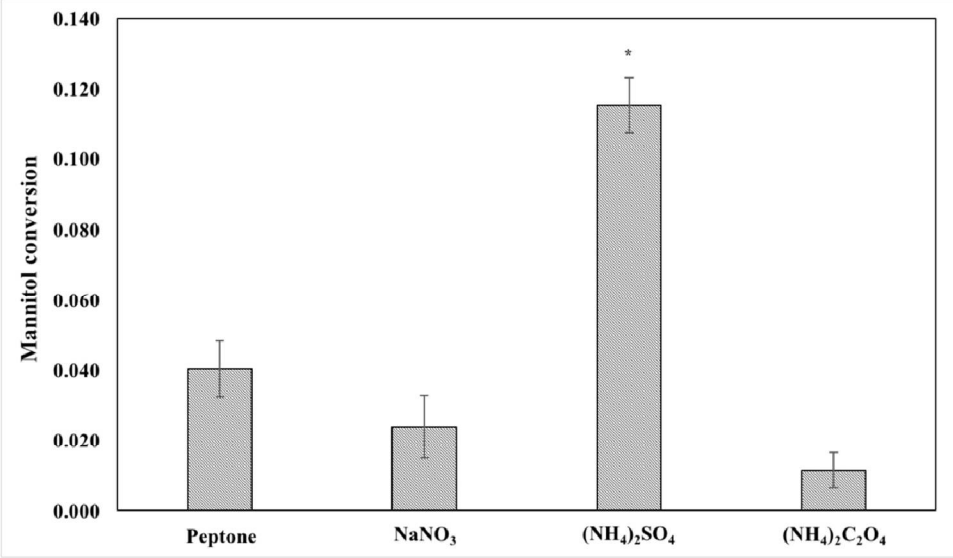

Figure 2. Effects of nitrogen sources on mannitol production by *A. thailandense* PBUAP70. Data were shown as average values. Bars represented one standard deviation derived from three replications. Asterisk indicated significantly different values ($p \leq 0.05$).

DISCUSSION

Different strains of *Aureobasidium* spp. had different capabilities of sugar alcohol production, both in term of diversity and yield (Table 1). Similar results have previously been reported that 10 strains of *Candida magnolaie* can produce various polyols with different production yields. It was suggested that the sugar alcohol production was dependent on several factors including yeast species and strains, the phase of growth and the carbon source (H. Van Eck et al., 1993; Savergave et al., 2013). In the case of mannitol, it was the major sugar alcohol produced by six out of the seven strains of *Aureobasidium* spp. tested in this study. It has been reported earlier that some Thai *Aureobasidium melanogenum* strains accumulated mannitol under stress condition (Yanwisetpakdee et al., 2016). Therefore, mannitol seems to be a universal sugar alcohol for this yeast genus and the ability to produce sugar alcohol may not be species specific.

Aureobasidium thailandense PBUAP70 preferred glucose to glycerol and molasses for mannitol production with the conversion efficiency as high as 16%. It seems that the carbon source preference is varied among yeast species and strains. *Candida magnolia* was able to produce mannitol at 45% conversion using fructose (15% w/v) as the substrate (Song et al., 2002) while *Yarrowia lipolytica* produced mannitol from glycerol (25% w/v) at 15% conversion (Tomaszewska et al., 2012). It has been reported that many microorganisms are able to produce mannitol by consuming substrates such as glucose, fructose, xylose, and glycerol (Dai et al., 2017). Occasionally, mannitol could be produced by a combination of substrates. For instance, *C. magnolia* produced mannitol at 71% conversion when a mixture of glucose (50% w/v) and fructose (25% w/v) was used (Lee and et al., 2003) while a combination of glucose (5% w/v) and fructose (10% w/v) as the substrates enhanced mannitol production by *Lactobacillus fermentum* up to 89.6 % conversion (Waymarn et al.,2002).

There have been report that $(NH_4)_2SO_4$, $NH_4(C_2H_4(OH)COO)$, KNO_3, casamino acids, corn steep liquor and urea were able to use for mannitol production (Onishi and Suzuki, 1970). In this study, we found that $(NH_4)_2SO_4$ was the best nitrogen source for mannitol production by *A. thailandense* PBUAP70 comparing with other nitrogen sources (Figure 2). Similarly, *Yarrowia lipolytica* used $(NH_4)_2SO_4$ as the nitrogen source gave the highest erytritol and mannitol yields (Rywinska et al., 2015). However, the presence of ammonium also causes the reduction of the medium pH during fermentation which might decrease polyalcohol yield (Onishi et al., 1959). Therefore, both

carbon and nitrogen sources are crucial factors that affect the microbial polyol production and they are strain-dependent preference. Further determination of the optimal glucose and $(NH_4)_2SO_4$ concentrations is required to enhance the mannitol production by *A. thailandense* PBUAP70.

CONCLUSION

Aureobasidium spp. produced various sugar alcohols, including mannitol, arabitol and glycerol and its production capability seemed to be strain dependent. Among the seven strains tested, *A. thailandense* PBUAP70 was the best strain with the highest percent mannitol conversion. Both of carbon and nitrogen sources strongly affected its mannitol production. A combination of glucose and $(NH_4)_2SO_4$ was suitable for mannitol production by *A. thailandense* PBUAP70 with the conversion rate around 12-16 %. Compared to some other fungi, the mannitol production efficiency of *A. thailandense* PBUAP70 was relatively moderate, but there are still more spaces to improve. Further optimization, i.e. mixed carbon source, optimal nutrient concentration and production period, might be able to enhance the production efficiency of this yeast strain and thus could improve its potential for the future commercial application.

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