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Trehalose 6-Phosphate Production with Energy Coupling Fermentation by Yeast Cells

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We tried a method for the production of trehalose 6-phosphate (T6P) with energy-coupling fermentation by baker's yeast. T6P was produced in a reaction mixture containing glucose, 5′-UMP, $MgSO_4$, inorganic phosphate, and dried cells of baker's yeast as the enzyme preparation. T6P was isolated from the reaction mixture and identified by TLC, HPLC, GC-MS, and enzymatic methods. The reaction conditions suitable for T6P production were investigated. The formation of T6P and its precursors, glucose 6-phosphate and UDPglucose, at various pHs and concentrations of substrates was examined. Accumulation of T6P was maximum with a reaction mixture containing 1 M glucose, 20 mM 5′-UMP, 20 mM $MgSO_4$, 400 mM sodium phosphate buffer (pH 6.2), and 100 mg/ml dried cells of baker's yeast shaken at 37°C for 6 h. The yield of T6P as a percentage of glucose was 11% (mol/mol) under these reaction conditions.

Key words: trehalose 6-phosphate; energy-coupling fermentation; baker's yeast

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is found in a variety of organisms, in which it is important as a protectant against stresses such as extreme temperatures and desiccation.[1] The compound may stabilize biological structures and molecules, for which reason its metabolism and functions are attracting much attention.

Trehalose 6-phosphate (T6P) is an intermediate in the pathway of trehalose synthesis[2] and also may be a precursor of trehalose esters of mycolic acid that are biologically important components of *Mycobacterium* and *Corynebacterium*.[3] In addition, T6P may help to regulate yeast glycolysis.[4] A method to prepare T6P is needed to facilitate further studies of trehalose. Several methods for the preparation of T6P have been described in the literature that involve enzymatic synthesis,[2,5] extraction from a mutant strain of *Saccharomyces cerevisiae*,[6,7] and chemical phosphorylation of trehalose.[8,9] However, these methods yield only small amounts of T6P.

T6P is synthesized from glucose and UMP in yeasts with consumption of ATP by the following sequence of reactions.

glucose + ATP → glucose 6-phosphate + ADP
(glucokinase, EC 2.7.1.2)

glucose 6-phosphate ⇆ glucose 1-phosphate
(phosphoglucomutase, EC 5.4.2.2)

UMP + ATP → UDP + ADP
(nucleosidemonophosphate kinase, EC 2.7.4.4)

UDP + ATP → UTP + ADP
(nucleosidediphosphate kinase, EC 2.7.4.6)

UTP + glucose 1-phosphate ⇆ UDPglucose + PP_i
(glucose-1-phosphate uridylytransferase, EC 2.7.7.9)

UDPglucose + glucose 6-phosphate → T6P + UDP
(α,α-trehalose-phosphate synthase (UDP-forming), EC 2.4.1.15)

The T6P synthesized is finally dephosphorylated by a specific phosphatase, trehalose-phosphatase, to form trehalose and inorganic phosphate.

T6P + H_2O → trehalose + P_i
(trehalose-phosphatase, EC 3.1.3.12)

In this paper, we report an efficient method for the production of T6P from glucose and UMP as substrates with dried cells of baker's yeast as the catalyst. This method depends on coupling reactions between T6P synthesis, which consumes energy, and glucose fermentation, which supplies energy. The reactions occur in yeast cells, in which ATP supplied by fermentation participates efficiently in the reactions for T6P synthesis. As is the sugar nucleotide fermentation by yeast cells reported previously,[10,11] the method described here seems to be an efficient and specific method to prepare T6P at low cost.

Materials and Methods

Chemicals and enzymes. All chemicals used were analytical grade reagents from Nacalai Tesque, Inc. (Kyoto, Japan). Enzyme preparations (trehalase, alkaline phosphatase, glucose oxidase, and peroxidase) were purchased from Sigma Chemical Co. (St. Louis, Mo).

Preparation of dried cells. Commercial compressed

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Abbreviations: G6P, glucose 6-phosphate; T6P, trehalose 6-phosphate

baker's yeast (Oriental Yeast Co., Ltd., Osaka, Japan) was crushed to fine pieces, spread on filter paper, dried at room temperature for 12 h with an electric fan, and desiccated overnight under reduced pressure over phosphorus pentoxide. The dried cells were stored at −20°C until use.

Production of T6P. The standard reaction mixture for T6P production contained 1 M glucose, 20 mM 5′-UMP, 20 mM $MgSO_4 \cdot 7H_2O$, 400 mM sodium phosphate buffer (pH 6.2), and 200 mg of dried baker's yeast in a total volume of 2.0 ml. The concentrations of glucose, 5′-UMP, $MgSO_4 \cdot 7H_2O$, phosphate buffer, and pH were as shown in the figures, and 5′-AMP was added when necessary. The reaction was at 37°C with shaking in a test tube containing all of the reaction mixture. The reaction was stopped by immersal of the test tube in boiling water for 3 min, and the reaction solution was cooled immediately on ice and centrifuged at $10,000 \times g$ for 10 min to remove cell debris. The precipitate was washed 3 times with 1 ml of water and the washings were added to the first supernatant. The combined solution was brought to 5 ml and analyzed for T6P and other products.

Analytical methods. Reaction products in the reaction solution were assayed by HPLC with a Shodex SP1010 column, Shimadzu RID-2A detector, and an eluent of H_2O for glucose and trehalose analysis. For analysis of T6P and glucose 6-phosphate (G6P), an AX Pak WA-624 column, Shodex RI SE-61 detector, and an eluent of an 8:2 (by vol.) mixture of 0.1 M NaH_2PO_4 (pH 3.0) and CH_3CN were used. For analysis of UMP, UDP, UTP, and UDPglucose, a Cosmosil $5C_{18}$ column, UV monitor SF-1205A as the detector, and an eluent of a 3:7 (by vol.) mixture of methanol and 0.02 M KH_2PO_4–0.005 M tetra-*n*-butyl ammonium phosphate were used.

Isolation of T6P. T6P was isolated from the reaction supernatant as follows.[12] To the supernatant, 1 M barium acetate was added until no more precipitate formed, and the abundant precipitate was removed by centrifugation. T6P barium salt, which is water-soluble, remains in the supernatant. The supernatant was adjusted to pH 8–9 with dilute ammonia. A magnesia mixture was prepared as follows. First, 50 g of $MgCl \cdot 6H_2O$ and 100 g of NH_4Cl were dissolved in 500 ml of water, and 10 ml of 2.5% ammonia was added to this mixture and left overnight before the mixture was filtered. The filtrate was adjusted to pH 5–6 with HCl and brought to 1 liter with H_2O. To the supernatant obtained above, this mixture was added to precipitate any excess inorganic phosphate and the new mixture was centrifuged. To the supernatant, ethanol was added to a final concentration of 80%. The alcoholic solution was left at 5°C for 48 h to allow the precipitation of T6P barium salt, which was then collected by centrifugation. The precipitate was dried overnight in a desiccator and suspended in water at 100°C to make the barium salt soluble. An ammonium sulfate solution was added to the T6P solution until no more barium sulfate precipitate formed, and the precipitate was removed by centrifugation. The clear supernatant obtained was adjusted to pH 8.2 and put on a Dowex-1 column (16 × 2 cm i.d.) equilibrated with 1 mM NH_4OH. The column was washed further with 1 mM NH_4OH until carbohydrates were not detected by the anthrone method in the effluent and then the column was eluted with 2.5 liters of 0.01 M potassium tetraborate containing 0.025 M ammonium chloride at a flow rate of 60 ml/h. Fractions of 14 ml were collected and then monitored by the anthrone method and HPLC for the detection of carbohydrates. The anthrone-positive fractions containing T6P were pooled and lyophilized. The borate in the lyophilized preparation was removed by repeated evaporation with addition of methanol under reduced pressure. The concentrated solution was dissolved in water, put on a Dowex-50 (H^+) column (16 × 2 cm i.d.), and eluted with water. Active carbon was added to the eluent (pH 2–3) to adsorb nucleotides and stirred at 5°C for 1 h. The active carbon was removed by filtration and the filtrate was neutralized and lyophilized.

Identification of T6P. From the reaction solution, a portion of T6P equivalent to about 5 μmol was measured out (assay, HPLC) and incubated at 37°C for 1 h with 10 U of alkaline phosphatase in 100 mM Tris-HCl buffer, pH 8.0. The reaction was stopped by heating of the mixture at 100°C for 5 min, and the inorganic phosphate liberated was assayed by the method of Fiske and Subbarow.[13] The liberated carbohydrate, trehalose, was identified by TLC (silica gel G plate; solvent mixture, n-butanol:acetone:0.1 M phosphate buffer, pH 5 in a 4:5:1 mixture), HPLC (Shodex SP1010 column and Shimadzu RID-2A detector), and GC-MS (Shimadzu QP-2000; Shimadzu Hi Cap-CBP5 fused silica capillary column, 0.2 mm i.d. × 25 m × 0.25 μfilm; carrier, helium). Trehalose (0.08 μmol or less) was incubated with 0.07 U of trehalase in 57 mM citrate-phosphate buffer, pH 5.7, and glucose liberated was assayed by the glucose oxidase-peroxidase method.[14]

Results and Discussion

Changes with time in T6P production

Figure 1 shows typical changes with time in T6P production by baker's yeast. UTP, UDPglucose, and G6P began to accumulate simultaneously with the consumption of UMP and glucose at an early stage of fermentation. After the maximum accumulation of G6P and UDPglucose, which are direct substrates for T6P synthesis, T6P increased simultaneously with the formation of trehalose. After 6 h of reaction, T6P was at its maximum concentration, 108 mM. With longer incubation, T6P decreased and trehalose increased; T6P had completely disappeared by 12 h.

Identification of T6P isolated

T6P was isolated from the reaction solution shown in Fig. 1 at 6 h and identified as follows. When the T6P preparation isolated was hydrolyzed by alkaline phosphatase, inorganic phosphate and a certain carbohydrate were liberated. The carbohydrate was identified as

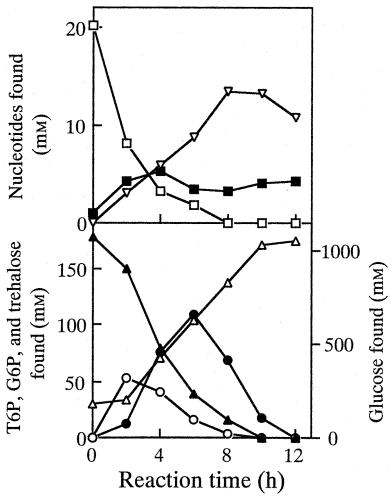

Fig. 1. Changes with Time in T6P Production by Baker's Yeast.
The reaction was under standard conditions. Symbols: □, UMP; ▽, UTP; ■, UDPglucose; ●, T6P; ○, G6P; ▲, Glucose; and △, Trehalose.

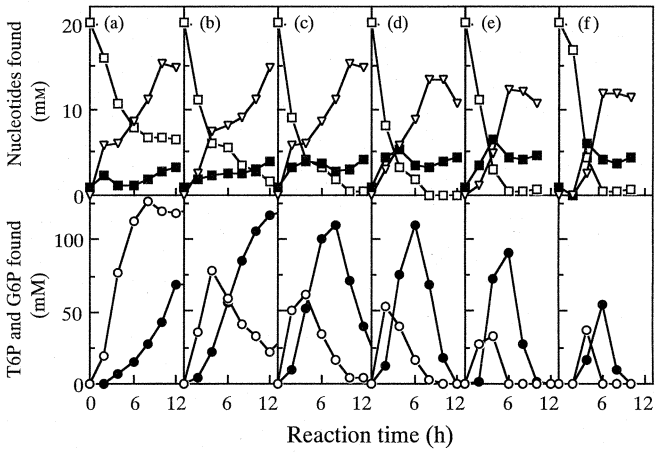

Fig. 2. Effects of pH on T6P Production.
Reactions were under standard conditions except that the pH was as follows: (a) 5.0, (b) 5.4, (c) 5.8, (d) 6.2, (e) 6.6, (f) 7.0. Symbols: □, UMP; ▽, UTP; ■, UDPglucose; ●, T6P; and ○, G6P.

trehalose by TLC, HPLC, and GC-MS. When the carbohydrate was further hydrolyzed by trehalase, a specific hydrolase of trehalose, glucose was liberated and identified by TLC, HPLC, GC-MS, and a glucose oxidase —peroxidase method (data not shown). Finally, the molar ratio of inorganic phosphate:glucose liberated from the T6P preparation was calculated to be 1:2. The chromatographic behavior of the compound isolated and its components liberated by enzyme treatment were identical with those of an authentic T6P preparation; therefore, we concluded that the reaction product was T6P.

Effects of various factors on T6P production

1) *Effects of aeration.* Glucose metabolism by yeast cells provides 2 mol of ATP by anaerobic glycolysis and 36 mol of ATP by aerobic respiration from 1 mol of glucose. Therefore, the effect of aeration on the reaction for T6P production was examined to find how aerobic and anaerobic reaction conditions affect T6P production. An anaerobic reaction was done in an evacuated Thunberg tube, and aerobic reaction was done with a Thunberg tube without evacuation. The formation of both G6P and T6P was maximum at about 50 mM at 4 h with the anaerobic reaction, and by the aerobic reaction, the maximum amount of T6P formed was about 100 mM at 6 h, with the maximum amount of G6P close to that in the anaerobic reaction (data not shown). The synthesis of T6P seemed to proceed better under anaerobic conditions.

2) *Effects of pH.* Figure 2 shows the effects of the initial pH of the reaction mixtures on T6P production when phosphate buffers of various pH are used. More UDPglucose accumulated by 4 h and later in the pH range 6.2–7.0 than at lower pH, but G6P, another precursor of T6P synthesis, accumulated more efficiently in the range of pH 5.0–6.2. The accumulation of T6P was maximum at pH 5.4–6.2. These results suggested that the ratio of UDPglucose and G6P in the reaction mixture would affect T6P production. The accumulation of T6P seemed slow in acidic reaction mixtures, so the effect of pH was further examined with longer incubation than 12 h. In the range of pH 4.6–5.4, the amount of T6P that accumulated was not decreased with long incubation (data not shown). This result suggested that the activity of trehalose-phosphatase and other phosphatases (alkaline phosphatase, etc.) was inhibited at the acidic pH. The maximum amounts of T6P at different pHs and 14 h were 120 mM (pH 5.4), 69 mM (pH 5.0), and 17 mM (pH 4.6).

3) *Effects of inorganic phosphate concentration.* The concentration of P_i in sugar nucleotide fermentation may be the most important factor in the phosphorylation of sugars and nucleotides.[10,11] Figure 3 shows the effects of P_i concentrations from 200 to 600 mM on the formation of T6P. At 200 or 300 mM, more UDPglucose accumulated as UMP was consumed, but at higher concentrations of P_i (400–600 mM) more UTP was formed instead. The production of T6P and G6P was maximum at 400 mM P_i; higher concentrations gave less of these sugar phosphate compounds.

4) *Effects of $MgSO_4$ concentration.* Some of the enzymes involved in glycolysis and respiration need Mg^{2+} as an activator. The phosphorylation of UMP and formation of T6P may be affected by the Mg^{2+} concentration. In fact, the amounts of T6P and G6P produced were affected little by concentration changes in $MgSO_4$ from 4 to 100 mM. The accumulation of T6P was lower than the maximum when 40 mM $MgSO_4$ or more was present (data not shown).

5) *Effects of glucose concentration.* Glucose is necessary in the reaction mixture for T6P production both as an energy source for ATP production for the phosphorylation of UMP and glucose, and also as a sugar substrate for G6P and UDPglucose production. Therefore, the formation of T6P by yeast cells may be affected by the glucose concentration. A high concentration of glucose favored the formation of G6P and T6P (Fig. 4). The yield of T6P to glucose (mol/mol) was 3.1%, 8.3%, 9.8%, and 8.8% at 0.5 M, 0.75 M, 1.0 M, and 1.25 M glucose, respectively. The optimum glucose concentration

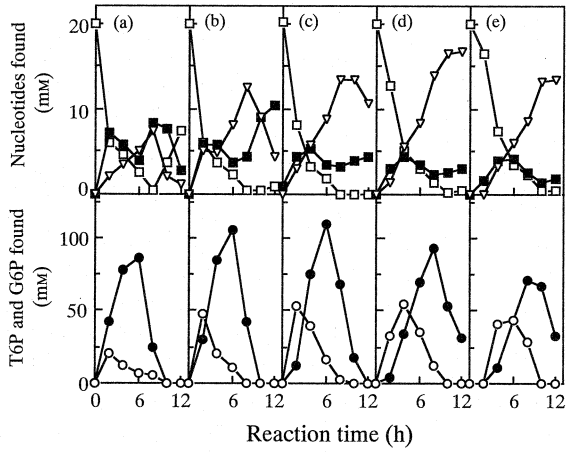

Fig. 3. Effects of Inorganic Phosphate Concentration on T6P Production.

Reactions were under standard conditions except that the inorganic phosphate concentration (mM) was as follows: (a) 200, (b) 300, (c) 400, (d) 500, (e) 600. Symbols: □, UMP; ▽, UTP; ■, UDPglucose; ●, T6P; and ○, G6P.

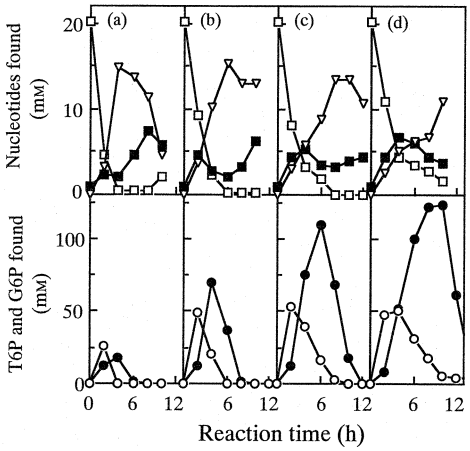

Fig. 4. Effects of Glucose Concentration on T6P Production.

Reactions were under standard conditions except that the glucose concentration (M) was as follows: (a) 0.50, (b) 0.75, (c) 1.00, (d) 1.25. Symbols: □, UMP; ▽, UTP; ■, UDPglucose; ●, T6P; and ○, G6P.

for T6P formation was about 1 M.

6) *Effects of 5′-UMP concentration.* The effects of the concentration of UMP, which is the precursor of UDPglucose, on the synthesis of T6P was investigated with the expectation that the small amount of UMP added to the reaction mixture would be converted to UDPglucose *via* UTP to form T6P and UDP, and that UDP would be recycled to regenerate UDPglucose. About 50 mM T6P accumulated without the addition of UMP, but with 5 mM UMP, almost twice as much T6P formed (Fig. 5). The maximum amount of T6P was almost identical at concentrations of UMP of 5 mM or more.

7) *Effects of 5′-AMP concentration.* The effects of addition of 5′-AMP, a precursor of ATP, on the production of T6P were examined. AMP was added to the standard reaction mixture at concentrations of 0, 2.5, or 5.0 mM. The maximum amount of G6P was greater with AMP (25, 70, and 83 mM in the presence of 0, 2.5, and 5.0 mM AMP, respectively), but the maximum amount of T6P was unaffected (114, 115, and 118 mM, respectively). Added AMP promoted G6P formation, but did not change the amount of T6P formed.

In this study, the enzymatic preparation of T6P from UMP and glucose as substrates by dried cells of baker's yeast as the enzyme source was investigated. This method is based on coupling reactions between T6P synthesis, which consume energy, and glucose fermentation, which supplies energy, with dried cells of yeast as catalyst. The yield of T6P was affected by certain reaction conditions, especially by the pH and the concentrations of reaction components. The maximum yield of T6P as a mole-to-mole percentage of added glucose was about 11% (45 mg of T6P per milliliter of reaction mixture) when 1 M glucose, 20 mM UMP, 20 mM $MgSO_4$, and 400 mM phosphate buffer (pH 6.2) were incubated aerobically at 37°C for 6 h with 100 mg/ml dried cells of baker's yeast. The amount of T6P in the reaction mixture declined by prolonged reaction under these conditions. One of the most important factors in T6P accumulation was the pH: in the acidic pH range (4.6–5.4), the T6P that had accumulated did not decrease even with 20 h of reaction. The optimum pHs of the enzymes involved in T6P synthesis and degradation are from pH 6.0 to 9.0, so the acidic pH may delay both T6P accumulation an degradation. If the reaction pH were controlled, the accumulation of T6P might be more stable.

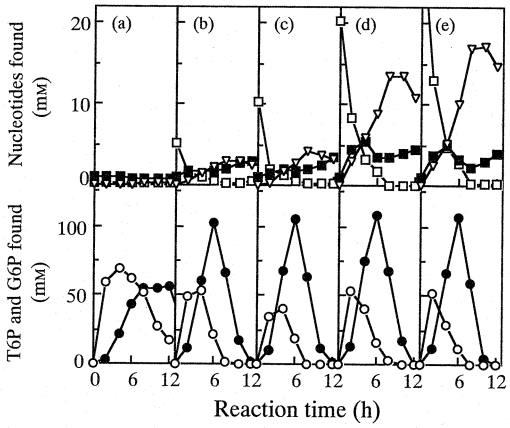

Fig. 5. Effects of 5′-UMP Concentration on T6P Production.

Reactions were under standard conditions except that the 5′-UMP concentration (mM) was as follows: (a) 0, (b) 5, (c) 10, (d) 20, (e) 30. Symbols: □, UMP; ▽, UTP; ■, UDPglucose; ●, T6P; and ○, G6P.

The method for the preparation of T6P described here was more efficient, specific, and inexpensive than the other methods mentioned in the Introduction. The production of large amount of T6P will facilitate the study of trehalose metabolism and glycolysis regulation.

T6P seems to be the precursor of trehalose esters such as code factors (trehalose lipid in *Mycobacterium* and *Corynebacterium*), and trehalose dimannosyl phosphate in *Mycobacterium* species. Some species of bacteria that can use hydrocarbons as energy and carbon sources produce surface-active compounds consisting of treha-

lose and fatty acids when grown in a medium containing hydrocarbons.[15] These products may be used as biosurfactants unlike the synthetic surfactants already being produced. We used yeast cells for the preparation of T6P, but microorganisms other than yeasts could be used as the enzyme source for synthetic reactions. Therefore, new and useful trehalose derivatives such as trehalose lipids, oligosaccharides containing trehalose, and trehalose fatty acid esters may come to be prepared efficiently from T6P produced by this method in combination with other synthetic enzyme reactions.

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