



Mannitol Production from Fructose by Using Resting Cells of Methylotrophic Yeasts

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Abstract

Background: Mannitol is a polyol sugar widely used in pharmaceutical and food industries which can be produced by bioconversion. Using of resting cells and methanol as a carbon source are strategies to increase the efficiency of mannitol production by increasing NAD(P)H needed in the reduction process. **Objectives:** This research aimed to optimize bioconversion condition by using resting cells of methylotrophic yeasts with methanol and fructose as carbon source and substrate, respectively. **Methods:** Several isolates were used including *Candida* sp, *Debaryomyces nepalensis* and *Debaryomyces hansenii* and three species suspected to be yeast isolated from a local paddy field. The methylotrophic characteristic of the yeasts was screened by turbidometry. The optimization of fermentation condition was conducted by varying cultivation time (24-96 hours), resting cell concentration (30-140 mg/mL), fructose concentration (7.5-15%), ammonium sulphate concentration (0.25-0.75%) and aeration condition (50-80%). Quantitative analysis of the mannitol was conducted by HPLC with NH₂ column and Refractive Index Detector. **Results:** *D. hansenii* showed the highest yield value in mannitol production (23.17%), followed by *D. nepalensis*, Isolate A and *Candida* sp. (6.52%, 6.50% and 4.38%, respectively). Variation of bioconversion condition using *D. hansenii* showed that the highest resting cell concentration (140 mg/mL) incubated for 72 hours, moderate fructose concentration (10%), the highest ammonium sulphate concentration (0.75%) and moderate aeration condition (70%) would result in the highest yield value of mannitol (60%). **Conclusion:** This finding showed the potency of *D. hansenii* in mannitol production and gave preliminary information of its optimum fermentation condition.

Keywords: resting cell, methylotrophic yeast, mannitol, fructose, methanol

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INTRODUCTION

Mannitol is a polyol sugar with the lowest glycemic index (Msomi *et al.*, 2021). Mannitol is used not only in the food industry but also in the pharmaceutical industry, as an active ingredient and excipient (Shi *et al.*, 2020; Yang *et al.*, 2021), particularly as tablet diluent. The advantages of mannitol as a diluent are its low hygroscopicity and pleasant taste (Kosugi *et al.*, 2020; Martau *et al.*, 2021). Currently, industrial production of mannitol is done with catalytic hydrogenation. However, this method provides low efficiency and comprises complex purification steps (Mérillon & Ramawat, 2018).

Bioconversion technology for producing mannitol using lactic acid bacteria, yeast and fungi has been studied to increase its efficiency on industrial scale. In microorganism cells, mannitol is produced with a reduction process from fructose, catalyzed by mannitol dehydrogenase (MDH) enzyme (Gonçalves *et al.*, 2019; Lu *et al.*, 2019).

Several research have been conducted to optimize the efficiency of the bioconversion by using methylotrophic yeasts, which are able to use methanol as carbon source (Yurimoto *et al.*, 2011). Compared to sugars, methanol has higher reduction degree by which the yield value will increase. In the bioconversion process, Nicotinamide Adenine Dinucleotide (Phosphate) Hydrogen (NAD(P)H) is required to reduce fructose into mannitol. Using methanol, NAD(P)H is produced from oxidation of methanol into formaldehyde catalyzed by methanol dehydrogenase (Wang *et al.*, 2019).

Another strategy to increase the efficiency of mannitol production is resting cells. Resting cells are cells that are not active in growing, but cell metabolisms are still going on. This characteristic allows minimum carbon and energy use for biomass production. Instead, those are used for oxidation-reduction (Jackson *et al.*, 2019; Ng, 2020). Mannitol production by resting cell of *Escherichia coli* BL21 and *Candida magnoliae* has been studied previously and showed 82% and 45% yield value, respectively (Khan *et al.*, 2009; Koko *et al.*, 2021). However, studies on utilizing the resting cell of methylotrophic yeast for mannitol production have not been published. Therefore, this research was performed to identify the ability of resting cells from several methylotrophic yeasts to make mannitol, including optimising the fermentation condition. The change in yield value with varying fermentation conditions showed the significance of each variable in mannitol production, by which efficiency of the mannitol

production could be increased, especially on an industrial scale.

MATERIALS AND METHODS

Materials

Three strain of yeasts were used in this study, including *Candida* sp. UICC Y216, *Debaryomyces hansenii* UICC Y276 and *Debaryomyces nepalensis* UICC Y328 (University of Indonesia Culture Collection). In addition, species expected to be yeast, isolated from local paddy field (Jl. Sindang Barang, Dramaga, Bogor Barat, Bogor, Indonesia), was also used.

Four types of medium were used in this study, including medium for isolation, cultivation, preculture and fermentation. An isolation medium was used for yeast isolation from paddy soil. It was a selective media, composed of 0.5 g of ammonium sulphate, 0.05 g of magnesium sulphate, 0.35 g of disodium hydrogen phosphate, 0.3 g of kalium dihydrogen phosphate, 0.05 g of chloramphenicol and 0.01 g of yeast extract (Asthana *et al.*, 1971). Chloramphenicol was added to the isolation media. The composition of each 100 mL of cultivation medium was 2 mL of glucose, 2mL of peptone, 1 g of yeast extract and 1.5 g of agar (Lai *et al.*, 2019).

According to Suryadi *et al.* (2000), the fermentation was done in two steps with some modifications. The media used in the first steps was preculture media composed of 1 mL of methanol, 1 mL of fructose, 0.5 g of ammonium sulphate, 0,05 g magnesium sulphate, 0.35 g disodium hydrogen phosphate, 0.3 g potassium dihydrogen phosphate and 0.01 g yeast extract in 100 mL of distilled water. At the second step, the media used was fermentation media composed of 1% methanol and 10% fructose.

Chemicals used in this study were methanol (JT Baker), acetonitrile (JT Baker), fructose (Merck), glucose (Merck), mannitol (Merck), ammonium sulphate (Merck), magnesium sulphate (Merck), dinatrium hydrogen phosphate (Merck), kalium dihydrogen phosphate (Merck), chloramphenicol, yeast extract (Bacto) and 1.5 g of agar (Wako), glucose (Merck), peptone (Liofilchem), yeast extract (Bacto) and 1.5 g of agar (Wako).

Instruments

Shaking bath incubator (Labline), pH meter (Eutech), microscope (Euromex), centrifuge (Kubota 6800), HPLC (Shimadzu LC-20AD), refractive index detector (Shimadzu RID-10A), degasser (Shimadzu DGU-20A5), HPLC oven column (Shimadzu CTU-

6AS), Carbohydrate Analysis NH₂ column (3,9 mm x 300 mm, 10 µm) (Waters) and Spectrophotometry UV-Vis (Shimadzu 1601).

Methods

Isolation of yeast from paddy soil

The soil was taken from 2-10 cm depth of the paddy field. Paddy soil was suspended with serial concentrations (10⁻¹, 10⁻², 10⁻³, 10⁻⁴ g/mL). The suspension (0.1 mL) was then incubated in an isolation medium for 2-3 days. Then, the cells grown were recultivated in new isolation medium with the addition of 1% methanol, for 2-3 days in at room temperature. The isolation medium was adapted from Asthana et al., (1971).

Screening of paddy soil isolates

Isolates grown from paddy soil were screened for the methylotrophic ability as well as the ability to produce mannitol. Screening for methylotrophic ability was conducted by cultivation of the isolates in the preculture media while screening for mannitol production was done in the preculture media containing 5% of fructose.

Determination of cell concentration

Cell suspension in the preculture media was centrifuged and the supernatant was filtered through 0.22 µm filter. The biomass was determined turbidometrically at 600 nm with Spectrophotometry UV-Vis (Shimadzu UV 1601) (Suryadi *et al.*, 2000). Dry cell weight was determined by centrifugation and wash of cell suspension. The supernatant was then dried at 110°C for 7 hours and then triplicate weighing was performed. From this procedure, one OD unit was shown corresponded to 2.26 mg/mL dry cells.

Fermentation

Three loopful of cells were cultivated in 100 mL of preculture media for 2 days with 175 rpm shaking in room temperature (Kasbawati *et al.*, 2022). Resting cells were then made from preculture cell suspension by centrifugation at 8000 rpm, 5°C for 10 minutes. The cells were resuspended in sterile NaCl 0.9% solution and then centrifuged at 8000 rpm, 5°C for 10 minutes. This procedure was done with two replicates to get clean resting cells (Khan *et al.*, 2009). The resting cells were then cultivated in the fermentation media, incubated at 175 rpm with shaking incubator in room temperature. Optimization of fermentation condition was conducted by the following factors: cultivation time (24, 48, 72 and

96 hours); resting cell concentration (300, 500, 100 and 1400 mg), fructose concentration (7,5; 10; 12.5 and 15%), ammonium sulphate concentration (0.25; 0.50 and 0.75%.) and aeration condition (varying the volume of fermentation media into 10, 15, 20 and 25 mL in 50 mL Erlenmeyer.).

Quantitative analysis of substrate and product

Fructose and mannitol calibration curves had previously been prepared. The substrate (fructose) and product (mannitol) were then quantified from 1 mL of fermentation culture. The culture was centrifuged at 6000 rpm for 10 minutes, and the supernatant was filtered through a 0.22µm filter. HPLC was then used to analyze the supernatant. (Shimadzu model LC-20AD), refractive index detector (Shimadzu RID-10A), degasser (Shimadzu DGU-20A5), oven column (Shimadzu CTU-6AS), Waters Carbohydrate Analysis NH₂ column (3.9 mm x 300 mm, 10 µm), with mobile phase acetonitrile:distilled water (97:3). The column was run at a temperature of 298 K, the flow rate of 1.0 mL/min and and injection volume of 20 µL.

RESULTS AND DISCUSSION

Macroscopic and microscopic visualization of isolates were depicted in Figure 1. All isolates have glossy surfaces and circular edges. The color of isolate A, B and C colonies were yellow, bright yellow and white, respectively. Microscopically, all isolates were circular. Similar media was used by other research previously and isolated 2 yeast species and 6 Actinomycetes bacteria (Asthana *et al.*, 1971).

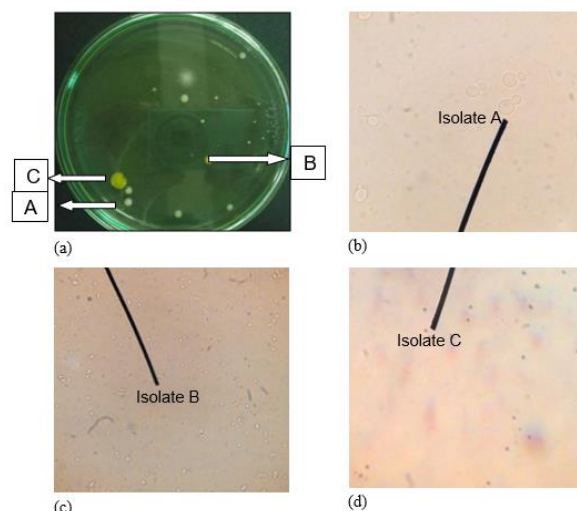


Figure 1. Yeast colonies (A) and microscopic (100 x magnification) examination of isolates (B, C, D)

Table 1. Yield value of mannitol from fermentation with different yeasts

Yeast	Yield Value
<i>Debaryomyces hansenii</i>	23.17%
<i>Debaryomyces nepalensis</i>	6.52%
Isolate A	6.50%
<i>Candida sp.</i>	4.38%
Isolate B	Undetected
Isolate C	Undetected

The cell's ability to use methanol as a carbon source was screened based on the change in Optical Density (OD) after incubation in preculture media for three days at room temperature. It was found that all isolates were methylotrophs. However, the ability to produce mannitol was only shown by UICC strains and isolate A. The highest mannitol yield was achieved by *D. hansenii* (23.17%), followed by *D. nepalensis*, isolate A and *Candida sp* (Table 1). The *D. hansenii* has long been known for its potency in polyol production, including arabitol and xylitol (López-Linares *et al.*, 2018; Mardawati *et al.*, 2019). The yeast was reported as a higher xylitol producer than *Candida sp* (Breuer & Harms, 2006; Loman *et al.*, 2018; Rice *et al.*, 2020).

As *D. hansenii* showed the highest yield value of mannitol among the 5 other tested yeasts, variation of fermentation condition was then only conducted with this microorganism. The result of bioconversion to produce mannitol with each fermentation condition variation using *D. hansenii* was presented in Table 2. Addition of the resting cell at the optimum concentration (140 mg/mL) leads to an increase in the mannitol yield from the first to the third day. However, the yield value of mannitol was decreased on the fourth day. On the contrary, the amount of fructose decreased from the first to the third day but increased on the fourth day (Figure 2), indicating that mannitol had been re-converted into fructose on day 4.

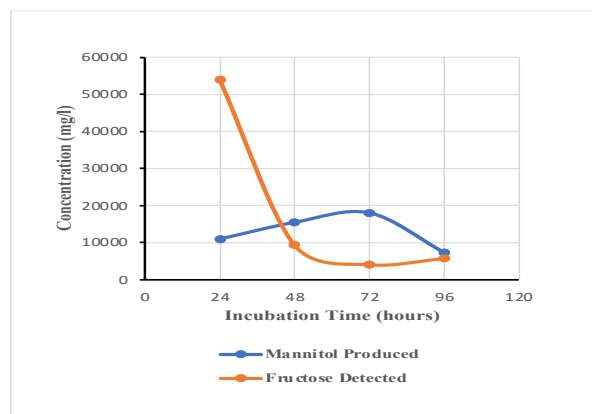


Figure 2. Mannitol and fructose concentration detected in fermentation culture of *Debaryomyces hansenii* with resting cell concentration 140 mg/mL

This is probably due to the negative feedback mechanism, in which the mannitol inhibits mannitol dehydrogenase, as reported by research (Lee *et al.*, 2003). This was supported by the different results obtained when less mannitol was produced in the third day of fermentation using less resting cell concentration. The addition of 30, 50 and 100 mg/mL resting cells caused the mannitol production on the third day to be smaller than the mannitol produced by 140 mg/mL resting cells. In this scenario, the mannitol yield value increased from the first to the fourth day, indicating that product inhibition was not shown with less mannitol (Figure 3).

Table 2. Yield value of mannitol from fermentation with varying resting cell concentration, fructose and ammonium concentration and aeration condition

Resting Cells Variation		Fructose Variation		Ammonium Sulphate Variation		Aeration Variation	
Resting Cell Concentration	Yield Value (%)	Fructose Concentration	Yield Value (%)	Ammonium Sulphate Concentration	Yield Value (%)	Aeration Concentration	Yield Value (%)
300 mg	5.24	7.50	15.65	0.25	3.47	80.00	10.67
500 mg	14.18	10.00	16.33	0.50	9.57	70.00	61.15
				0.75	40.99		
1000 mg	18.44	12.50	11.0			60.00	31.85
1400 mg	20.49	15.00	9.35	-	-	50.00	6.14

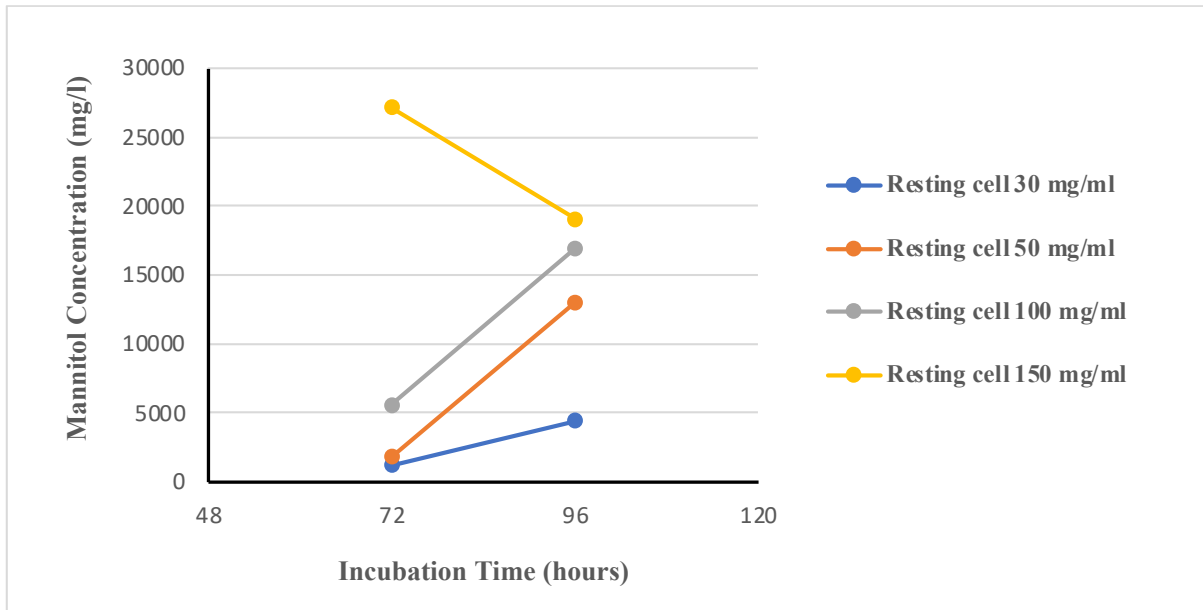


Figure 3. Mannitol concentration detected in fermentation culture of *Debaryomyces hansenii* with different resting cell concentration

The effect of biomasses variation on mannitol yield valuhas been studied previously in mannitol dehydrogenase-transfected *Escherichia coli* BL21. In this study, increasing biomass from OD (600 nm) 20 to 60% decreased mannitol production, which may have been caused by lower substrate availability with higher cell amount (Koko *et al.*, 2021). This study did not examine fermentation with resting cell concentrations greater than 140 mg/mL.. However, given the behavior of decreased mannitol yield value on the fourth day compared to the third day, increasing resting cell concentration to more than 140 mg/mL without increasing substrate concentration may not result in increasing product yield value.

The decrease in mannitol yield value was also obtained by using fructose concentration higher than 10%. This was probably because of the fructose osmotic effect on biomass production. Osmotic pressure equivalent to NaCl 3-5% w/v has been reported as the ideal osmotic condition for *D.hansenii* growth as moderate halophilic yeast (Breuer & Harms, 2006; Navarrete *et al.*, 2022). The fructose is an osmotic agent. Optimal growth will shift the use of substrate to fulfil the biomass production, therefore, the mannitol yield value was decreased. This was supported by less amount of fructose detected on the fourth day of fermentation using fructose 12.5%, compared to 7.5 and 10% fructose. Moreover, fructose detected on the fourth day of fermentation was higher than the added amount in fermentation using 15% fructose, indicating the high use of fructose caused mannitol to be converted into fructose

(Figure 4). Previous research studied galacticol production by yeast *Rhodospiridium toruloides* IFO0880 with variations in galactose concentration (20, 40 and 60 g/L). This research reported that increasing galactose concentration up to 60 g/L increased biomass production. However, the yield value of galactitol was increased only by increasing galactose concentration up to 40 g/L but decreased by higher galactose. This occurrence indicated that increasing biomass production by increasing galactose concentration did not correlate with galactitol yield value (Jagtap *et al.*, 2019).

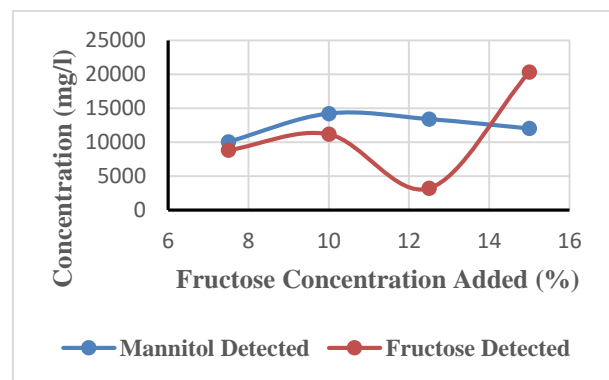


Figure 4. Mannitol and fructose concentration detected in fermentation culture of *Debaryomyces hansenii* with different resting cell concentration

Optimization of aeration level was conducted by varying the volume of culture media (10, 15, 20 dan 25 mL) with the use of the same amount of *resting cell* (500 mg), the same concentration of fructose (10%) and methanol (1%) and the same Erlenmeyer volume (50

mL). The variation in culture media volume will allow different aeration level (80%, 70%, 60% and 50% for media culture volume of 10, 15, 20 and 25 mL, subsequently). Based on this optimization, the highest mannitol yield value was achieved by the moderate aeration (70%, resulted from the use of 15 mL media in 50 mL Erlenmeyer flask).

This founding was in concordance to research conducted previously on bioconversion of xylose to xylitol by *D. hansenii* (Breuer & Harms, 2006). In the highly aerobic condition, the yield value of xylitol was decreased because the reoxidation of NADH into NAD⁺ needed for biomass production was increased with the high availability of oxygen in the respiratory chain. In contrast, with moderate aeration, the available oxygen was only used for the regeneration of NADH into NAD⁺ required for xylitol production. In minimal oxygen, xylitol yield value is low, owing to low NADH-dependent *Xylitol Dehydrogenase* activity (Breuer & Harms, 2006).

CONCLUSION

Debaryomyces hansenii is a promising microorganism for mannitol production. Variations in fermentation conditions will highly determine the yield value of mannitol production. The balance between cell amount and substrate will provide an optimum number of cell production and substrate availability for mannitol production. Limiting biomass production will direct the use of substrate for metabolism to produce mannitol rather than cell growth. Therefore, the use of resting cell and applying osmotic and aeration condition that is less in favor of biomass production will increase mannitol production efficiency.

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AUTHOR CONTRIBUTIONS

Conceptualization, N. Y. S., K. B., H. S.; Methodology, N. Y. S., K. B., H. S.; Software, N. Y. S., K. B., H. S.; Validation, N. Y. S., K. B., H. S.; Formal Analysis, N. Y. S., K. B., H. S.; Investigation, N. Y. S., K. B., H. S.; Resources, N. Y. S., K. B., H. S.; Data Curation, N. Y. S., K. B., H. S.; Writing - Original Draft, N. Y. S.; Writing - Review & Editing, H. S.; Visualization, N. Y. S., K. B., H. S.; Supervision, H. S.;

Project Administration, N. Y. S., K. B., H. S.; Funding acquisition, N. Y. S., K. B., H. S.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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