

Full Length Research Paper

Optimization of fermentation conditions for trehalose production by a marine yeast

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Accepted 20 January, 2012

The culture conditions for the production of trehalose by *Rhodotorula* sp. strain were optimized. The optimum medium contained (g/L) 20.1 sucrose, 8.15 yeast extract, 8.15 peptone, 1.81 NH₄Cl, 1.66 KH₂PO₄, 1.66 Na₂HPO₄, 0.84 MgSO₄, and 0.84 CaCl₂. In a shake flask, the optimal culture conditions were as follows: initial pH 5.5, temperature 28°C and medium volume 75 mL. Under optimum conditions, the production of trehalose was increased 2.3 times through optimization and comprised 193 mg/g biomass. In the case of a 5 L fermentor, the optimum culture time was 54 h and under optimal conditions, the production of trehalose was 2.45 g/L.

Key words: Trehalose, *Rhodotorula* sp., medium, culture conditions, optimization.

INTRODUCTION

Trehalose is a non-reducing disaccharide that is widely present in microorganisms and invertebrates. It has many physiological functions in cells, such as carbohydrate storage and as a highly efficient protectant, enhancing cell resistance against adverse conditions such as high temperature, freezing, low hydration, high osmotic pressure and high concentrations of ethanol (Chi et al., 2003). Among the three possible isomers (α/α , α/β , and β/β trehalose), the only naturally occurring form is the α/α isomer (Cesaro et al., 2008). Trehalose has many applications e.g. as a cryoprotectant for preserving cells in medicine and microbiology, as an effective component in cosmetics, as a stabilizer in clinical reagents and bioproducts, or even as a preservative for fresh foodstuff. Recently, trehalose has been accepted as a safe food ingredient by the European regulation system, following approval by the U.S. Food and Drug Administration (Cho et al., 2006; Schiraldi et al., 2002). Thus, much attention has been directed to devising inexpensive means for producing trehalose.

In the early 1990s, the cost of commercial trehalose

was as high as 700 US\$ kg⁻¹. The most interesting approaches to production have been filed as patents and successfully exploited in production processes leading to a major reduction in the commercial price of trehalose to 5 to 6 US\$ kg⁻¹ (Schiraldi et al., 2002). Various methods for large-scale production of trehalose are available. These include: (1) accumulation of trehalose from glucose by fermentation using *Saccharomyces cerevisiae* and Basidiomycotinous yeast (Chi et al., 2009; Miyazaki et al., 1996; Schiraldi et al., 1995). (2) Production of trehalose from starch or maltose by thermostable enzymes from thermophilic bacteria (Mukai et al., 1997; Yoshida et al., 1997). (3) Synthesis of trehalose from glucose and glucose-1-phosphate by trehalose phosphorylase from *Pichia fermentans* (Mukai et al., 1997; Yoshida et al., 1997). In this paper, a trehalose producing yeast strain was selected from seawater media. Anticipating possible industrial applications, culture conditions were optimized to improve the yield of trehalose.

MATERIALS AND METHODS

Yeast strain

Rhodotorula sp., a trehalose producing strain, was screened from

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seawater.

Media and culture conditions

1) Solid medium (g/L): 200 potato, 20 glucose, 20 agar, boiled filtration seawater 1000 mL, and natural pH.

2) Seed medium (g/L): 30 sucrose, four yeast extract, four ammonium sulfate, three potassium phosphate monobasic, five potassium phosphate sodium, two magnesium sulfate, boiled filtration seawater 1000 mL, and pH 6.5.

3) Basic medium (g/L): 30 sucrose, four yeast extract, four ammonium sulfate, three potassium phosphate monobasic, five potassium phosphate sodium, two magnesium sulfate, boiled filtration seawater 1000 mL, and pH 6.5

Fermentation

Seed cultures were prepared by inoculating *Rhodotorula* sp. cells grown on a solid medium agar slant into a 500 mL Erlenmeyer flask that contained 180 mL of seed medium and subsequently incubating this at 28°C for 30 h with shaking (140 rpm). The fermentation was carried out in a 5 L automatic fermentor. 180 mL of seed culture was transferred into 3500 mL of basic medium at 28°C with an agitation rate of 350 rpm for 60 h.

Trehalose extraction and assay

Trehalose in the yeast cells was extracted with trichloroacetic acid and the trehalose content in the mixture was assayed by the Anthrone method (Stewart, 1982).

Determination of cell dry weight

The yeast cells from 5 mL of culture were harvested by centrifugation at 4000 rpm for 5 min, washing three times with distilled water, and drying at 100°C to a constant weight.

Determination of sucrose concentration

Sucrose concentration in the fermented media was determined by Anthrone method (Zhong et al., 1995).

Optimization of medium composition

Screening of carbon sources and the level of the optimal carbon source

Glucose, maltose, and sucrose were selected as substrates for the production of trehalose. The level of the carbon sources was set at 30 g/L. The level of the optimal carbon source (sucrose) was tested at 10, 20, 30 and 40 g/L, respectively (Duan et al., 2008).

Screening of organic nitrogen sources

Yeast extract, peptone and beef extract were selected as three organic nitrogen sources. The level of organic nitrogen sources was set at 10 g/L.

Screening of compound nitrogen source

Ammonium chloride, ammonium oxalate, and urea were selected

as inorganic sources. Combinations of nine different organic and inorganic nitrogen sources were investigated. The ratio of organic nitrogen to inorganic sources was 9:1 and the concentration of total nitrogen sources was 10 g/L.

Screening of the ratio of carbon to nitrogen

The ratios of carbon and nitrogen investigated were 40:10, 30:10, 30:20, 20:10, 10:10, 10:20, and 10:30.

Determination of culture conditions

Seed cultures were prepared by inoculating *Rhodotorula* sp. cells grown on a solid medium agar slant into a 250 mL flask that contained 50 mL of seed medium and incubating this at 28°C for 30 h with shaking (140 rpm). A seed culture (2.5 mL) was then transferred into a 250 mL flask that contained 50 mL of basic medium. The flasks were incubated at 28°C for 60 h with shaking (140 rpm).

RESULTS AND DISCUSSION

Effect of carbon source on the production of trehalose

Effect of different carbon source on the production of trehalose

When glucose, sucrose and maltose were used as carbon source, the cell dry weights were 3.4, 3.4 and 2.4 g/L respectively, trehalose production were 68.8, 84.3 and 73.8 mg/g respectively. With sucrose, trehalose production was higher than those using the other two carbon sources. Therefore, sucrose was the optimum carbon source.

Effect of carbon source concentration on the production of trehalose

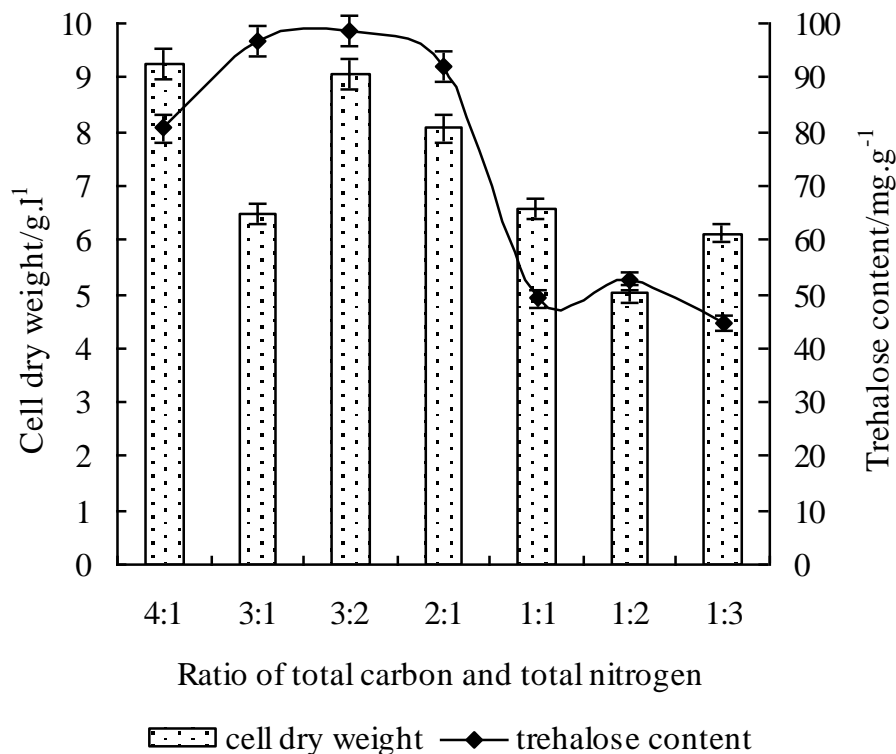
When concentration of sucrose was 10.0, 20.0, 30.0, and 40.0 g/L, the cell dry weights were 6.4, 7.5, 7.4, and 7.0 g/L respectively, trehalose production were 50.2, 69.4, 73.5, and 54.1 mg/g respectively. The dry cell weight increased as the concentration of sucrose increased from 10.0 to 20.0 g/L. However, trehalose content in the yeast strain increased from 50.9 to 73.5% (w/w) of dry cell weight when the concentration of sucrose increased from 10.0 to 30.0 g/L. In contrast, when the concentration of sucrose increased from 30.0 to 40.0 g/L, trehalose content in the yeast cells decreased from 73.5 to 54.1% of cell dry weight. Therefore, the optimum concentration of sucrose was judged to be 30.0 g/L.

Effect of nitrogen source on the production of trehalose

Results of experiments show that when a single organic

Table 1. Effect of nitrogen sources on trehalose production.

| N source | Cell dry weight/g.l ⁻¹ | Trehalose content/mg.g ⁻¹ |
|--------------------------------|---|--------------------------------------|
| Peptone + yeast extract + | NH ₄ Cl | 7.45 |
| | (NH ₄) ₂ C ₂ O ₄ | 6.73 |
| | (NH ₄) ₂ SO ₄ | 6.87 |
| | Urea | 5.35 |
| Beef extract + yeast extract + | NH ₄ Cl | 6.15 |
| | (NH ₄) ₂ C ₂ O ₄ | 7.01 |
| | (NH ₄) ₂ SO ₄ | 6.40 |
| | Urea | 3.69 |
| Peptone + beef extract + | NH ₄ Cl | 6.16 |
| | (NH ₄) ₂ C ₂ O ₄ | 6.85 |
| | (NH ₄) ₂ SO ₄ | 6.29 |
| | Urea | 3.29 |

**Figure 1.** Effect of C/N source concentrations and their ratios on trehalose production.

nitrogen source was used, the dry weight of yeast cells and the yields of trehalose were lower than those obtained with a complex nitrogen source, so a complex nitrogen source was chosen for subsequent experiments. The data in Table 1 indicates that trehalose production and yeast cell dry weight were highest when yeast extract, peptone and ammonium chloride were used as the complex nitrogen source. The highest production of trehalose was 101 mg/g, so an appropriate composition

of the complex nitrogen source consisted of peptone, yeast extract and ammonium chloride.

Effect of the carbon source to nitrogen source ratio on trehalose production

The results in Figure 1 show that cell growth and the production of trehalose were higher when the level of

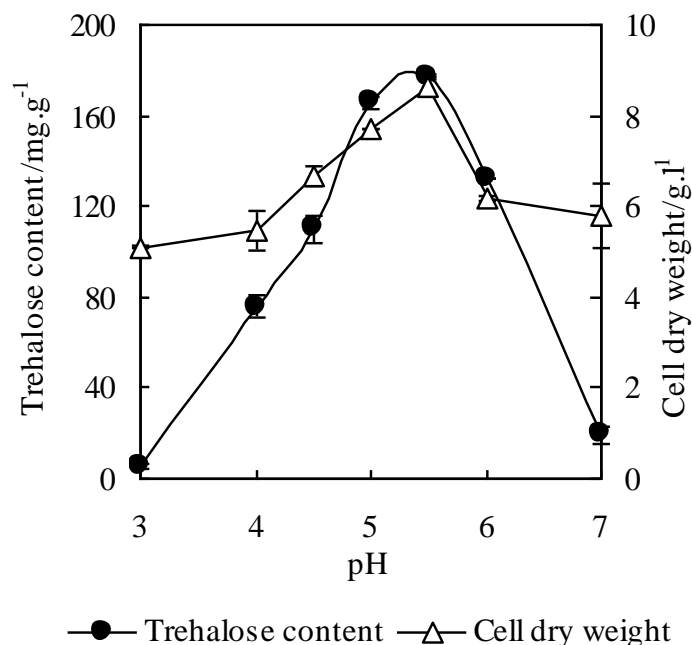


Figure 2. Effect of pH on trehalose production.

carbon was higher than that of nitrogen in the medium. When the ratio of carbon to nitrogen was 4:1, the yeast cell dry weight reached 9.25 g/L. However, when the ratio of carbon to nitrogen was 3:2, the production of trehalose was maximal at 99 mg/g cell and the cell dry weight was 9.06 g/L. Therefore, a carbon to nitrogen ratio of 3:2 was optimal, so a complex medium comprising 30 g/L sucrose, 9 g/L yeast extract, 9 g/L peptone and 2 g/L ammonium chloride were optimal for trehalose production by the yeast strain.

Effects of initial pH on trehalose production

As can be seen in Figure 2, the yield of trehalose in yeast cells increased rapidly with increase in initial pH. Trehalose production (176.89 mg/g) and cell dry weight (8.65 g/L) reached their highest values when the initial pH of the growth medium was 5.5. The production of trehalose decreased rapidly when the initial pH was greater than 5.5. The results shown in Figure 2 indicate that the effects of initial pH on cell growth and trehalose production were significant and therefore the initial pH should be strictly controlled in the cultivation process.

Effects of temperature on trehalose production

The results in Figure 3 indicate that the cell dry weight and yield of trehalose increased sharply as temperature increased. Trehalose production (192.53 mg/g) and cell dry weight (7.53 g/L) reached their highest values when

the cultivation temperature was 28°C. The cell dry weight was still higher when the culture temperature was 37°C, but the yield of trehalose decreased sharply beyond 37°C. These results indicate that a higher long-term cultivation temperature was not suitable for the production of trehalose, while higher, short-term cultivation temperatures supported the production of trehalose because this stimulates the yeast cell stress response to protect itself.

Effects of medium volume on trehalose production

As can be seen in Figure 4, the dry cell weight and yield of trehalose reached their highest values at a medium volume of 75 mL, but the effect of medium volume on cell growth was insignificant. However, the effect of medium volume on trehalose production was significant. Trehalose production in the flask was 193 mg/g cell which was 2.3 times that achieved previously.

Time course of cell growth and trehalose production during 5 L fermentation

The results in Figure 5 show that the sucrose content in the medium sharply decreased and the cell growth rate sharply increased when the culture time was over 10 to 20 h. At culture times beyond 20 h, the cell growth entered the stationary phase. The yield of trehalose was low when culture time was over 10 to 20 h. The yield of trehalose increased gradually with the depletion of

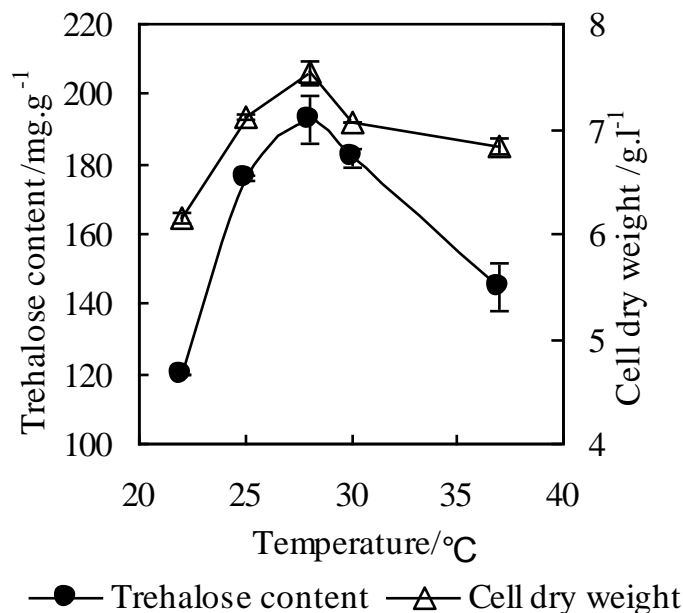


Figure 3. Effect of temperature on trehalose production.

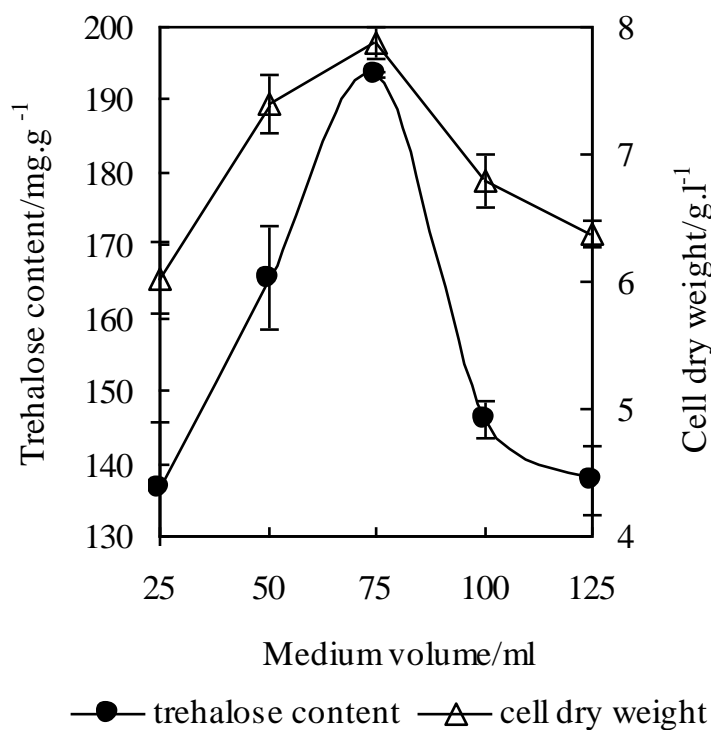


Figure 4. Effect of medium volume on trehalose production.

sucrose when the culture time was over 20 to 54 h. The yield of trehalose was the highest (189 mg/g cell) at 54 h, with a trehalose content in the culture medium of 2.45 g/L. The trehalose yield decreased sharply beyond 54 h because sucrose and other components in the medium

were seriously depleted, resulting in trehalose hydrolysis to maintain cell viability. In addition, yeast autolysis affected the trehalose yield. Therefore, the optimal incubation time in the 5 L automatic fermentor was 54 h (Zhao et al., 2010).

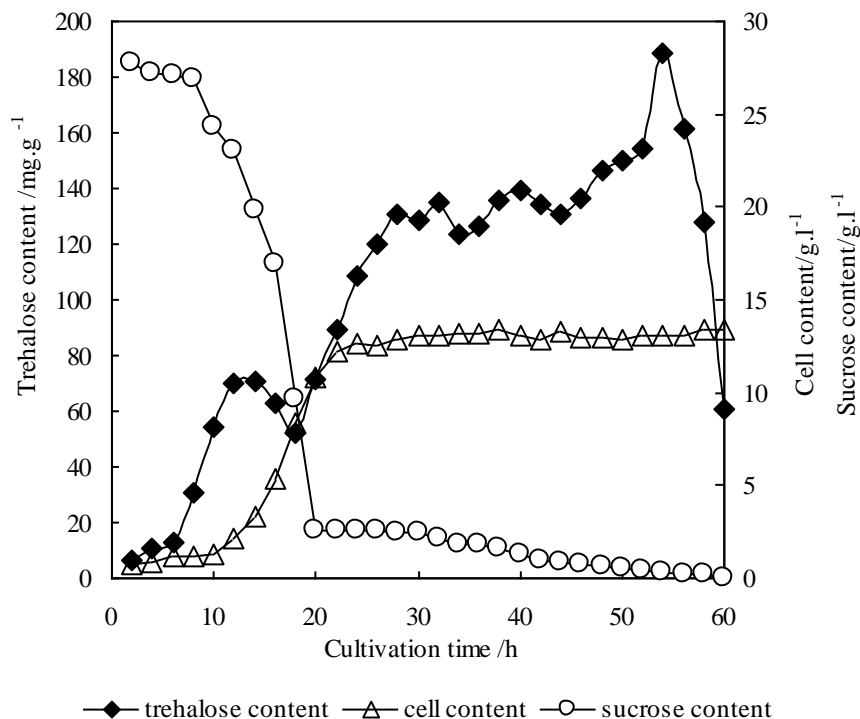


Figure 5. Progress curves for cell growth and trehalose production (Zhao et al., 2010).

Conclusions

The trehalose production conditions by *Rhodotorula* sp. were investigated for the first time. Culture conditions in the flask showed that the yeast growth and trehalose production were sensitive to the initial pH, possibly because trehalose synthesis in the cell occurred in a small range around the optimal pH (5.5). The effect of culture temperature on cell growth was insignificant but its effect on trehalose yield was significant. The protection of cells and trehalose synthesis displayed an optimum culture temperature. The optimum medium volume for cell growth and production of trehalose by red yeast was 75 mL (250 mL flask). Results indicate that the yeast cells had sufficient oxygen, similar to the oxygen requirement of other yeasts. Results in the 5 L automatic fermentor showed that the yield of trehalose increased sharply in the stable phase and with the depletion of sucrose. Results indicate that a depletion of the carbon source could stimulate cells to produce trehalose. Although production of trehalose in cells did not increase, the cell dry weight increased in the 5 L automatic fermentor (13 g/L). Culture in the 5 L automatic fermentor was superior to that in the shake flask.

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