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Short communication

Impact of hyperosmotic condition on cell physiology and metabolic flux distribution of *Candida krusei*

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Abstract

The cell physiology and metabolic flux distribution of the osmophilic yeast *Candida krusei* under hyperosmotic (NaCl as the osmoticum) and normal conditions (as a control) were compared. For the cells in exponential phase (12th hour) under hyperosmotic condition, the glycerol concentration was doubled than the control and the trehalose content reached 0.124 g/g dry cell weight. Metabolic flux analysis indicated that the carbon flux flowed toward glycerol-3-phosphate (G3P) to form glycerol from dihydroxyacetone phosphate (DHAP) and the glyceraldehydes-3-phosphate (GAP) node increased 67.2% while that toward TCA cycle decreased 26.9%. At the same time, the carbon flux flowed toward trehalose synthesis from glucose-6-phosphate (G6P) node was 2.6-fold that of the control. The metabolic energy flux analysis also suggested that the hyperosmotic condition efficiently improved the cellular glycerol and trehalose metabolism. Furthermore, the hyperosmotic condition also altered key enzyme activities. Compared with the control, G3P dehydrogenase (ctGPD) activity showed a rapid increase under hyperosmotic condition. For the activity of trehalose-6-phosphate synthase (T-6-P synthase), it slightly decreased but could keep at a higher level in the late stage of fermentation. This work is considered helpful to the further understanding of the metabolism of the osmophilic yeast under osmotic stress environment and also useful for the metabolic flux control of *C. krusei* for efficient production of glycerol and trehalose.

Keywords: *Candida krusei*; Enzyme activity; Glycerol; Hyperosmotic condition; Metabolic flux; Trehalose

1. Introduction

In recent years, glycerol production by osmophilic yeasts, such as *Candida*, *Debaryomyces*, *Hansenula*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*, has received attention [1]. Glycerol plays an essential role as a compatible solute during osmoregulation in yeasts. For example, in response to the decreased extracellular water activity, *Saccharomyces cerevisiae* greatly increases the glycerol formation rate to protect the cell itself [2]. Trehalose is another important metabolite of yeasts. Under heat stress, *S. cerevisiae* can accumulate a large amount of trehalose [3].

Metabolic flux analysis (MFA) is important to evaluate fluxes in metabolic pathways. A lot of reports have indicated that the comparison of metabolic flux distribution in different environmental conditions can help to better understand the cellular physiology and metabolism [4–6]. However, until now there have been no reports regarding the metabolic flux responses of yeast cells to osmotic stress although it is an important environmental factor.

Candida krusei belongs to osmophilic yeast that can produce glycerol in a high yield and productivity [7,8], but its detailed metabolic characteristics are not yet clarified. This work aims to reveal the cell physiology and main metabolic flux responses of *C. krusei* under osmotic stress. For this purpose, the study on cell

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physiology under normal and hyperosmotic conditions was carried out in 5 l bioreactors. Detailed metabolic flux and dynamic activities of key enzymes in glycerol and trehalose biosyntheses were analyzed. This work is considered helpful to establish a rational osmotic strategy for efficient production of the useful metabolites (glycerol and trehalose) by the yeast in a large-scale bioreactor.

2. Materials and methods

2.1. Organism and medium

C. krusei (ICM-Y-05) was routinely maintained on a solid medium containing 200 g/l of glucose, 3 g/l of urea, 3 g/l of corn steep slurry, and 20 g/l of agar. For seed cultures, the medium composition was: 100 g/l of glucose, 3 g/l of urea, and 3 g/l of corn steep slurry. For fermentation, the medium consisted of glucose (150 g/l), urea (2.5 g/l), KH_2PO_4 (0.3 g/l), and $MgSO_4{\cdot}7H_2O$ (0.25 g/l).

2.2. Culture condition

C. krusei stored in a refrigerator was inoculated to a slant and incubated at 35 °C for 24 h. Then, the seed was transferred to 500 ml shaking flasks with 50 ml medium and cultivated at 35 °C and 150 rpm for 24 h. After centrifugation at 4600 × *g* and 4 °C for 10 min, the cells were collected, and washed with cold distilled water before being inoculated into a bioreactor.

2.3. Bioreactor cultivation

The 5 l whisk bioreactor (Zhenjiang Dongfang Co., Jiangsu, China) with 3 l medium was used. The cultivation temperature was 35 °C. The aeration rate and agitation speed was controlled at 1.0 vvm and 500 rpm, respectively.

2.4. Products analysis

A Shimadzu 10AVP HPLC system (Shimadzu, Japan) with a RID-10A refractive index detector was used for determination of glycerol, glucose, trehalose and pyruvate [7]. An LKM 2000-03 Exhaust Gas Analyzer (Lokas Automation Corp.) was used to detect CO_2. The trehalose was analyzed according to the literature [7].

2.5. Preparation of cell extracts and analyses of enzyme activities

Cells extracts were prepared according to the work by Liu et al. [7]. Activity of glycerol-3-phosphate dehydrogenase (ctGPD) (EC 1.1.1.8) and trehalose-6-phosphate synthase (T-6-P synthase) (EC 2.4.1.15) was analyzed according to the work by Gancedo et al. [9] and Panek et al. [10], respectively.

Protein concentration was estimated by Bradford's method [11] with bovine serum albumin (Sigma) as standard. The reagents were purchased from Sigma Co.

2.6. Metabolic flux analysis

Detailed construction of the metabolic flux analysis is the same as described in the references [12,13]. Trehalose is also considered as a product. The metabolic flux is defined as mmol/(g cell h). The same reaction models were used for all metabolic flux analyses, while the PP pathway was not taken into account at the product synthesis phase because the cell growth was stable at that period. The formulation resulted in a set of linear equations that can be expressed as $A \times v = q$, where A $(m \times n)$ is the stoichiometric matrix, v $(n \times 1)$ represents metabolic flux vector and q $(m \times 1)$ represents the measured net accumulation or consumption specific vector. Both in the cell growth phase ($n = 19$, $m = 18$) and the production synthesis phase ($n = 10$, $m = 5$) of normal and hyperosmotic conditions, the systems are mathematically determined. The calculated amount of CO_2 is derived from biosynthesis process and metabolic pathways. Energy metabolic net is also involved. Since the energy consumed in the trans-membrane activities and any other maintenance are unknown, a metabolic reaction that consumes excess ATP (to ADP) is set up to balance the formulated energy. The metabolic flux of monomers required to synthesis the biomass of *C. krusei* cells and the flux reactions involved in the analysis of cell growth and product synthesis phase were listed as Appendices II and III.

3. Results and discussion

3.1. Physiological response of exponential phase cells to hyperosmotic condition

Because the *C. krusei* cells in the exponential phase were most sensitive to the osmotic stress (data not shown), the hyperosmotic condition was set by adding NaCl (at a final concentration of 0.34 M) to the medium when the cells were cultivated to exponential phase (at 12th hour). The cell growth, glucose consumption and the extracellular products syntheses are compared in Fig. 1. The cell growth was affected only a little and the glucose consumption showed a little bigger decrease under the hyperosmotic condition. Prista et al. [14] studied the cell growth of *S. cerevisiae* and *D. hansenii* at different osmotic stresses. In their case, when the concentration of NaCl was less than 1 mol/l, the cell growth of *D. hansenii* was not disturbed, while the cell growth of *S. cerevisiae* decreased at an even lower concentration of NaCl. The information obtained in this work and from the literature suggests that different yeasts had quite different responses to the osmotic stress.

Glycerol production by the *C. krusei* cell was greatly enhanced under the hyperosmotic condition. The extracellular glycerol concentration reached 57.8 g/l at 96 h and it was two-fold that of the control (normal cultivation). Enhancement of the glycerol production under osmotic stress was also observed in *S. cerevisiae* [15]. In contrast, the extracellular pyruvate production decreased nearly half and the CO_2 evolution also declined correspondingly.