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Optimization of cultivation conditions for the production of 1,3-dihydroxyacetone by *Pichia membranifaciens* using response surface methodology

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Abstract

By isolating soil samples, we obtained a novel *Pichia* strain (sp. ZJB-0009) capable of producing 1,3-dihydroxyacetone from glycerol. Based on the physiological, biochemical characteristics, and ITS rDNA gene sequence analysis, the strain was identified as *Pichia membranifaciens* and subsequently named *P. membranifaciens* ZJB-0009. A mathematical model was developed to investigate the influences of various fermentation parameters and to predict the optimum fermentation conditions for 1,3-dihydroxyacetone production. The maximal concentration of 1,3-dihydroxyacetone (12.91 g/L) was predicted to occur when the pH was 6.74, the incubation temperature was 29.6 °C, and the growth time was 48.8 h. A repeat fermentation of 1,3-dihydroxyacetone by *P. membranifaciens* ZJB-0009 was carried out in a 15-L fermenter under the optimized conditions for the verification of optimization. The maximum concentration of 1,3-dihydroxyacetone was 13.57 g/L, which was significantly higher than that obtained under unoptimized conditions.

Keywords: *Pichia membranifaciens*; 1,3-Dihydroxyacetone; Identification; Medium optimization; Response surface methodology; Box–Behnken design

1. Introduction

1,3-Dihydroxyacetone (DHA) is a commercially important chemical used in cosmetics, pharmaceuticals, and as a food additive. It is also employed as an intermediate in the synthesis of organic chemicals such as surfactants. Since the microbial formation of DHA from glycerol was discovered by Bertrand [1], many investigators have reported the bioconversion of glycerol to DHA by acetic acid bacteria [2–6]. DHA is produced industrially by a fermentation process using *Gluconobacter* strains and a glycerol substrate in much the same way as first reported [1]. Glycerol can be directly oxidized into DHA via a glycerol dehydrogenase. The microbial strains that produce glycerol dehydrogenase include *Acetobacter xylinum* [4], *Acetobacter suboxydans* [5] and *Gluconobacter oxydans* [7]. Glycerol dehydrogenase is a membrane-bound and PQQ dependent [8] enzyme. DHA is produced by batch fermentation at pH 6 and at a high aeration rate to maintain an adequate oxygen supply.

One major problem with this fermentation process is enzyme inhibition due to increasing concentration of product, a classic example of feedback inhibition. Moreover, high concentration of DHA may cause irreversible cell damage, and inhibit the activity of the pentose cycle [9] and the glycerol facilitator [8]. Thus, it is evident that the bioconversion process of glycerol to DHA requires further optimization.

Since the production of DHA by microorganisms is strongly influenced by many factors, such as carbon source, nitrogen source, growth factors, inorganic salts, and cultivation conditions, it is crucial to search for the key influencing factors among many related ones. To perform such work using conventional techniques such as the 'one-factor-at-a-time' method is extremely laborious and time-consuming; moreover, such methods do not guarantee the determination of optimal conditions and are unable to detect synergistic interactions between two or more factors [10].

The present study explores the suitability of a newly isolated strain of yeast (*P. membranifaciens*) for the production of DHA. The strain was firstly isolated from soil and identified by the analysis of the ITS rDNA region sequence. Then response surface methodology (RSM) was employed to opti-

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mize fermentation parameters for the production of DHA. Three variables, including pH, temperature, and time course, were selected as process (independent) variables while DHA was the response (dependent variable). An empirical model including the effects of independent variables has also been developed through SAS software to represent the response surface.

2. Materials and methods

2.1. Materials and microorganisms

Pichia sp. ZJB-0009 cells that were isolated and stored in our lab, were grown aerobically at 30 °C for 24 h in 50 mL seed cultures containing glycerol (4%, w/v), yeast extract (0.5%, w/v), peptone (0.2%, w/v) and distilled water. The pH was adjusted to 6.5. The medium was placed into 250 mL flasks on a rotary shaker incubator at 200 rpm for 18 h. All other chemicals were of analytical grade.

2.2. Isolation and identification of strain Pichia sp. ZJB-0009

Different strains including *Pichia* sp. ZJB-0009 were isolated from soil samples based on their morphology. The identification of the strain was based on standard morphological characterization [11] and nucleotide sequence analysis of the enzymatically amplified internal transcribed spacer (ITS) region including 5.8S rDNA. For the morphological characterization, observations were made with both an optical microscope and a scanning electron microscope. For the sequence analysis, chromosomal DNA was isolated by the method of Reader and Broda [12]. Amplification using primer set pITS1 (5′-TCCGTAGGTGAACCTGCCG-3′) and pITS4 (5′-TCCTCCGCTTATTGATATGC-3′) was carried out in a thermal cycler (Bio-Rad, USA) under the following conditions: 3 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 53 °C, 2 min at 72 °C and one final step of 10 min at 72 °C. The resulting PCR fragment was ligated with pMD18-T (Takara, Japan) using the T/A cloning procedure [13]. The constructed vector was transformed to *E. coli* JM109 according to the method of Chung et al. [14]. A positive clone designated *E. coli* JM109/pMD18-T-DHA was subsequently obtained via blue/white screening. DNA from both strands was sequenced using an Applied Biosystems Model 377 automatic DNA sequencer and a dye-labeled terminator sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequences obtained were compiled and compared with sequences in the GenBank databases using the BLAST program. Sequence analysis was performed using the SEQBOOT, NEIGHBOR-JION and DNASENSE of Phylips (version 3.572) software (Department of Genome Sciences, Department of Biology, University of Washington) and FITCH, DRAWGRAM, and alignment matching was then used to construct the neighbor-joining phylogenetic tree. Evaluations of the MicroSeq database system and comparisons to other frequently used systems are from DDBJ/EMBL/GenBank.

2.3. Inoculum preparation and flask culture

For production of the inoculum, *Pichia* sp. ZJB-0009 was transferred from a slant culture into an Erlenmeyer flask (250 mL) containing the seed medium. The seed cultures were grown at 30 °C on a rotary shaker incubator at 200 rpm for 24 h.

All basic fermentation cultures were carried out in 250 mL Erlenmeyer flasks containing 50 mL fermentation medium whose composition was formulated based on previous experimental results: glycerol (4%, w/v), yeast extract (0.5%, w/v), peptone (0.2%, w/v) and distilled water. Different culture conditions are described in Table 2. All media were autoclaved for 20 min at 121 °C. Values given are the means of experiments repeated two or more times.

2.4. Batch fermentation

The batch fermentation was carried out in a 15-L fermenter (Biostat C10-3, B. Braun, Germany) equipped with three 6-bladed disc impellers and electrodes for measuring oxygen and pH. Fermentation was performed under the following general conditions: medium volume 10 L, inoculation volume 2% (v/v), aeration rate 0.8 vvm, and agitation speed 300 rpm. Temperature, initial pH and time course for fermentation were set up based on the optimized cultivation conditions by RSM model.

2.5. Biomass measurement

The biomass of *P. membranifaciens* grown under different conditions was measured gravimetrically after separation from the fermentation broth by filtration or centrifugation, washed twice with distilled water, and dried at 105 °C to a constant weight.

2.6. Dihydroxyacetone assay

Our two-step determination method was performed as follows: DHA was assayed with diphenylamine reagent [15] containing diphenylamine (0.6 g), sulfuric acid (6 mL) and acetic acid (54 mL). 0.5 mL Samples containing 0.0–0.3 g/L DHA were mixed with 4.5 mL diphenylamine reagent and incubated in closed tubes for 20 min in boiling water. The samples were then cooled to room temperature and the absorbance at 615 nm was measured immediately. The samples were also tested qualitatively by thin-layer chromatography (TLC) [16]. Aliquots (2 μL) were added to silica gel TLC plates (10 cm × 20 cm), and chromatographed over an 18 cm path length using a mobile phase consisting of 80:19:1 (v/v/v) chloroform/methanol/water. The DHA spots were visualized on the plate by rapidly dipping the plate into a solution containing 0.3% (w/v) phosphomolybdic acid and 5% (v/v) H_2SO_4 in MeOH. The plate was dried and heated to 120 °C for 10 min. The plates were analyzed qualitatively using a UV plate scanner.