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Effects of nitrogen source on ethanol production in very high gravity fermentation of corn starch

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ABSTRACT

Nitrogen sources, the critical media component, were optimized to enhance ethanol production by *Saccharomyces cerevisiae* in very high gravity (VHG) fermentation of corn starch (340 g/l). Screening experiments revealed yeast extract as an ideal nitrogen source for ethanol production. When yeast extract concentration was controlled at 2%, ethanol yield and fermentation efficiency reached approximately 20.3% and 84.5%, respectively, after 72 h of fermentation. To reduce ethanol production cost, yeast extract supplementation was partially replaced with less expensive nitrogen sources, namely urea and ammonium sulfate. Combined effects of the three nitrogen sources on ethanol production were determined through central composite design. The optimum combination of nitrogen sources (0.6% yeast extract, 69 mM urea, and 26 mM ammonium sulfate) enabled ethanol yield and fermentation efficiency comparable to those supplemented with 2% yeast extract, indicating that urea and ammonium sulfate synergistically enhanced ethanol production by *S. cerevisiae* in VHG fermentation.

1. Introduction

Fuel ethanol is a sustainable energy source that is intended to provide a more environmentally friendly alternative to fossil fuels such as diesel and gasoline [1–3]. World ethanol production for transport fuel has increased more than four-fold in the past decade and reached about 93 billion liters in 2014. In conventional alcoholic fermentation, a substrate containing 180–220 g/l total sugars is used to achieve ethanol concentration of 10–14% (v/v) [4]. Very high gravity (VHG) fermentation technology can considerably increase both fermentation productivity and ethanol concentration while consuming less water and energy [5,6]. VHG is defined as "the preparation and fermentation to completion of mashes containing 27 g or more dissolved solids per 100 g mash" [7]. An important consideration for VHG fermentation is that a high final ethanol concentration subjects yeast to ethanol stress, which decreases its growth and cell viability [8].

Saccharomyces cerevisiae (*S. cerevisiae*), the budding yeast, is used universally for industrial production of fuel ethanol because of its ability to produce high ethanol concentrations [9–14]. Yeast requires an adequate supply of nutrients to grow. In addition to fermentable sugars and inorganic sources, the nitrogen source is an essential component of yeast growth media [15]. Lack of nitrogen source leads to a significant reduction in ethanol yield, and such negative effect cannot be omitted particularly in VHG fermentation [16,17]. *S. cerevisiae* is able to use a wide variety of nitrogen sources for growth, such as organic nitrogen, inorganic nitrogen, or a combination of both. Previous studies have shown that ammonium ion [18], urea [18,19], peptone [20], yeast extract [20], corn steep liquor [19], free amino acid [21], spent brewer's yeast [22], and other nitrogen sources [23], could improve the growth of yeast cells and increase ethanol production; however, not all nitrogen sources contribute to yeast growth equally well [24,25]. In addition, the nitrogen source is usually the most expensive component of microbial growth media [26,27]. Because the nitrogen source is one of the main contributors in the total material cost, there is great need to replace costly nitrogen sources with less expensive ones for ethanol production.

In this study, we investigated VHG fermentation of corn starch (340 g/l, w/v) and compared the effects of the readily available and often-used nitrogen sources (ammonium sulphate, urea, peptone, and yeast extract) on ethanol production by *S. cerevisiae*. Furthermore, a central composite design (CCD) was used to investigate the possibility of replacing high-cost nitrogen sources with more economical ones.

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2. Materials and methods

2.1. Materials

Normal corn starch was obtained from National Starch Food Innovation, now Ingredion Inc. (Bridgewater, NJ). Active dry yeast (*S. cerevisiae*, Red Star Ethanol Red) was obtained from Lesaffre Yeast Corp. (Milwaukee, WI). The yeast contained $> 20 \times 10^9$ viable cells/g [22,28]. Thermostable α-amylase (Liquozyme SC DS) and glucoamylase (Spirizyme SC DS) were obtained from Novozymes (Franklinton, NC). Enzyme activity of Liquozyme SC DS was 240 kilo Novo units (KNU)/g, and one KNU was defined as the amount of enzyme that hydrolyzed 5.26 g of starch (soluble starch) per hour under Novo Nordisk's standard conditions for α-amylase determination (37 ± 0.05 °C, 0.3 mM Ca^{2+}, and pH 5.6). Enzyme activity of Spirizyme SC DS was 750 AGU/g, and one AGU is defined as the amount of enzyme that hydrolyzed 1 μM maltose/min under standard conditions (37 ± 0.05 °C, pH 4.3, 23.2 mM maltose, and reaction time of 5 min). Yeast extract (5.1% of assimilable nitrogen), the water-soluble portion of autolyzed fresh yeast, and peptone (3.7% of assimilable nitrogen), a mixture of peptides and free amino acids from pancreatic digest of casein, were purchased from Thermo Fisher Scientific (Santa Clara, CA). Ammonium sulfate (21.0% of assimilable nitrogen), urea (46.5% of assimilable nitrogen), and other general chemicals were also purchased from Thermo Fisher Scientific.

2.2. Liquefaction of normal corn starch

A pressure reactor from Parr Instrument Company (Moline, IL) was used for the liquefaction of normal corn starch. Corn starch was mixed with distilled water to obtain starch slurry at 340 g/l (w/v). Corn starch slurry was adjusted to about pH 5.8 with 0.1 N HCl and thermostable α-amylase (Liquozyme SC DS, 9 KNU/100 g dry starches) was then added to the corn starch slurry. The mixture of corn starch and enzyme was placed in a beaker in the pressure reactor. The temperature of the mixture was ramped from 25 to 90 ± 2 °C with continuous agitation at 200 rpm in about 30 min. After the temperature reached 90 °C, the mixture was kept at this temperature and agitating rate of 200 rpm for 90 min for starch liquefaction. The liquefied solution was then used for ethanol fermentation.

2.3. Reducing sugar analysis

Reducing sugar contents of liquefied samples were determined by a Nelson–Somogyi method [29]. Glucose was used as a standard solution.

2.4. Determination of molecular weight distribution by gel permeation chromatography (GPC)

The molecular weight distribution of the liquefied corn starch samples, after dissolving in dimethyl sulfoxide (DMSO), was determined by a GPC instrument (PL-GPC220, Polymer Laboratory, Amherst, MA) equipped with a refractive index detector and a set of three Phenogel columns (300×7.8 mm, 10 μm, 105 Å, 103 Å, and 100 Å, Phenomenex, Torrance, CA) and eluted with 0.80 ml/min DMSO containing 5 mM $NaNO_3$. The oven and detector temperatures were controlled at 80 °C. Dextran standards were used to determine the relative molecular size.

2.5. Simultaneous saccharification and fermentation

For the preparation of inoculums, active dry yeast (1 g) was dispersed in 19 ml of a preculture broth containing glucose (20 g/l), peptone (5 g/l), yeast extract (3 g/l), KH_2PO_4 (1 g/l), and $MgSO_4 \cdot 7H_2O$ (0.5 g/l) and incubated at 38 °C for 30 min in an incubator shaking at 200 rpm. The fermentation broth containing the liquefied sample (100 g), activated yeast culture (1 ml), glucoamylase (Spirizyme SC DS, 2 U/g dry starches), K_2HPO_4 (1 g/l), $CaCl_2$ (0.2 g/l), and a nitrogen source were adjusted to pH 4.2 with 2 M HCl and added to each flask, which was subsequently sealed with an S-shaped airlock filled with about 2 ml of mineral oil. Ethanol fermentation was performed in an incubator shaker (model I2400, New Brunswick Scientific, Edison, NJ) at 30 °C for 72 h with continuous shaking at 150 rpm. The fermentation process was monitored by measuring the total weights of the fermentation flasks because the weight loss by CO_2 evolution was proportional to the amount of ethanol produced during ethanol fermentation. Ethanol yield was defined as the ethanol concentration in fermentation broth.

Fermentation efficiencies were calculated as a ratio of the experimentally determined ethanol yield to the theoretical ethanol yield. The total starch contents in the samples were used to calculate theoretical ethanol yields, assuming that 1.0 g of starch converts to 1.11 g of glucose and 1.0 g of glucose generates 0.511 g of ethanol.

2.6. Experimental design and optimization

Ethanol production experiments were carried out with a sole nitrogen source (ammonium sulfate, urea, peptone, or yeast extract) at different concentrations in fermentation broth. All experiments were conducted in triplicate.

A central composite design (CCD) with five coded levels (−1.41, −1, 0, +1, and +1.41) was used to investigate the possibility of partially replacing yeast extract with ammonium sulfate and urea. The concentration of yeast extract in the fermentation broth was controlled at 0.6%. The two independent variables used at five different levels were ammonium sulfate (X_1: 2, 10, 30, 50, and 58 mM) and urea (X_2: 4, 20, 60, 100, and 116 mM) concentrations. According to this design, the total number of treatment combinations was $2^k + 2k + n_o$, where k is the number of independent variables and n_0 is the number of repetitions of experiments at the center point. Experimental results of the CCD were used to fit with a second-order polynomial equation by a multiple regression technique (Eq. 1):

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_{ii}^2 + \sum_{i<j}^{k} \sum \beta_{ij} x_i x_j \quad (1)$$

Where Y is the predicted response, β_0 is the offset term, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient, and β_{ij} is the ijth interaction coefficient.

3. Results and discussion

3.1. Liquefaction of normal corn starch

When the initial concentration of starch slurry was controlled at 340 g/l (w/v), the reducing sugar contents of corn starch hydrolysate increased gradually with liquefaction time. After the liquefaction of corn starch at 90 °C for 90 min, reducing sugar content reached approximately 12%. GPC analysis showed that the molecular weight distribution curve of starch hydrolysate had two peaks: one was located in the higher molecular weight region (about 16,000 g/mol), and the other was located in the lower molecular weight region (about 1000 g/mol) (Fig. 1). Furthermore, the peak located in the low molecular weight region was much higher than that in the high molecular weight region (Fig. 1), suggesting that the liquefaction of corn starch solution could result in a very high proportion of maltodextrin with low molecular weight