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Yeast cell-free enzyme system for bio-ethanol production at elevated temperatures

Waleed Ahmad Khattak, Mazhar Ul-Islam, Muhammad Wajid Ullah, Bowan Yu, Shaukat Khan, Joong Kon Park*

Department of Chemical Engineering, Kyungpook National University, Daegu 702-701, Republic of Korea

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ABSTRACT

A yeast cell-free enzyme system containing an intact fermentation assembly and that is capable of bio-ethanol production at elevated temperatures in the absence of living cells was developed to address the limitations associated with conventional fermentation processes. The presence of both yeast glycolytic and fermentation enzymes in the system was verified by SDS-PAGE and LC–MS/MS Q-TOF analyses. Quantitative measurements verified sufficient quantities of the co-factors ATP (1.8 mM) and NAD^+ (0.11 mM) to initiate the fermentation process. Bio-ethanol was produced at a broad temperature range of 30–60 °C but was highly specific to a pH range of 6.0–7.0. The final bio-ethanol production at 30, 40, 50, and 60 °C was 3.37, 3.83, 1.94, and 1.60 g/L, respectively, when a 1% glucose solution was used, and the yield increased significantly with increasing cell-free enzyme concentrations. A comparative study revealed better results for the conventional fermentation system (4.46 g/L) at 30 °C than the cell-free system (3.37 g/L); however, the efficacy of the cell-free system increased with temperature, reaching a maximum (3.83 g/L) at 40 °C, at which the conventional system could only produce 0.48 g/L bio-ethanol. Successful bio-ethanol production using a single yeast cell-based enzyme system at higher temperatures will lead to the development of novel strategies for efficient bio-ethanol production through SSF.

1. Introduction

Bio-ethanol production from different feedstocks using microbial cells (mainly yeast) has received a great deal of attention in the last few decades [1]. However, conventional microbial fermentation processes have faced obstacles that restrict cost-effective and eco-friendly bio-ethanol production. One such obstacle is the adverse effects of excessive glucose concentrations on the microbial fermentation process [2]. Attempts to resolve this problem have been made by employing simultaneous saccharification and fermentation (SSF) in which the production and consumption of glucose occurs concurrently [3,4], yet SSF is hampered by differences between the temperature optima of the saccharification and fermentation processes. Specifically, the optimum temperature of saccharifying enzymes is 45–50 °C, temperatures that are too high for fermentation and microbial growth, which are optimal at 25–35 °C [5,6]. Therefore, developing an approach that can overcome the temperature difference between the saccharification and fermentation processes is of utmost importance [7,8].

Cell-free fermentation could be a possible strategy to resolve this dilemma because it bypasses the restrictions of cell growth and viability at high temperatures [7–10]. In contrast to the conventional processes of bio-ethanol production, cell-free systems have several advantages, including prolonged and continuous bio-ethanol production, controlled variables, such as the pH, ionic strength [11], maintenance of cofactor concentrations, and bypassing the abnormal accumulation of intermediary metabolites [12]. Similar to a high glucose concentration, the accumulation of excessive ethanol in the culture broth also has an inhibitory effect on yeast cell viability [13]. The ratio of two important fatty acids in the plasma membrane (palmitoleic acid and oleic acid) is changed with ethanol accumulation that leads to decreased membrane fluidity, resulting in cell death due to the blockage of the nutrient barrier [14]. Thus, a cell-free enzyme system is considered to be an effective system for bioethanol production under such conditions [8]. Without any surrounding boundaries (cell wall or membranes), a cell-free system possesses the complete set of machinery required for the cascade of reactions required to metabolize glucose into bio-ethanol [7,8]. Furthermore, some previous studies have suggested that fermentation at an elevated temperature using a thermophilic yeast cell-free enzyme system would overcome the problem of ethanol inhibition [15]. Cell-free systems obtained from crude cell extracts have previously shown remarkable utility as a research

* Corresponding author. Tel.: +82 950535621; fax: +82 539506615.
E-mail address: parkjk@knu.ac.kr (J.K. Park).

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tool in such ground-breaking experiments as the discovery of the genetic code and the production of proteinaceous antibiotics, vaccines, hormones, and non-proteinaceous bio-fuels [16,17].

Regardless, it has long been unclear whether cell-free enzyme systems could be more economical for bio-ethanol production than conventional yeast fermentation. Some investigations based on process modeling have suggested that the cell-free fermentation process is more efficient than conventional processes, though an accurate economic analysis of cell-free bio-ethanol production is currently difficult [18]. Welch and Scopes (1985) described a reconstituted cell-free glycolytic system possessing a series of 12 purified enzymes (from yeast, rabbit muscle), *ATPase* (potato), arsenate (substitute for ATPase, Sigma Chemical Co.), and cofactors (Sigma Chemical Co.) and evaluated it for bio-ethanol production using 18% glucose [12,19]. The system successfully fermented glucose with a 50% yield of bio-ethanol. Although the approach was successful, the bio-ethanol production was inadequate given the expensive purified enzymes and cofactors, thus preventing its industrial scale up. Moreover, the fermentation was conducted at 30 °C, which is within the optimum fermentation temperature range of most microbes; therefore, the study did not provide any information that would help resolve the limitations associated with SSF [8–10,12].

We previously produced bio-ethanol from waste of beer fermentation broth (WBFB) at a high temperature and also found that the microbial cell wall burst at high temperatures, releasing the cellular contents, including fermentation enzymes, to the surrounding medium [9,10]. These enzymes are expected to play an important role in performing the fermentation process without the use of live cells. Considering the these observations, it was necessary to develop a system consisting of purified yeast cell glycolytic enzymes with cofactors and verify that the entire fermentation operation could be conducted without live yeast cells, and it was also essential to compare the efficacy of such a system with the conventional yeast fermentation system. Therefore, we developed a complete cell-free enzyme system from a single yeast cell line to evaluate the role of cell-free enzymes in bio-ethanol production. The presence of the glycolytic and fermentation enzymes and cofactors involved in the fermentation process were identified in the cell-free extract, and the system was evaluated for bio-ethanol production at elevated temperatures using pure glucose as the substrate. This system has a potential role in enhancing bio-ethanol production and might be effective in future development of SSF processes.

2. Materials and methods

2.1. Collection and processing of WBFB

WBFB was obtained from the Hotel Aryana Beer Industry, Daegu, Korea, and stored as previously described [20]. Prior to use, the solid residue and supernatant were separated by centrifugation at 3500 rpm for 20 min.

2.2. Glass bead pretreatment

Glass beads (Sigma, 9268) with a diameter of 425–600 microns were used for the bead-beating experiments [21,22]. Prior to use, the beads were soaked in concentrated HCl for 16-h and then rinsed thoroughly with distilled water until they were completely neutralized; the beads were then autoclaved at 121 °C and 15 psi for 20 min and chilled at 4 °C until use.

2.3. Preparation of yeast cells for the bead-beating experiment

Yeast cells present in WBFB [20] were spread on YM selective medium (3 g/L glucose, 10 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L dextrose, and 20 g/L agar) and incubated at 30 °C for two days until complete growth of the yeast colonies. The yeast colonies were then separated from the other microorganisms on the basis of their specific morphology. The yeast colonies were grown repeatedly three times on fresh YM selective medium agar plates to obtain pure colonies [20,23]. Next, a pre-culture of *Saccharomyces cerevisiae* at a density of 2.45×10^7 cells/mL was prepared by inoculating yeast colonies into 100 mL YM broth (pH 6.0) in a 250-mL flask and incubating the samples at 30 °C and 150 rpm for 24 h. To obtain dense cultures, the freshly prepared yeast pre-culture (50 mL) was centrifuged at 3500 rpm for 10 min, and the pellets obtained were resuspended in 5 mL YM broth. The concentration of the dense yeast culture was determined to be 2.21×10^8 cells/mL based on observations using a Neubauer-improved hemocytometer (Paul Marienfeld, Germany).

2.4. Development of the yeast cell-free system

The freshly prepared dense yeast culture (5 mL) was placed in a sterilized glass vial with 5 mL chilled glass beads, and the samples were vortexed for 15.0 min to rupture the cells. To avoid the thermal denaturation of the proteinaceous yeast cell internal matrix, the samples were chilled on ice after regular intervals of 3 min. The yeast lysates were then collected with a sterile syringe and concentrated using a 10-kD spin column (Abcam, England; Product # ab93349).

2.5. SDS-PAGE analysis

The presence of yeast glycolytic and fermentation enzymes after bead beating was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% polyacrylamide gradient gel under reducing conditions with a Bio-Rad mini-gel apparatus [24]. Thermo Scientific PageRuler™ protein ladders with a range of 10–170 kDa were used as standards, and the protein bands were visualized by staining with Coomassie brilliant blue R-250 (Sigma).

2.6. Liquid chromatography–mass spectrometry/mass spectrometry hybrid quadrupole time of flight (LC–MS/MS Q-TOF) analysis

A nano-LC–MS/MS Q-TOF analysis of the yeast lysates was performed using a nano-HPLC system (Agilent, Wilmington, DE). A nano-chip column (Agilent, Wilmington, DE, 150 mm × 0.075 mm) was used for peptide separation. The mobile phase A for LC separation was 0.1% formic acid in deionized water, and mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was designed for a linear increase from 3% B to 40% B in 80 min, then to 60% B in 10 min, 95% B in 10 min, and 3% B in 20 min; during the analysis, the flow rate was maintained at 400 nL/min. The product ion spectra were collected in the information-dependent acquisition (IDA) mode and analyzed by Agilent 6530 Accurate-Mass Q-TOF using continuous cycles of one full scan of TOF MS from 350 to 1200 m/z (1.0 s) plus three product ion scans from 100 to 1700 m/z (1.5 s each). Precursor m/z values were selected, starting with the most intense ion, using a selection quadrupole resolution of 4 Da. The rolling collision energy feature, which determines the collision energy based on the precursor value and charge state, was used. The dynamic exclusion time for the precursor ion m/z values was 30 s.

2.7. Database searching

The mascot algorithm (Matrixscience, USA) was used to identify peptide sequences present in a protein sequence database. The