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Production of D-Talitol from D-Tagatose by *Aureobasidium pullulans* Strain 113B

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***Aureobasidium pullulans* strain 113B transformed D-tagatose to D-talitol at a faster rate in the presence of glycerol in the reaction mixture. The transformation rates were 93.0, 72.0 and 68.0% respectively when 0.5, 1.0 and 2.0% substrate was used. Cells grown on D-glucose were found to have the most potential for obtaining maximum transformation. In a flask reaction, about 0.58 g D-talitol crystals were recovered from 1 g of D-tagatose after various product purification treatments. Considerable transformation (70.0%) was also observed when substrate was added to the cell cultivation medium. Cells stored at −20°C showed the same transformation activity as intact cells.**

Exploitation of microorganisms for the production of useful materials is an age-old process. Our laboratory is concerned with the production of rare sugars or polyols by microbial cell or enzyme reactions. In our previous communications, we have reported biocatalytic processes for the large-scale production of rare sugars like D-tagatose, D-sorbose, D-psicose and L-xylulose (1–5). The chemical production of these rare sugars has been attempted by several workers (6, 9). However, in most cases chemical production methods were found to be disadvantageous because of low yield/purity and high cost (10; Jozef, K. *et al.*, Czech CS. 21,039, 1986). The main problem in the extensive utilization of unnatural sugars/polyols is the high expense, since they are not abundant in nature and the existing commercial production methods are inadequate. Despite their expense, however, unnatural sugars/polyols are important because they have the potential for use as safe non-calorie sweeteners and as precursors for many natural products (11; Zehner, L. R., Eur. patent 257626, 1988; Biospherics, Inc., JP. 82,129,671, 1982, [in Japanese]).

D-Tagatose, a rare ketohexose, is being produced in adequate amounts in our laboratory by microbial cell reaction. Here, we describe the production of a rare polyol, D-talitol, from D-tagatose by using microbial cells. So far as we know, this is the first report to describe a biocatalytic process for the production of D-talitol.

MATERIALS AND METHODS

Microorganism *Aureobasidium pullulans* strain 113B, an isolate from soil, was used and the culture was maintained at 4°C on 2% potato dextrose agar (PDA) slants.

Chemicals Authentic D-tagatose crystals were purchased from Sigma Chemical Co. (USA). Potato dextrose agar (PDA) was purchased from Nissui Seiyaku Co. (Tokyo). D-Talitol was obtained from Hayashibara Biochemical Laboratories, Inc. (Okayama). Yeast extract was purchased from Oriental Yeast Co. (Tokyo). Other chemicals were obtained from Wako Pure Chemical Industry (Osaka) and were certified as reagent grade.

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Analytical methods Reduction of ketose (D-tagatose) in the reaction mixture was detected by the method of Dische and Borenfreuind (12), and after the complete disappearance of the ketose in the reaction mixture product accumulation was determined by high performance liquid chromatography (HPLC, Nihonbunko HPLC 880 PU liquid chromatography, Shimadzu RID-6A refractive index detector and Shimadzu CR-6A Chromatopac) using a Hitachi HPLC column (GL-611). Separation was achieved at 60°C using 10^{-4} M NaOH at a flow rate of 1.0 ml/min.

Determination of a suitable carbohydrate for transformation Cells of the selected organism were grown aerobically with continuous shaking for 48 h in 500-ml Erlenmeyer flasks containing medium (100 ml) of the following composition: 1.0% Polypepton, 0.5% yeast extract, 0.5% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$ and 1% or 3% carbohydrate. The culture medium was inoculated with 100 μl of preculture grown in the same medium without carbohydrate. After growth, the cells were harvested by centrifugation at 9,000 rpm for 10 min. The collected cells were washed with Na-phosphate buffer (0.05 M) pH 7.0, and centrifuged for 10 min at 9,000 rpm. After washing twice, the cells were checked for the transformation of D-tagatose to D-talitol.

Transformation reaction conditions A suspension of the washed cells was made in 0.05 M Na-phosphate buffer (pH 7.0) which had an absorbance of 40 at 600 nm. Transformation was carried out at 30°C with shaking in L-tubes containing a reaction mixture of the following composition: deionized water 2.5 ml, washed cell suspension 2.5 ml and 25 mg D-tagatose as substrate (the final absorbance of the reaction mixture was 20 at 600 nm). Samples were taken from the reaction mixture at different times to determine the reduction of ketose and transformation rate. For substrate concentration effect experiment, 50 mg and 100 mg of D-tagatose were added to the reaction mixture. Cells grown on D-glucose for 48, 72 and 96 h were used to observe the effect of culture time on the transformation.

Determination of a carbohydrate to accelerate reaction speed To the reaction mixture described above, 0.5% various carbohydrates were added. The progress of the reactions and accumulation of the product in the reac-

tion mixture were observed as described above under 'analytical methods'.

Transformation in flask After determining a suitable growth carbon for transformation and a suitable carbohydrate to accelerate the reaction speed, cells were cultivated aseptically as described earlier for 48 h at 30°C in Erlenmeyer flasks containing 100 ml of medium with 3% carbon source. The cells were harvested and washed twice with 0.05 M Na-phosphate buffer (pH 7.0) as described above. A 50-ml suspension of harvested cells was made in 0.05 M Na-phosphate buffer (pH 7.0) which had an absorbance of 40 at 600 nm. The transformation reaction was carried out in an Erlenmeyer flask at 30°C with shaking (170 rpm). The composition of the reaction mixture was as follows: 1 g D-tagatose, 1 g glycerol, 50 ml deionized water and the cell suspension made previously (total volume 100 ml).

Transformation during cultivation A solution of D-tagatose was sterilized by milipore filtration and added to sterile medium, which was used for cultivation during the study. The inoculation and cultivation conditions were as described earlier. Samples were taken aseptically at different times to monitor the reduction of D-tagatose. After the complete disappearance of ketose in the cultivation medium, product accumulation was determined by HPLC analysis.

Transformation at different cell concentrations The absorbance of the reaction mixture was adjusted to 10, 15, 20 and 30 at 600 nm by the cells of strain 113B. Transformation conditions, determination of substrate reduction and product accumulation in the reaction mixture were as described earlier.

Transformation by cells kept at −20°C A suspension of cells grown as described earlier was made in 0.05 M Na-phosphate buffer (pH 7.0) and kept at −20°C. After 7 d, the frozen cell suspension was thawed at room temperature and used for the transformation of D-tagatose to D-talitol in an L-tube. The composition of the reaction mixture and the reaction conditions were as same as mentioned earlier.

Product recovery After the transformation, the cells were removed by centrifugation at 9,000 rpm for 10 min and kept at 4°C. The supernatant fluid was then treated with activated charcoal and filtered after centrifugation at 12,000 rpm for 30 min to remove the charcoal. The filtrate was treated with a microacilyzer (Asahi Kasei, Model G-1) for deionization. Microacilyzer treated sample was again deionized with a mixture of diaion (SK1B, H^+) and amberlite (IRA-411, CO_3^{2-}) ion exchange resins. This deionized sample was then treated with 40% ethanol and stirred to precipitate polysaccharide. The polysaccharide precipitate was then removed by centrifugation at 12,000 rpm and filtration. The resulting fluid was then evaporated several times under vacuum at 40°C with deionized water and finally concentrated. After concentration, the sample was applied to a column of Dowex 50W-X2 in the Ca^{2+} form. The column was eluted with deionized water and 8 ml fractions were collected. The fractions were analyzed by HPLC as described above. The fractions containing D-talitol were pooled and concentrated by evaporation under vacuum at 40°C until a viscous mass was obtained. Then, a very small amount of authentic D-talitol crystals were added to the viscous mass and it was kept in a desiccator. After a few days, the crystals that formed were washed, dried in a desiccator, and weighed.

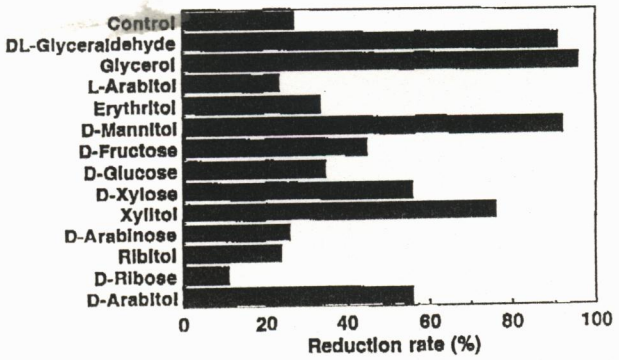

FIG. 1. Effect of addition of different carbohydrates in the reaction mixture for D-talitol production from D-tagatose.

Determination of product The product was identified by HPLC analysis, infrared spectra and optical rotation measurement with authentic D-talitol. Infrared spectra were measured with a Nihonbunko infrared spectrophotometer (model A-302) using KBr tablets.

RESULTS

Effect of growth carbon source *A. pullulans* strain 113B was grown on various carbohydrates, and its ability to transform D-tagatose to D-talitol was observed. D-Glucose, D-mannose, D-fructose, L-arabinose, D-xylose, D-arabitol and D-mannitol supported very good growth of the microorganism. Cells grown on D-glucose showed the highest transformation activity, followed by those grown on D-fructose and D-mannitol (data not shown). However, the rate of transformation of D-tagatose to D-talitol was slow.

Carbohydrate to accelerate transformation Since the transformation rate of D-tagatose to D-talitol was slow, various carbohydrates were added to the reaction mixture to try and obtain an improvement of the reaction speed. The speed was significantly influenced by the addition of glycerol, D-mannitol and DL-glyceraldehyde. Almost 94.0, 92.0 and 91.0% transformations were observed in a reaction of 9-h when glycerol, D-mannitol and DL-glyceraldehyde were added to the reaction mixture (Fig. 1). In contrast, only 27.0% substrate was reduced after 9-h reaction in the control (only the substrate D-tagatose and cells).

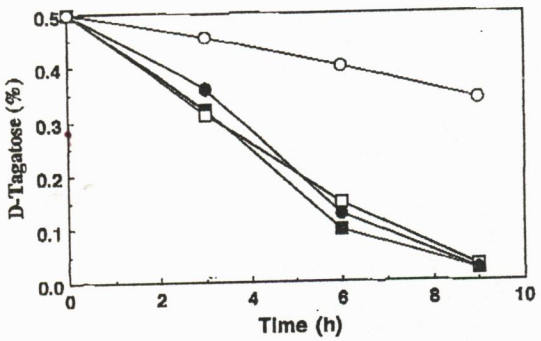

FIG. 2. Effect of glycerol concentration on the production of D-talitol from D-tagatose by strain 113B. Symbols: ○, 0%; ■, 0.5%; □, 1%; ●, 2%.

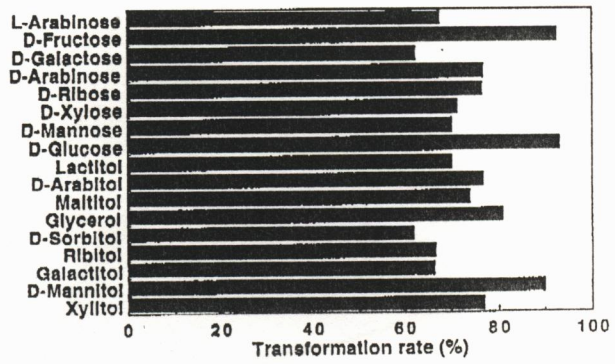

FIG. 3. D-Talitol production potential of strain 113B (in presence of glycerol) grown on different carbohydrates.

As the addition of glycerol to the reaction mixture accelerated the reaction speed most effectively, we tried to find a suitable concentration of glycerol for transformation. Figure 2 shows that 0.5, 1.0 and 2.0% glycerol has almost same effect on the transformation of 0.5% D-tagatose to D-talitol.

Transformation in presence of glycerol Following the determination of a suitable carbohydrate to accelerate the reaction speed significantly, cells of the strain 113B were cultivated on various carbohydrates and used in a reaction mixture containing 0.5% D-tagatose and 0.5% glycerol. After 9-h reaction, cells grown on D-glucose was found to achieve the best transformation (93.0%), followed by D-fructose and D-mannitol, although the transformation activities of cells grown on each of these three carbohydrates did not differ markedly (Fig. 3). D-Glucose was selected as the carbohydrate for the culture, and the concentration needed to obtain optimum transformation was investigated. Similar transformation activities and cell yields were obtained when cells were grown on D-glucose at concentrations from 3–20% (data not shown).

Transformation at different substrate concentrations D-Tagatose at concentrations of 0.5, 1.0 and 2.0% was added to L-tubes, and transformation was carried out in the presence of glycerol by cells grown on D-glucose. Complete transformation of 0.5, 1.0 and 2.0% D-tagatose took about 9, 20 and 45-h, respectively (Fig. 4). A decrease in product accumulation was observed with an increase of substrate concentration. The transformation rates were about 93.0, 72.0 and 68.0% at 0.5, 1.0 and 2.0% substrate concentrations, respectively. Transformation rates were derived from the amount of product accumulated in the reaction mixture from a given concentration of substrate.

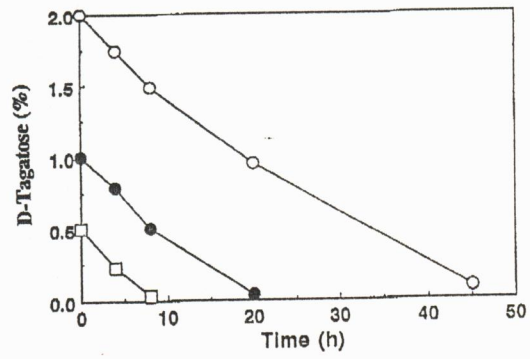

FIG. 4. D-Talitol production from different concentrations of D-tagatose by strain 113B. Symbols: □, 0.5%; ●, 1%; ○, 2%.

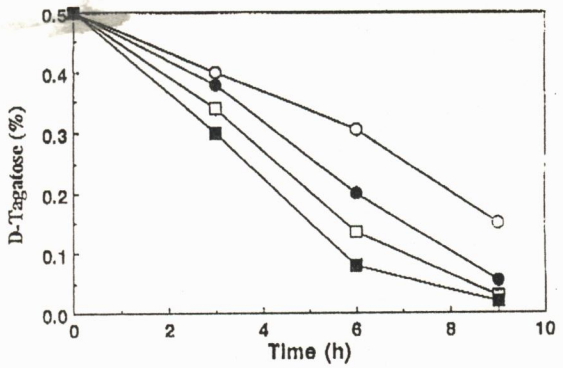

FIG. 5. Effect of cell concentration on the production of D-talitol from D-tagatose by strain 113B. Symbols: ○, Ab 10; ●, Ab 15; □, Ab 20; ■, 30.

Flask reaction Cells grown on 3.0% D-glucose were used for transformation of 1.0% D-tagatose to D-talitol in the presence of glycerol. The complete transformation of D-tagatose took 19-h. The transformation rate was little higher than that of the L-tube reaction (73.0%). After various treatments for product purification, about 0.58 g of pure D-talitol crystals were obtained, which indicated a net yield of 58.0%.

Cell concentration Transformation of 0.5% D-tagatose to D-talitol in the presence of glycerol was performed at different cell concentrations. Figure 5 shows that the reaction speed increased with increased cell concentration. However, the reduction rates were similar after 9-h reaction when the cell absorbance was 20 and 30 at 600 nm.

Effect of cultivation time Cells grown for 48, 72 and 96-h were used to observe the effect of culture time on the transformation of D-tagatose to D-talitol. Figure 6 indicates that cells grown for 48–72 h had very good transformation activity. A culture time longer than 72-h was found to be unsuitable for good transformation.

D-Talitol production during cultivation We tried to transform 1.0% D-tagatose to D-talitol by strain 113B during cultivation in an L-tube with 3.0% D-glucose or 3.0% glycerol used as the growth carbon. Glycerol was

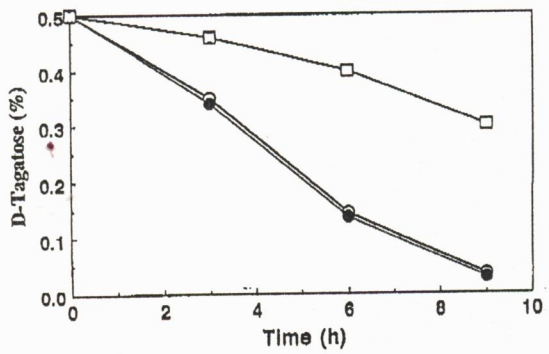

FIG. 6. Effect of culture time on the transformation of D-tagatose to D-talitol by strain 113B. Symbols: ○, 48 h; ●, 72 h; □, 96 h.

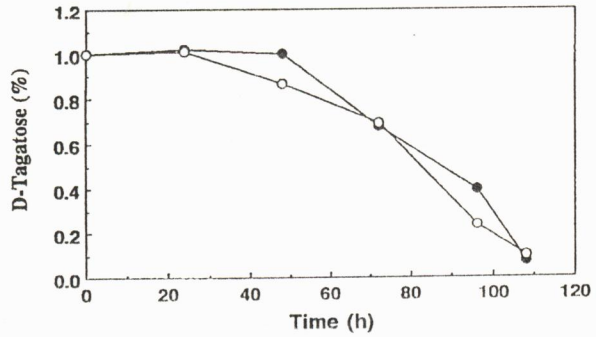

FIG. 7. Transformation of D-tagatose to D-talitol in cultivation medium during growth of strain 113B. Symbols: ●, glycerol; ○, D-glucose.

used since it accelerated the reaction speed significantly when it was added in the reaction mixture during the washed cell reaction. In the case of the glycerol-containing medium no reduction of D-tagatose was observed until 48-h of cultivation, which might be due to the low cell concentration at 48-h, whereas in the case of the D-glucose-containing medium almost 15% of the substrate was reduced at 48-h (Fig. 7). The highest product accumulation was observed by HPLC analysis after 108-h of cultivation. The amounts were about 70.0 and 64.0% of the substrate respectively for the D-glucose and glycerol media.

Effect of storage at −20°C Cells kept at −20°C for 7 d were investigated for the transformation of D-tagatose to D-talitol. Intact cells and cells stored at the low temperature showed similar transformation activities (data not shown).

Identification of the product The HPLC retention time, infrared spectrum (Fig. 8) and specific optical rotation (α_D+3.7) of the isolated crystals were identical to those of authentic D-talitol. On the basis of these results, the product formed from D-tagatose in the reaction mixture was identified as D-talitol.

DISCUSSION

D-Talitol is a rare hexitol, and chemical reduction of D-tagatose can yield a mixture of D-talitol and galactitol. To our knowledge there has hitherto been no report on the microbial production of D-talitol. Rare sugars/polyols are expensive since they do not exist in nature in adequate amounts. It can be presumed from the results of some recent studies that rare carbohydrates have potential organoleptic and other biochemical properties (11, 13, 14). However, to obtain these rare sugars/polyols in amounts large enough for their utilization, a less expensive production method with high yield is essential, which, in turn, is dependent on extensive studies in this area.

The organism used during this study has considerable potential for the transformation of D-tagatose to D-talitol. The transformation rate was as high as 93.0% when a low concentration of substrate (0.5%) was used. However, at 1.0 and 2.0% substrate concentrations 72.0 and 68.0% transformation was obtained. Cells grown on D-glucose and D-fructose showed very high transformation activity, which is indeed advantageous since this organism does not require any expensive growth carbon/inducer for obtaining better transformation. Addition of glycerol/D-mannitol/DL-glyceraldehyde accelerated the reaction speed to more than three times than that of the control. The faster reduction of the substrate in the presence of above-mentioned carbohydrates in the reaction mixture might be due to the activity of a dehydrogenase coupled with NADH generation when the glycerol/D-mannitol/DL-glyceraldehyde was oxidized. Vongsuvanlert and Tani (15) reported that methanol yeast can effectively reduce L-sorbose to L-iditol with D-sorbitol dehydrogenase coupled with NADH generation under methanol oxidation. In another study, Martin and Mortlock (16) reported that a mutant of *Klebsiella aerogenes* could reduce L-fuculose to 6 deoxy L-talitol when reducing power was provided by coupling the reduction of L-fuculose with the oxidation of glycerol to dihydroxyacetone.

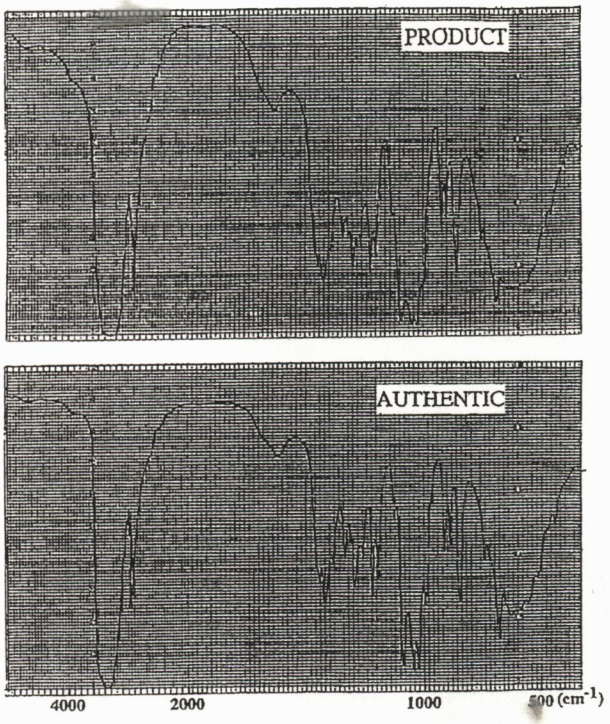

FIG. 8. Infrared spectra of authentic D-talitol and product.

Frozen cells exhibited similar transformation activity after thawing to that of intact cells, which is advantageous in the storage of cells for practical use. Considerable transformation also occurred when substrate was used in the cultivation medium.

From the findings of the present study it can be concluded that strain 113B is potentially of considerable importance as an efficient producer of D-talitol from D-tagatose.

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