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Microbial Production of D-Mannitol and D-Fructose from Glycerol

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Summary

In view of the recent development that some petrochemical products are efficiently available as substrates for the fermentation industry, glycerol manufactured from propylene by chemical synthesis would also be hoped for the purpose. This paper describes some of the factors influencing mannitol production from glycerol by *Torulopsis* yeasts and a microbial conversion of glycerol to D-fructose via mannitol, in which two sequential steps of yeast and *Acetobacter* fermentation are involved. *Torulopsis mannitofaciens* CBS 5981 and *Torulopsis versatilis* CBS 1752, exceptionally good mannitol producers, were selected for the study. High concentrations of nitrogen sources and KH_2PO_4 in the medium markedly decreased mannitol yield in spite of good utilization of the substrate. *T. mannitofaciens* produced mannitol in yield of 31% of the glycerol consumed at optimal condition. The fermentation by washed yeast cells gave much higher mannitol yield of more than 50%. A sequential fermentation process was carried out without isolation and purification of the intermediate and yielded 51.7% D-fructose from the glycerol.

INTRODUCTION

In the previous paper,[1] the authors reported that erythritol, D-arabitol, D-mannitol (mannitol) and a heptitol-like compound were produced from glycerol by aerobic fermentation of yeasts; *Trigonopsis variabilis* produced erythritol, *Candida polymorpha* ATCC 20213 produced D-arabitol and *Torulopsis mannitofaciens* CBS 5981 produced mannitol in yields of 12.6%, 28.6% and 31.0% of the glycerol consumed respectively.

Recently, it has been demonstrated that some petrochemical products are efficiently available as substrates for fermentation industry; L-glutamic acid is produced from ethanol[2] and acetic acid[3,4,5] in good yield of about 60% by microbial fermentation.

Glycerol, synthetically manufactured from propylene,[6] is also expected to be a useful substrate for fermentation.

The present paper describes influences of cultural conditions on mannitol production from glycerol by *Torulopsis* yeasts and a microbial conversion of glycerol to D-fructose via mannitol, in which two sequential steps are involved.

MATERIALS AND METHODS

Culture

A total of 222 yeast strains were screened for their ability to produce mannitol from glycerol.[1] *Torulopsis mannitofaciens* CBS 5981 and *Torulopsis versatilis* CBS 1752 were selected for further study. *Acetobacter suboxydans* ATCC 621 was employed for oxidation of mannitol to D-fructose.

Media

The composition of the standard medium for yeast fermentation was as follows: 10% glycerol, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.01% NaCl, 0.4% vitamin-free Casamino Acids (Difco) and 0.05% Yeast Extract (Difco), pH 5.0. Some changes in the medium were also made and departures from the medium were noted in text or tables.

Fermentation Conditions

Growing Culture. A 0.2-ml amount of a 3-day yeast culture in the standard medium was inoculated into 500-ml shake flasks each containing 50 ml of medium. The flasks were shaken on a reciprocal shaker operating at 140 rev/min with a stroke of 7.5 cm. Fermentation temperature was 30°C.

Fermentation by Washed Yeast Cells. Yeast cells, aerobically cultivated in a glucose medium (instead of 10% glycerol, 5% glucose was added to the standard medium) at the same condition as stated above, were repeatedly centrifuged and washed three times with water. Shake flasks containing 50 ml of suspension of the washed cells in glycerol solutions of various concentrations, were shaken on a reciprocal shaker at the same condition as that of growing culture.

Acetobacter fermentation. After yeast fermentation, the broth was adjusted to pH 6.0 with NaOH without removing yeast cells and

autoclaved at 120°C for 15 min. This is used for *Acetobacter* fermentation at the same cultural condition as that of the yeast fermentation.

Analytical Methods

After removal of yeast or *Acetobacter* cells by filtration or centrifugation, the cleared fermented broth was analyzed for sugar and polyalcohol by the method of Neish.[7] Paper chromatography was performed by the ascending method on Whatman No. 1 filter paper by using a solvent system of n-propanol–ethylacetate–water (7:1:2 v/v). Reducing sugar was detected by spraying with aniline hydrogen phthalate reagent[8] and polyalcohol by KIO_4-tetrabase reagent.[9]

Mixture of mannitol and glycerol, or D-fructose and dihydroxyacetone were separated by chromatography on sheets of Whatman No. 3MM filter paper. Each section containing the glycitols or sugars was cut from the sheets and eluted with water and then subjected to analysis.

Isolation and identification of mannitol from the fermented broth were previously reported[1] and D-fructose was isolated and identified as follows: After removing microbial cells by centrifugation, the cleared broth was concentrated in vacuum at 45°C to syrup. The syrup was extracted with hot 99% ethylalcohol. The extract gave a crystalline product, and pure D-fructose was obtained by recrystallization from ethylalcohol. The melting point was 102–104°C and no melting point depression was observed when the crystal was mixed with authentic D-fructose. Elementary analysis gave the following results: $C_6H_{12}O_6$; calculated: C, 40.00%, H, 6.72%; found: C, 39.96%, H, 6.35%. Optical rotation, as $[\alpha]_D^{20}$, was -93.9 ($c = 5.08$ in water), and the infrared spectrum was indistinguishable from that of authentic D-fructose.

RESULTS

Effect of Cultural Condition on Mannitol Production

Several nitrogen sources were tested for their suitability for mannitol production from glycerol. As shown in Table I, ammonium nitrogen, amino nitrogen and nitrate nitrogen were found to be available for mannitol production and organic nitrogen sources gave rather higher yield.

TABLE I

Effect of Nitrogen Sources on Mannitol Production by *Torulopsis mannitofaciens* CBS 5981[a]

Nitrogen source	Glycerol		Mannitol produced g/100 ml	Yield of mannitol based on glycerol consumed %
	Initial g/100 ml	Final g/100 ml		
Casamino Acids, 0.4%	10.92	3.50	1.96	26.4
Corn steep liquor, 0.4%	10.85	5.34	1.54	27.9
Urea, 0.05%	10.85	5.73	1.16	22.6
Ammonium lactate, 0.4%	10.85	1.09	2.04	20.9
Ammonium sulfate,[b] 0.2%	11.25	4.66	1.24	18.8
Potassium nitrate, 0.1%	10.92	5.28	0.92	16.3

[a] Media: each nitrogen source was added to standard medium without Casamino Acids. Fermentation time: 7 days.

[b] Two and one half ml of a buffer solution consisting of 2.5% citric acid and 10% potassium citrate was added to 100 ml of medium.

TABLE II

Effect of Concentration of Casamino Acids and Corn Steep Liquor upon Yield of Mannitol

Nitrogen source[a]		Fermentation time days	Glycerol		Mannitol produced g/100 ml	Yield of mannitol based on glycerol consumed %
			Initial g/100 ml	Final g/100 ml		
Casamino Acids	0.1%	7	10.43	8.50	0.94	48.7
	0.4%	7	11.26	3.72	2.34	31.0
	1.0%	7	10.43	2.13	0.30	3.6
	2.0%	7	10.52	1.19	0	0
Corn steep liquor	0.5%	7	10.70	4.63	1.16	19.1
	1.0%	7	10.70	1.20	1.18	12.4
	2.0%	5	10.83	2.57	0.62	7.5
	4.0%	5	11.01	3.75	0	0

[a] Media: each nitrogen source was added to standard medium without Casamino Acids.

It has been previously reported that the C:N ratio of medium significantly affects polyalcohol production from glucose.[10,11] When the effect of casamino acids and corn steep liquor on mannitol production was examined, it was found that at the highest level, substrate utilization was fast but mannitol yield markedly decreased (Table II). At the lowest level, mannitol yield was high but fermentation was slow. The phenomenon was analogous to that in production from glucose.

It has been shown that an excess of inorganic phosphate has a detrimental effect upon the yield of polyalcohol in some yeasts.[12,13] The previous paper[11] showed that even at the highest level used (2% KH_2PO_4), mannitol yield from glucose did not drop. However, mannitol could not be produced from glycerol at the high concentrations of 1–2% KH_2PO_4 (Table III).

Effects of aeration, temperature and pH of medium were found to be substantially similar to those in the production from glucose, which was previously reported.[11]

TABLE III

Effect of Phosphate Concentration on Mannitol Yield[a]

KH_2PO_4 added to the basal medium	Glycerol		Mannitol produced	Yield of mannitol based on glycerol consumed
	Initial g/100 ml	Final g/100 ml	g/100 ml	%
No addition	11.18	9.74	0.58	40.2
0.01%	11.18	3.35	2.10	26.8
0.1%	11.18	3.92	1.84	25.3
1.0%	11.18	2.16	0	0
2.0%	11.18	3.13	0	0

[a] Standard medium without KH_2PO_4 was employed as the basal medium. Fermentation time: 7 days.

Fermentation by Washed Cells

As one of trials for increasing mannitol yield, fermentation by washed cells was examined. Some preliminary experiments showed that *Torulopsis versatilis* gave rather better yield than *Torulopsis mannitofaciens* and almost the same yield was obtained regardless of

precultural conditions in a glucose or a glycerol medium. Thus, the experiment shown in Table IV was carried out using washed cells of *T. versatilis* cultivated in a glucose medium. As addition of 0.1% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ to a yeast suspension gave no favorable effect, the washed cells were suspended in just glycerol water solution. The result showed that the yield was much enhanced to more than 50% of the glycerol consumed.

TABLE IV

Mannitol Production from Glycerol by Washed Cells of *Torulopsis versatilis* CBS 1752[a]

Glycerol				Mannitol produced		
0 hrs g/100 ml	24 hrs g/100 ml	46 hrs g/100 ml	72 hrs g/100 ml	24 hrs g/100 ml	46 hrs g/100 ml	72 hrs g/100 ml
5.81	1.46	—	—	2.34 (53.7%)	—	—
10.76	8.07	3.73	—	2.24 (83.2%)	3.64 (51.7%)	—
17.06	—	10.36	7.37	—	2.72 (40.5%)	3.90 (41.6%)

[a] Cell concentration (as dry matter): 20.5 mg/ml.
Parentheses show mannitol yield based on glycerol consumed.

Production of D-*Fructose from Glycerol by a Sequential Fermentation Process*

It has been known that mannitol could be converted quantitatively to D-fructose by *Acetobacter* fermentation.[14] So, it was expected that D-fructose would be produced from glycerol in approximately 50% yield by a sequential application of the yeast and *Acetobacter* fermentation processes.

The process was carried out without isolation and purification of the intermediate product (mannitol). After 45 hrs fermentation by the washed cells of *T. versatilis*, 7.78 g of glycerol was consumed per 100 ml of the broth and 4.06 g of mannitol was accumulated. Then, the fermented broth was adjusted to pH 6.0 with NaOH without removing yeast cells and autoclaved at 120°C for 15 min. The broth was thus enriched with the nutrients favorable to the growth of

TABLE V

Production of D-Fructose from Glycerol by a Sequential Fermentation Process.

(1). The first step: mannitol production by washed cells of *Torulopsis versatilis*[a].

Fermentation time hrs	Glycerol in the broth g/100 ml	Glycerol consumed g/100 ml	Mannitol produced g/100 ml	Yield of mannitol based on glycerol consumed %
0	8.83	—	—	—
28	3.90	4.93	2.64	53.5
45	1.05	7.78	4.06	52.1

[a] Cell concentration (as dry matter): 21.5 mg/ml.

(2). The second step: conversion of mannitol to D-fructose by *Acetobacter suboxydans*.

Fermentation time hrs	Mannitol in the broth g/100 ml	D-fructose produced g/100 ml	Yield of D-fructose based on mannitol consumed %	Yield of D-fructose based on glycerol consumed %
0	4.06	—	—	—
42	0	4.03	99.2	51.7

As the broth contained 1.05 g glycerol per 100 ml, dihydroxyacetone was formed after oxidation. D-fructose and dihydroxyacetone were separately determined as described in "Materials and Methods".

Acetobacter suboxydans, which oxidized the mannitol almost quantitatively to D-fructose in 42 hrs at 30°C.

A 4.03-g amount of D-fructose was produced from 7.78 g of glycerol with a final yield of 51.7% (Table V). D-Fructose was isolated in pure crystalline form from the broth and identified by melting point, elementary analysis, optical rotation and infrared spectrum.

DISCUSSION

The data presented in this paper demonstrate the practicability of D-fructose production from glycerol, which is a petrochemical product. The process was carried out by means of two sequential steps

(glycerol → mannitol → D-fructose) without isolation and purification of the intermediate and yielded 52% D-fructose from glycerol.

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