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# Production of mannitol from raw glycerol by *Candida azyma*

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**To promote the effective use of raw glycerol, 13 yeast strains with the ability to produce mannitol from glycerol were isolated from environmental samples. Of the 13 strains, strain 7-12G was selected as an efficient mannitol producer from 25% (w/v) glycerol and was identified as *Candida azyma* by morphological, physicochemical, and phylogenetic analyses. When the ability to produce mannitol from raw glycerol in flask culture was compared among strains 7-12G, NBRC10406 (the type strain of *C. azyma*), and related strains, strain NBRC10406 exhibited the highest production level (31.8 g/l). Culture in jar fermentors was next investigated, and mannitol production reached 50.8 g/l over 7 days, corresponding to 0.30 g/g-glycerol. To the best of our knowledge, this is the highest reported level of mannitol produced by a microbe from glycerol under batch-type culture conditions.**



[**Key words:** Mannitol; *Candida azyma*; Raw glycerol; Sugar alcohol; Biodiesel]

A shift from the use of petroleum to that of biomass will become essential if industrial societies are to be sustainable and effectively manage greenhouse gas emissions (1). The production of biodiesel fuel (BDF), fatty acid methyl esters derived from plant oils, has increased dramatically over the past decade. Production of BDF and some oleochemicals is based on ester exchange reactions between triglycerides and alcohols (e.g., methanol) under alkaline conditions, resulting in the formation of crude (raw) glycerol as a by-product. The increase in BDF production worldwide means that surplus glycerol has become an alternative feedstock, and much effort has been devoted to the conversion of glycerol into value-added chemicals (2,3). The quality of raw glycerol is usually rather low; refining is necessary to obtain pure glycerol. However, this process is costly and requires energy. Thus, it is important to develop processes whereby raw (i.e., non-refined) glycerol can be used to produce valuable chemicals.

Sugar alcohols, including erythritol, arabitol, xylitol, and mannitol, are often produced by osmophilic yeast to oppose osmotic pressure exerted across the cell membrane, and some of these alcohols have applications in the food and pharmaceutical industries (4–6). Of these alcohols, mannitol, a C6 sugar alcohol present in many fruits, vegetables, and mushrooms, has a sweet taste but low caloric value, and is a valuable additive used in food and pharmaceutical preparations (7). Because mannitol plays an important role in the stress tolerance of microorganisms, lichens, and higher plants due to its function as a compatible solute and free radical scavenger (8), mannitol can be applied in some industries. In addition, mannitol is recently reported to be a feedstock for bioethanol production (9).

Many reports on the biotechnological production of mannitol by lactic acid bacteria [LAB; for a review, see Ortiz et al. (10)], osmophilic yeast species (11–14), and fungi (15,16) have been published. Mannitol production by LAB has been intensively investigated over the past decade because these organisms are very efficient producers of mannitol from D-fructose (10). In one recent report, the production of mannitol upon glycerol fermentation by a mutant of *Lactobacillus brevis* was 215 g/l over 98 h under optimal conditions (17). However, D-fructose is relatively expensive to prepare via glucose isomerization; thus, other methods of mannitol production from inexpensive substrates are required. Although raw glycerol is a cheap by-product of BDF production, only two reports on mannitol production from glycerol have been published (11,18). No report has yet addressed the microbial production of mannitol using raw glycerol as a feedstock. Raw glycerol frequently inhibits microbial growth because it contains impurities, including non-glycerol organic materials (e.g., tar and methanol), sodium or potassium salts, and/or heavy metals. Hence, it is necessary to identify novel microbial strains capable of producing mannitol from raw glycerol.

In the present study, we first searched for microbial strains that could produce high levels of mannitol from raw glycerol. Among the tested yeast candidates, a strain from the NITE Biological Resource Center (NBRC), *Candida azyma* NBRC10406, was finally selected as the strain that most effectively produced mannitol from 25% (w/v) glycerol, and jar fermentation of glycerol to produce mannitol was optimized.

## MATERIALS AND METHODS

**Microorganisms and culture conditions** Three type strains, *C. azyma* NBRC10406, *Candida vanderwaltii* NBRC10319, and *Candida magnoliae* NBRC0705,

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**TABLE 1.** Components of the raw glycerol used in the present study.

| Compound | Concentration (%) |
|---|---|
| Glycerol | 85 |
| Organic impurities | 2 |
| Inorganic impurities | 2 |
| ($Na_2SO_4$) | (>0.5) |
| $H_2O$ | 11 |

were obtained from the NBRC (Chiba, Japan). Strain 7-12G, which was isolated in the present study, has been deposited as FERM P-21419 at the NITE Patent Microorganisms Depository (Chiba, Japan). The yeasts were maintained on YM agar plates [1% (w/v) glucose, 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, and 1.5% (w/v) agar]. Raw glycerol, by-product of an oleochemical production from virgin plant oil by Lion Corp. (Tokyo, Japan), was used as the substrate for mannitol production following the removal of impurities by passage through a column packed with granular activated charcoal. The composition is shown in Table 1.

**Screen for microorganisms capable of producing mannitol from glycerol** Screening for mannitol-producing microorganisms was done using the enrichment medium [25% (w/v) glycerol, 0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 0.5% (w/v) peptone, 0.5% (w/v) $Na_2SO_4$, 50 μg/ml streptomycin, and 50 μg/ml chloramphenicol]. Environmental samples were added to test tubes containing 5 ml of this medium, followed by incubation on a reciprocal shaker (180 strokes/min) at 28°C for 3–7 days. Next, 100 μl of culture was inoculated into 96-well deep-well plates containing 1 ml/well of the same medium and incubation continued on a reciprocal shaker (150 rpm) at 28°C for 3 days. The mannitol levels in each culture broth were calculated using paper chromatography, and cultures that yielded mannitol were appropriately diluted and spread on YM agar plates. Individual colonies were re-inoculated into deep-well plates containing the same medium and mannitol production upon further cultivation was checked using paper chromatography.

**Paper chromatography** To detect mannitol, 10 μl of each culture was subjected to paper chromatography using 1-propanol/ethyl acetate/water (7:1:2, v/v/v) as the mobile solvent. Mannitol was detected using Yoda's reagent (5 g/l $KIO_4$ and 150 g/l $MnSO_4$/*p*,*p*′-tetramethyl diaminodiphenylmethane saturated with 2 M acetic acid [1:1, v/v]) (19).

**High-performance liquid chromatography (HPLC)** HPLC was used to quantify the mannitol and glycerol levels using a SUGAR SC1011 column (8 mm × 30 cm; Showa Denko, Tokyo, Japan) operating at 80°C and a refractive index detector (RID-10A; Shimadzu, Kyoto, Japan). The column was eluted with $H_2O$ (1 ml/min). The mannitol concentration was determined with reference to calibration curves prepared using authentic material, which was purchased from Wako Pure Chemical Industries, Ltd.

**Molecular phylogenetic analysis** Strain 7-12G was grown in YM medium at 25°C and DNA was extracted using a DNeasy Plant Mini-Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The D1/D2 region of the 26S ribosomal RNA (rRNA) gene was amplified by PCR using primers NL1, NL2, NL3, and NL4 (20) and puRe*Taq* Ready-To-Go PCR beads (GE Healthcare Life Sciences, Buckinghamshire, UK). Initial denaturation at 95°C for 7 min was followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, with a final 7-min extension at 72°C. An ABI PRISM BigDye Terminator Kit (v. 3.1) (Applied Biosystems, Foster City, CA, USA) was used as described by the manufacturer. The products were sequenced on an ABI PRISM 3100 genetic analysis platform (Applied Biosystems). The sequence of the D1/D2 region of the 26S rRNA gene of strain 7-12G was compared with those of closely related species using information from the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp) using the BLAST search program followed by alignment with the aid of ClustalW software (21). A phylogenetic tree was constructed using TreeView software; *C. magnoliae* served as the outgroup. The nucleotide sequence for the D1/D2 region of strain 7-12G was registered in the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession number AB855789.

**Physiological tests** Strain 7-12G was evaluated in terms of the assimilation of various carbon and nitrogen sources, drug resistance, vitamin auxotrophy, urease production, and reaction with diazonium blue B (the DBB test). All test procedures were standard (22,23). Morphological characteristics were examined via BX51 optical microscopy (Olympus, Tokyo, Japan).

**Production of mannitol by newly isolated and related strains** A single colony of each strain growing on YM plates was inoculated into a test tube containing 5 ml of the seed culture medium [1% (w/v) glycerol, 0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, and 0.5% (w/v) peptone]. After incubation on a reciprocal shaker (180 strokes/min) at 28°C for 1 day, 1 ml of each culture was inoculated into 300-ml Erlenmeyer flasks containing 30 ml of the raw glycerol medium [30% (w/v) raw glycerol, 0.6% (w/v) yeast extract, and 0.1% (w/v) $KH_2PO_4$; pH 6.0] unless otherwise noted. The cultures were further incubated on a rotary shaker (250 rpm) at 28°C for 7 days and the concentrations of mannitol and glycerol were determined by HPLC.

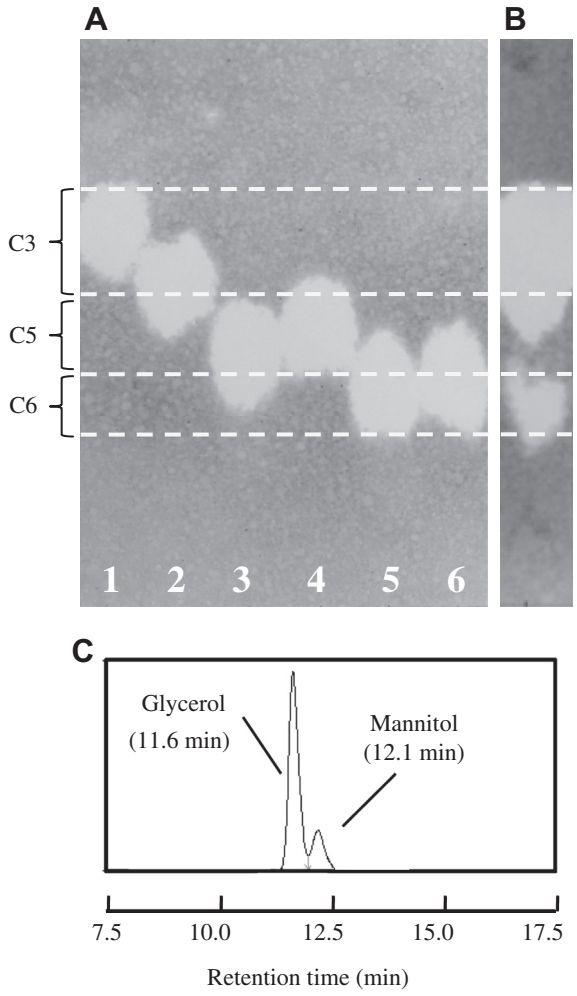


FIG. 1. Detection and quantification of mannitol produced in microbial cultures. An example of a paper chromatographic profile is shown. (A) Standard samples. (B) Culture broth of strain 7-12G. (C) HPLC profile of the culture broth of strain 7-12G. Cultivation was performed in the enrichment medium. The solvent for paper chromatography was 1-propanol/ethyl acetate/water (7:1:2, v/v/v). Detection was achieved by spraying 150 g/l $MnSO_4$/*p*,*p*′-tetramethyl diaminodiphenylmethane saturated with 2 M acetic acid (1:1, v/v) after prior spraying with 5 g/l $KIO_4$. Lane 1, glycerol; lane 2, erythritol; lane 3, xylitol; lane 4, D-arabitol; lane 5, sorbitol; lane 6, mannitol. C3, C5, and C6 refer to the respective spot regions of 3-, 5-, and 6-carbon sugar alcohols.

**Optimization of the culture conditions for mannitol production in flask culture** A single colony of *C. azyma* NBRC10406 was inoculated into a test tube containing 5 ml of the seed culture medium. After incubation on a reciprocal shaker (180 strokes/min) at 28°C for 1 day, 1 ml of this pre-culture was inoculated into a 300-ml Erlenmeyer flask containing 30 ml of the basic culture medium A [25% (w/v) glycerol, 0.1% (w/v) yeast extract, 0.1% (w/v) $KH_2PO_4$, and 0.5% (w/v) $Na_2SO_4$; pH 6.0] or the basic culture medium B [25% (w/v) glycerol, 0.6% (w/v) yeast extract, 0.1% (w/v) $KH_2PO_4$, and 0.5% (w/v) $Na_2SO_4$; pH 6.0], modified as described below, and further incubated on a rotary shaker (250 rpm) at 28°C for 7 days.

To explore the effects of nitrogenous and organic nutrients, the basic culture medium A was supplemented with yeast extract, corn steep liquor, peptone, malt extract, or casamino acids [0.5% (w/v)]. To examine the effect of the yeast extract concentration, 0.1%, 0.3%, 0.5%, or 0.7% (w/v) yeast extract was added to the basic culture medium A. To explore the effect of the initial glycerol concentration, 15%, 20%, 30%, or 35% (w/v) glycerol was used to replace the 25% (w/v) glycerol in the basic culture medium B. Also, the effect of pH was investigated with the basic culture medium B. To examine the effects of minerals, manganese chloride, calcium chloride ($CaCl_2$), ferrous chloride, or zinc sulfate [0.5% (w/v)] was added to the basic culture medium B. To explore the effect of calcium, 0.05%, 0.1%, 0.2%, 0.4%, and 0.6% (w/v) $CaCl_2$ was added to the basic culture medium B. The concentrations of mannitol and glycerol in all cultures were determined by HPLC. All data shown are averages and standard deviations derived from three independent experiments.

**Mannitol production from raw glycerol upon jar fermentation** A single colony of *C. azyma* NBRC10406 was inoculated into a test tube containing 5 ml of the