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Journal of Molecular Catalysis B: Enzymatic 43 (2006) 137–141
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Production of trehalose by permeabilized *Micrococcus QS412* cells

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Available online 15 September 2006

Abstract

Permeabilized *Micrococcus QS412* cells were used to produce trehalose from starch through catalysis of maltooligosyl trehalose synthase and maltooligosyl trehalose trehalohydrolase in the cells. The permeabilized cells could omit the enzyme purification and simplify the immobilization of intracellular enzymes. The reagent, reagent dosage and time of cell permeabilization treatment were determined. The maximum trehalose biosynthesis activity was obtained after the cells were treated with 5% (w/v) of toluene at 30 °C for 40 min. Reaction conditions of trehalose synthesis of permeabilized cells were optimized. The yield of trehalose was up to 188 mg/g wet permeabilized cells in pH 8.0, 100 mmol/l phosphate buffer at 30 °C after 12 h reaction. Batch reactions showed that the permeabilized cells could be reused for 16 cycles in the biosynthesis reaction. The total trehalose yield was up to 2.5 g/g wet permeabilized cells. Development of permeabilized cells provide a new cheaply alternative technology for trehalose production.

Keywords: Batch reactions; Enzymatic hydrolysis; Permeabilized cells; Starch; Trehalose

1. Introduction

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a non-reducing disaccharide with two glucose residues bound by an α-1,1 linkage [1]. Trehalose can be produced by many biosynthesis method [2–5]. Among them, the reaction system composed of two enzymes, maltooligosyl trehalose synthase (MTSase) and maltooligosyl trehalose trehalohydrolase (MTHase), made mass production of trehalose become possible and economical. Trehalose was produced from starch through this enzymatic system (Fig. 1) [6–8]. The industrial trehalose production was achieved by purification and immobilization of these two intracellular enzymes.

Sometimes biocatalysts in the form of whole cells have an advantage over purified intracellular enzymes in many industrial bioconversion processes. In these application, cells might be treated with permeation reagent to increase permeability of substrate through cell membrane [9–11]. Permeabilized cells might be more suitable for two enzyme reaction of MTSase and MTHase than for single enzyme reaction. Permeabilized cells could omit the purification and immobilization of the two enzymes, and the whole cell could be used as immobilization enzymes.

In this study, *Micrococcus QS412* which could produce MTSase and MTHase was used to prepare whole cell biocatalysts. Five reagents were used to treat the cells, and the effects of permeabilization treatments on the reactivity were investigated. The obtained permeabilized cells could be used many times. This method provided a new cheap alternative for trehalose production.

2. Materials and methods

2.1. Chemicals

Trehalose were purchased from Sigma, USA. Glucose, peptone, yeast extraction, α-amylase, soluble starch, and agar were purchased from Beijing Double-Spin Microbial Medium Manufacturer. All other chemicals were of analar grade.

2.2. Microorganism and culture conditions

Micrococcus QS412 was used to produce MTSase and MTHase, which was screened from soil by our lab.

Micrococcus QS412 was cultivated at 30 °C. The medium contained 20 g/l glucose, 5 g/l yeast extract, 10 g/l peptone, 1 g/l K_2HPO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$. The cells were harvested by centrifugation after 72 h incubation with shaking. Then the bacteria pellet was washed twice with 20 mmol/l phosphate buffer (pH 7.0) and centrifuged.

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doi:10.1016/j.molcatb.2006.07.014

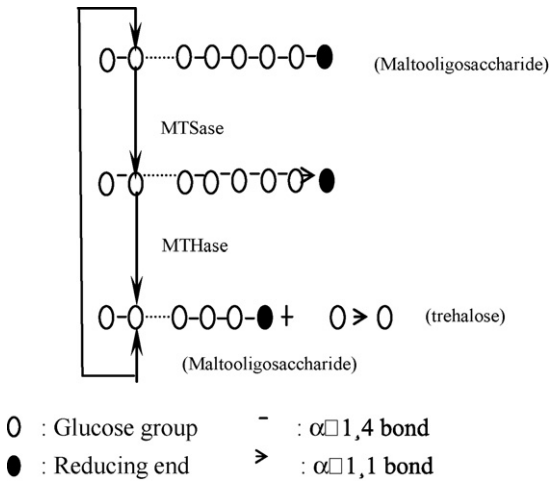

Fig. 1. Scheme of trehalose production by MTSase and MTHase.

2.3. Permeabilization procedure and trehalose synthesis

Micrococcus QS412 cells (20 g wet weight) were suspended in 80 ml of potassium phosphate buffer (50 mmol/l, pH 7.0) and the permeabilizing reagent was added at 30 °C. The cells were collected by centrifugation (5000 × *g*, 10 min) at 4 °C. The cells were further washed with potassium phosphate buffer (50 mmol/l, pH 7.0) and resuspended in 80 ml of the same buffer. The cell suspension was used as enzyme catalyst for trehalose synthesis.

Liquefied starch solution was used as the substrate to determine trehalose biosynthesis activity of the permeabilized cells. Permeabilized cells suspension was added into 10% (w/v) of liquefied starch solution (20DE) at the ratio of 1:1. The reaction mixture was incubated with shaking for 20 h at 30 °C. The supernatant was collected to measure the amount of trehalose by centrifugation at 10,000 × *g* for 10 min.

Trehalose was assayed by high performance liquid chromatography (HPLC). The conditions of analysis were as following: ZORBAX-NH_2 column; acetonitrile–water eluent (70:30, v/v); 1 ml/min of flow velocity; 35 °C column temperature; differential refractometer detector.

The trehalose biosynthesis ability of permeabilized cells was defined as the trehalose production by 1 g of wet permeabilized cells under specified conditions.

One unit of trehalose biosynthesis activity (U_{pc}) was defined as the trehalose production per hour by 1 g of wet permeabilized cells under specified conditions:

$$U_{pc}\,(\mathrm{mg/g\,h}) = \frac{C_t V}{W_{pc} T} \quad (1)$$

C_t is the concentration of trehalose after reaction, V is the reaction volume, W_{pc} is the weight of permeabilized cells used in the reaction and T is the reaction time.

2.4. Permeabilization conditions

The permeability barrier of cell wall and membrane for substrates and products may cause low reaction rates of whole cells catalyst. Therefore, it is important to find an effective method to overcome the permeability barrier to prepare whole cell biocatalysts with high activities. Herein, toluene, carbon tetrachloride, glutaraldehyde, Tween-80 and Span-20 were used as permeabilizing reagents to treat cells. The activity of permeabilized cells was also influenced by different dosage and treatment time of permeabilizing reagent [12–17].

The effect of permeabilizing reagents on enzymes was also investigated. The cells were suspended in 50 mmol/l phosphate buffer (pH 7.0) at 10% (w/v) ratio. Then the cells was disrupted in ice bath by sonication. After centrifugation at 12,000 × *g* for 10 min, the supernatant was obtained. The permeabilizing reagents were added into the supernatant and incubated at 30 °C for 1 h. Then the solution was used to synthesize trehalose from liquefied starch.

In order to investigate whether MTHase and MTSase could be released from cell after permeabilization, it is necessary to determine trehalose synthesis activity of extracellular solution and permeabilized cells. The concentration of released protein was determined by assay method of [18]. Bovine serum albumin was used as standard.

2.5. Enzyme reaction conditions

The reaction conditions of permeabilized cells included ionic strength, pH, reaction temperature and time. Reaction temperature was determined between 25 and 45 °C. The reaction was carried out in 50 mmol/l phosphate buffer (pH 7.0) for 20 h at different temperature.

Influence of ionic strength was studied with different concentration of pH 7.0 phosphate buffer. The reaction was carried out at 30 °C for 20 h with shaking.

The effect of pH was also studied by using citrate–phosphate buffer (100 mmol/l, pH 6–7.5), and phosphate buffer (100 mmol/l, pH 7.0–9.0). Reaction was carried out in 100 mmol/l phosphate buffer for 20 h at 30 °C.

At last, the influence of reaction time was determined after other conditions were optimized.

2.6. Reuse of the permeabilized cells

100 ml of permeabilized cells suspension was incubated with 100 ml of 10% (w/v) liquefied starch in phosphate buffer (100 mmol/l, pH 8.0) at 30 °C. Then the permeabilized cells were collected by centrifugation (5000 × *g*) for 10 min, washed with phosphate buffer, and resuspended in 180 ml of freshly prepared substrate to start a new reaction.

Two forms of batch reactions were studied. One reaction pattern was carried out according to the following procedure: 12 h of each reaction, 6 batches, 72 h of total time; and the other procedure was as following: 4 h of each reaction, 16 batches, 64 h of total time.

3. Results and discussion

3.1. Reaction activity of permeabilized cells

A simple trehalose biosynthesis method by catalysis of permeabilized cells was expected, which might substitute

Table 1
Released protein and trehalose biosynthesis activity of treated cells in 11 batches

Batch	Released protein concentration (mg/ml)	Trehalose production by extracellular enzymes (mg/ml)	Trehalose production by permeabilized cells (mg/ml)
1	0.069	1.12	30.72
2	0.065	0.65	34.84
3	0.051	0.23	33.65
4	0.003	0	33.41
5	0	0	32.28
6	0	0	31.75
7	0.009	0	31.55
8	0.036	0	30.54
9	0.012	0	31.12
10	0.011	0	30.87
11	0.001	0	32.84

Reaction conditions: 10% (w/v) cells; 5% liquefied starch; pH 7.0; 30 °C; 20 h.

purification and immobilization of two intracellular enzymes.

Due to permeability barrier of the cell membrane against the substrate natural cells showed no measurable trehalose biosynthesis activity. After the cells were treated with 1% (w/v) toluene in 50 mmol/l phosphate buffer (pH 7.0), the significant biosynthesis activity of trehalose was observed (Table 1). The extracellular solution of permeabilized cells also showed low trehalose biosynthesis activity. A small quantity of MTHase and MTSase might be released into extracellular from some over-damaged cells. The concentration of released protein also indicated that the released protein from cells was very little. The cells could be used many times, and each reaction batch lasted 4 h. After several reuse cycles of permeabilized cells, the extracellular solution could not catalyse the production of trehalose, but the activity of cells did not decrease. It indicated that MTHase and MTSase were located in the permeabilized cells and could not go through permeabilized cells membrane. Only a few over-damaged cells could release a small amount of enzymes. The liquefied starch could pass the treated cell membrane and then be catalysed by intracellular enzymes.

3.2. Permeabilization conditions

Toluene, CCl_4, glutaraldehyde, Tween-80 and Span-20 were used to treat cells. Among them, toluene resulted in higher enzyme activity than non-ionic detergents. Tween-80 and Span-20 also could be used as effective reagents to permeate micrococcus cells. Treatment with CCl_4 and glutaraldehyde leaded to very low trehalose biosynthesis activity of whole cells (Table 2). The effect of permeabilizing reagents on crude enzymes (the supernatant of cell lysate) was shown in Table 3. Except for glutaraldehyde, other reagents had no obvious influence on the enzyme activity. Two percent of glutaraldehyde could decrease enzyme activity by about 70%. Glutaraldehyde could result in enzyme inactivation. Herein, toluene was chosen to treat cells in further experiment.

In order to determine the optimal conditions for toluene permeabilization, toluene concentration and treatment time were studied. The effect of toluene concentration on permeabilization indicated that the trehalose biosynthesis activity

Table 2
Trehalose biosynthesis activity of cells treated with various permeabilizing reagents

Permeabilizing reagents	Dosage (w/v)	Trehalose concentration (mg/ml)
Toluene	2%	15.82
CCl_4	2%	0
Glutaraldehyde	2%	1.29
Tween-80	2%	9.97
Span-20	2%	11.32

Reaction conditions: 10% (w/v) cells; 5% liquefied starch; pH 7.0; 30 °C; 20 h.

increased above 2% toluene. At 5% toluene the concentration of trehalose was 12.76 mg/ml, the yield of trehalose from starch was 25.5%, and the trehalose biosynthesis activity of cells was up to 10.63U_{pc} (Fig. 2). Above 10% toluene, a visible decrease in trehalose biosynthesis activity was observed. This might be attributed to the inactivation of enzymes by toluene. In this experiment, released protein in treatment was also assayed. As shown in Fig. 2, maximum protein content was obtained at 5% toluene. The variation trends of trehalose biosynthesis activity of permeabilized cells and released protein were accordant. Further tests were examined under different treatment times based on 5% of toluene dosage at 30 °C. 40 min treatment time of cells with toluene was required for the best permeabilization effect. Under these conditions the final concentration of trehalose was 14.22 mg/ml, the yield of trehalose from starch was 28.4%, and the trehalose biosynthesis activity of the permeabilized cells was up to 11.85U_{pc}. A further increase in incubation time

Table 3
Effects of various permeabilizing reagents on enzyme activity

Permeabilizing reagents	Dosage (w/v)	Trehalose concentration (mg/ml)
Toluene	2%	10.15
CCl_4	2%	9.87
Glutaraldehyde	2%	3.02
Tween-80	2%	10.87
Span-20	2%	11.08
Blank	–	11.01

Reaction conditions: 5% liquefied starch; pH 7.0; 30 °C; 20 h.

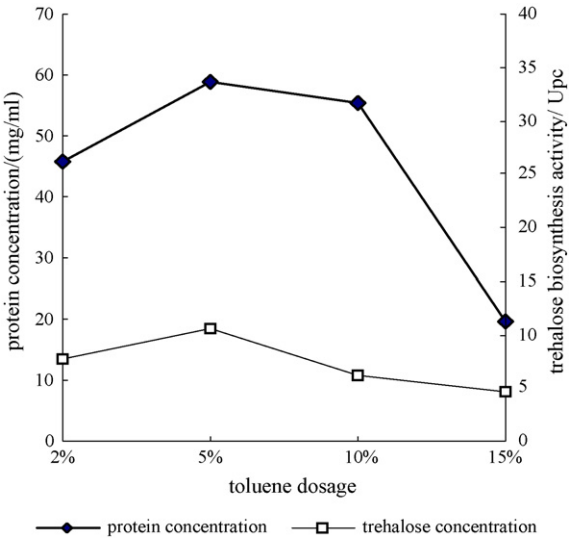

Fig. 2. Effects of toluene dosage on permeabilization treatment. Reaction conditions: 10% (w/v) cells; 5% liquefied starch; pH 7.0; 30 °C; 20 h.

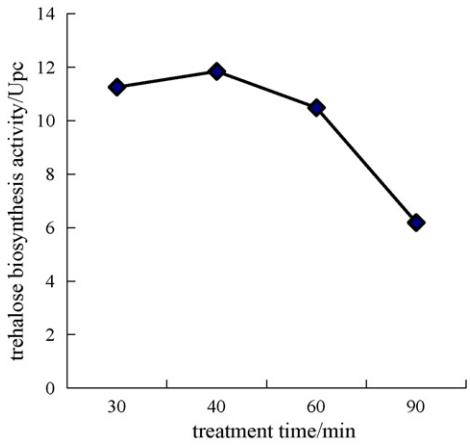

Fig. 3. Effects of toluene permeabilization time on trehalose biosynthesis activity. Reaction conditions: 10% (w/v) cells; 5% liquefied starch; pH 7.0; 30 °C; 20 h.

resulted in a slight decrease in trehalose biosynthesis activity (Fig. 3).

3.3. Optimization of reaction conditions

The influence of ionic strength, pH, reaction temperature, and time on trehalose productivity of permeabilized cells was determined. As shown in Fig. 4a, 30 °C was the optimal temperature for the trehalose biosynthesis reaction. Above 30 °C the yield of trehalose decreased significantly.

Ionic strength had an obvious effect on enzyme activity of MTHase and MTSase. When phosphate buffer was kept at 100 mmol/l, the highest yield of trehalose was obtained (Fig. 4b). As shown in Fig. 4c, the optimal trehalose biosynthesis ability was obtained in the pH 8.0 phosphate buffer (Fig. 4c).

The yield of trehalose increased with extension of reaction time before 12 h (Fig. 4d). The highest trehalose yield of

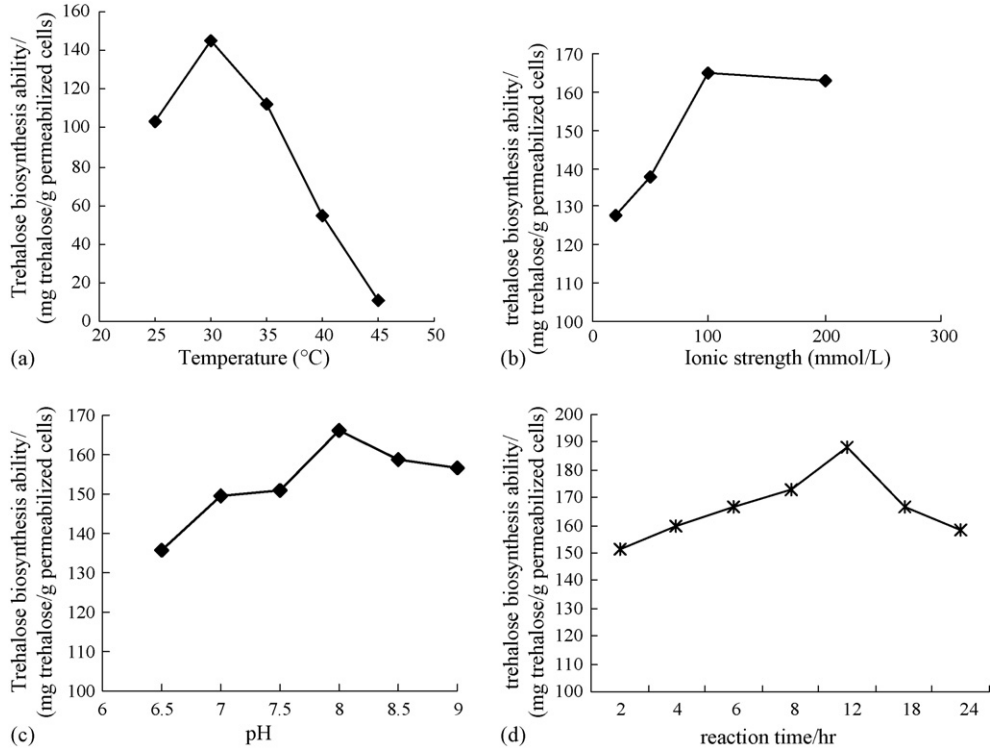

Fig. 4. Optimization of reaction conditions: (a) effect of temperature on the trehalose biosynthesis ability; (b) effect of ionic strength on the trehalose biosynthesis ability; (c) effect of pH on the trehalose biosynthesis ability; (d) effect of incubation time on the trehalose biosynthesis ability. Reaction conditions: 10% (w/v) cells; 5% liquefied starch; (a) 50 mmol/l potassium phosphate buffer, pH 7.0, 20 h; (b) 30 °C, pH 7.0, 20 h; (c) 30 °C, 100 mmol/l potassium phosphate buffer, 20 h; (d) 30 °C, 100 mmol/l potassium phosphate buffer, pH 8.0.

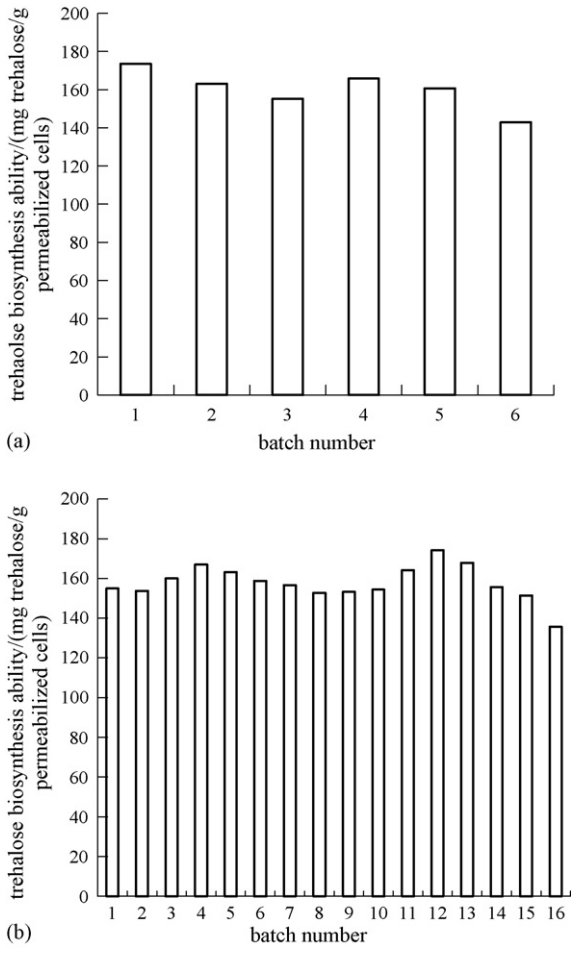

Fig. 5. Reuse of the permeabilized cells: (a) 12 h of each reaction time, 6 batches, 72 h of total time; (b) 4 h of each reaction time, 16 batches, 64 h of total time. Reaction conditions: 10% (w/v) cells; 5% liquefied starch; pH 8.0; 30 °C.

188 mg/g wet permeabilized cells was obtained at 12 h of reaction time and the yield of trehalose from starch was 37.6%. A further increase of incubation time resulted in a slight decrease. As the increase of trehalose was slow after 4 h reaction, less than 12 h of practical reaction time might be adopted, which could increase the time yield of trehalose.

3.4. Reuse of permeabilized cells

The reuse of cells was used in two patterns. In one pattern, each reaction lasted 12 h. In the other pattern, each reaction lasted 4 h. In both patterns, the permeabilized cells were recycled until their trehalose biosynthesis ability decreased to 80% activity in the first batch (Fig. 5a and b). Permeabilized cells could be recycled six times and still keep steady high trehalose biosynthesis activity in first pattern. The total trehalose yield in the second pattern was up to 2.5 g/g permeabilized cells, which was 1.65 times as much as that in the first one. The cells could be used many times without obvious decrease of activity.

4. Conclusion

Toluene can effectively improve the permeability of cell membrane of *Micrococcus QS412*. The treated cell membrane can prevent intracellular MTHase and MTSase from being released into the extracellular solution, and liquefied starch can pass through the treated cell membrane into the cells.

The permeabilized cells can be used to product trehalose from liquefied starch through catalysis of MTHase and MTSase in the cells. Trehalose biosynthesis ability was up to 188 mg/g wet permeabilized cells. The cells can keep steady and high trehalose biosynthesis for a long time and can be reused many times. In our experiment, the total trehalose yield was up to 2.5 g/g wet permeabilized cells. This method omits purification and immobilization of MTHase and MTSase, and provide an economical route for trehalose production.

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