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Bio-ethanol production through simultaneous saccharification and fermentation using an encapsulated reconstituted cell-free enzyme system

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ABSTRACT

An encapsulated reconstituted cell-free enzyme system was developed through liquid-droplet forming method by using endogenous glycolytic and fermentation enzymes from yeast cells and exogenously added saccharification enzymes, cofactors, and ATPase. It was evaluated for bio-ethanol production through simultaneous saccharification and fermentation (SSF) at various temperatures, pH, and cell-free enzyme and substrate concentrations. Using 1% starch as substrate, encapsulated system illustrated maximum efficiency at 45 °C and pH 7.0. SSF with encapsulated and bare reconstituted cell-free enzyme systems produced 3.47 g/L and 2.98 g/L bio-ethanol corresponding to 62% and 53% of maximum theoretical yield, respectively. It was explicable that encapsulated system provided better substrate utilization and product formation at elevated temperatures than bare system. Kinetic profile of SSF process of both systems was affected differently by variations in pH, temperature, and substrate and cell-free enzyme concentrations. Under appropriate conditions, system retained 90%, 64%, and 40% of initial enzyme concentration and produced 3.21, 2.24, and 0.83 g/L bio-ethanol after 5, 10, and 15 consecutive batches, respectively. The current system offered several advantages and was superior compared to previously reported SSF systems. This system can effectively overcome the major barriers associated with successful development of SSF processes for bio-ethanol production on an industrial scale.

1. Introduction

Global warming and energy crises are two major present-day problems [1]. Other problems associated with industrialization that need to be addressed include production of toxic gases by combustion of fossil fuels, industrial waste management, and depletion of natural fossil fuel resources [2,3]. These problems can be partially solved by using starch-based materials for production of bio-ethanol [4]. Natural starches are pretreated to yield different end products of various industrial applications. However, the traditional pretreatment methods such as acid hydrolysis and heating [5], are currently in no real practice since these cause degradation or loss of carbohydrate and formation of inhibitory by-products [6,7]. These problems have been aimed to overcome through the development of effective pretreatment strategies such as enzymatic hydrolysis [8]. This strategy offers several advantages in addition to yield, such as low cost, short hydrolysis time, low corrosion, low utility, moderate, specificity, and low toxicity, etc., and it has been employed in more than 75% of recent starch hydrolysis processes [6].

Simultaneous saccharification and fermentation (SSF) avoids the product inhibition observed during separate enzymatic hydrolysis and fermentation [8]. However, enzymatic hydrolysis occurs at 50–55 °C, whereas the optimum microbial growth and fermentation temperature is 30–35 °C [9]. In addition, microbial cells are vulnerable to growth inhibition and cell viability [9,10], and the presence of higher concentrations of glucose and ethanol in the culture medium [11] can disrupt cellular membrane fluidity [12,13]. The difference between the temperature optima for the saccharification enzymes and microbial cell growth and fermentation enzymes is a major hurdle for the production of bio-ethanol via SSF [9,14]. Therefore, as a compromise, SSF is consequently carried out at 35–37 °C [15]. To overcome these problems and improve the stability of the system, earlier attempts utilized cell immobilization technology, which avoided the growth inhibition during

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fermentation due to toxic lignocellulosic material and improved cell stress tolerance [16]. However, this immobilized cell system encountered several problems, including the substrate undergoing more than one biochemical reaction, accumulation of impurities, and retarded growth rates, patterns, and metabolism. Some of these problems were partially overcome by using thermotolerant yeast strains, which offered resistance to elevated temperatures and higher ethanol and sugar concentrations on an industrial scale. However, even thermotolerant and thermophilic yeast strains that tolerate temperatures as high as 42–45 °C [17] are still adversely affected at the temperatures suitable for SSF. Besides this difference in temperature optima [9,14], there are several other problems associated with yeast cell fermentation. For example, most of the supplied energy is utilized as feed for cell growth, survival, and proliferation instead of being converted to the desired product. In addition, undesirable products are formed that increase the downstream processing cost of an industrial product [18]. Furthermore, the microbial cell membrane often acts as barrier for both the substrate and product, preventing the efficient conversion of substrate into product.

To overcome the major problems faced by using SSF, modified biological systems are currently under development. The most promising approach is a cell-free enzyme system, which represents the state-of-the-art in biotechnological conversion of substrate into product [19]. Previously reported cell-free enzyme systems offered certain salient features such as improved bio-ethanol production, controlled parameters like pH, ionic strength, maintenance of cofactor concentrations (ATP and NADH etc.), and prevention of abnormal accumulation of intermediary metabolites [20–22]. Our own study of a single cell-based cell-free enzyme system showed bio-ethanol production at elevated temperatures [23]. These cell-free and immobilized enzyme systems offer several advantages such as inhibitor tolerance, maximum enzyme-substrate interaction, limited enzyme lost, and lower mass transfer resistance, etc. [16,24,25]. However, the major problem associated with cell-free systems is the non-reusability of expensive enzymes [26]. This problem was overwhelmed through encapsulation of cell-free enzymes. During this strategy, the enzymes are completely confined in a semi-permeable membrane [24]. With such a system, the enzymes can be retained and reused for several batches. Besides, it offers several other advantages, including the large space for cellular growth, maximum enzyme-substrate interaction in the presence of appropriate cofactors, and minimal enzyme loss, etc. On the other hand, during entrapment enzymes are embedded in the support matrix and only a small percentage of enzyme molecules may be exposed at the surface to interact with the substrate [27]. Moreover, the cofactors and apoenzyme are localized and cannot move freely for moderate interaction [28]. The advantages conferred by the encapsulation make this strategy a preferable choice for bio-ethanol production using cell-free enzyme system.

The results of our previous studies of a yeast cell-free enzyme system for bio-ethanol production at elevated temperatures gave us the confidence to extend the utility of the system for SSF with certain modifications. In the current study, we attempted to advance our yeast cell-free enzyme system for SSF by reconstituting it with saccharification enzymes and cofactors and subsequently confining the assembly into calcium alginate capsule. A multi-step enzymatic reaction was carried out to produce bio-ethanol via SSF at elevated temperatures, as encapsulation conferred additional thermal stability to the system. We evaluated and compared the thermal stability, and the efficacy at various pH, and enzyme and substrate concentrations of the encapsulated and bare reconstituted cell-free enzyme systems in terms of the bio-ethanol produced through SSF in repeated batches.

2. Materials and methods

2.1. Materials

Yeast *Saccharomyces cerevisiae* was isolated from waste of bear fermentation broth (WBFB) obtained from the Hotel Aryana Beer Industry, Daegu, Korea and cultured on YM selective medium plates containing (gL^{-1}): glucose, 3.0; yeast extract, 10; malt extract, 3; peptone, 5; dextrose, 10; and agar, 20. The glass beads (425–600 μ) (Cat. No. 9268) and other reagents including; α-amylase (Porcine pancreas; A6255), β-amylase (Barley; A7130); β-glucosidase (Almonds; G0395), pullulanase (*Klebsiella pneumoniae*; P1067), ATP (A6559), NADH (N704), and ATPase (Porcine cerebral cortex; A7510) were supplied by Sigma (Sigma Chemical Co. US). Other chemicals were purchased from commercial suppliers; calcium chloride (Dae Jung; Korea), sodium alginate (Yakuri Pure Chemicals Co., Ltd., Kyoto, Japan), Xanthan gum (Lavendersoap, USA), and Tween 20 (Duksan Pharmaceutical Co., Ltd., Korea).

2.2. Preparation and permeability testing of calcium alginate capsule

The liquid-droplet forming method was used to prepare calcium alginate capsules as described previously [16,28]. The starch permeability of the prepared capsules was characterized through equilibrium swelling analysis as previously described [29]. The permeability of the capsules to starch solutions of different concentrations (1%, 2%, 3%, 4%, and 5% w/v) was determined. In parallel experiments, equal weights of freeze-dried calcium alginate capsules were placed in 10 mL starch solutions at different concentrations in deionized distilled water. The solutions were stirred using a magnetic stirrer, and the temperature was maintained at 25 °C. At specified time intervals, the capsules were weighed after removing the excess water or starch solution from the surface by the sieve-shake method [30] until the weight of the capsule was constant. Finally, the equilibrium uptake (*EU*) for water and starch was calculated using the following equation [31]:

$$EU = \left[\frac{(W_s - W_d)}{W_s}\right] \times 100\%$$

where W_s is the weight of the swollen capsule due to starch or water uptake, and W_d is the weight of the freeze-dried capsules. The results are expressed as average equilibrium uptake for each concentration of starch solution, and distilled water was used as a reference.

2.3. Development and encapsulation of the reconstituted cell-free enzyme system

Few colonies from pure yeast culture plates were inoculated into 100 mL YM broth (pH 6.0) in 250 mL Erlenmeyer flask and incubated overnight at 30 °C under shaking conditions (150 rpm). The culture was sampled after 4, 8, 12, and 24 h and analyzed for variation in cell density, protein and metabolite contents. The density of the culture broth reached to 1.16×10^7 cells/mL and highest specific protein, ATP, and NADH contents per cell after 8 h (Table 1S). Therefore, we selected the 8 h to be the appropriate cultivation time of yeast cell for preparation of cell lysate. A 50 mL culture was harvested after 8 h and centrifuged at 3500 rpm for 15 min to obtain a concentrated yeast cell culture. The obtained pellet was again resuspended in 5 mL supernatant. The density of concentrated culture rose to 2.33×10^8 cells/mL as determined by Neubauer-improved heamocytometer. Thereafter, the yeast cells were lysed via bead beating to obtain the cell lysate persisting of endogenous glycolytic and fermentation enzymes. The cell lysate was concentrated to 5 mg/mL