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Short communication

Efficient production of acetoin by the newly isolated *Bacillus licheniformis* strain MEL09

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ABSTRACT

In this study, a new bacterial strain MEL09, which produces acetoin at high concentrations, was isolated from solid cultures of traditional Chinese vinegar. Based on physiological and biochemical characteristics as well as the 16S rDNA gene sequence, strain MEL09 was identified as *Bacillus licheniformis*. To improve acetoin production by *B. licheniformis* MEL09, medium composition and culture conditions were optimized by varying one factor at a time and using orthogonal array tests. Under these optimized conditions, the maximum acetoin concentration achieved was 41.26 g l^{-1}, with 41.26% glucose conversion efficiency (84.39% of theoretical glucose conversion efficiency). This increase is 84.86% over the initial condition and is, to our knowledge, the highest acetoin level ever reported using fermentation methods.

1. Introduction

Acetoin (3-hydroxy-2-butanone) is a naturally occurring flavor in wine, honey, cocoa, butter, coffee, strawberry and currants. It is also the common intermediate for diacetyl and 2,3-butanediol syntheses [1]. Because acetoin is useful as a food additive and is an intermediate in chemical synthesis, consumer demand has increased; thus, there is interest in alternative methods of acetoin production [2]. There are several chemical synthetic methods for acetoin preparation [3,4], but many recent efforts have been toward the development of natural acetoin production using fermentative or enzymatic technologies. Fermentative production of acetoin by microorganisms is more environmentally friendly and more cost-effective than petroleum-based chemical processes.

Acetoin can be synthesized by many microorganisms, including *Saccharomyces carlsbergensis* [5], *Salmonella* [6], *Lactobacillus casei* [7], *Leuconostoc citrovorum* [8], *Lactococcus lactis* [9], *Leuconostoc mesenteroides* [10] and *Hanseniaspora guilliermondii* [11,12]. However, acetoin production by most of these strains is less than 1.0 g l^{-1}. Efficient production of acetoin was observed in only the following three bacteria: *Klebsiella pneumoniae* (17–19 g l^{-1} with glucose as carbon resource) [13,14], *Enterobacter aerogenes* (10–12 g l^{-1} with glucose as carbon resource) [15] and *Bacillus subtilis* (37.9 g l^{-1} with molasses as carbon resource) [16,17]. Therefore, it is important to explore new microorganisms for efficient industrial acetoin production.

Bacillus licheniformis is a non-pathogenic bacterium that is used extensively in the large-scale fermentation industry. It can secrete large quantities of protein including α-amylase, penicillinase, and pentosanase [18]. Peptide antibiotics as well as organic acids and polymers (citric acid, inosinic acid, poly gamma glutamic acid and extracellular polymers) have also been produced [19,20], and recently the biological synthesis of gold nanocubes [21] and bioflocculant ZS-7 [22] has been demonstrated. However, *B. licheniformis* has only been shown to synthesize acetoin at a very low concentration (0.7 g l^{-1}) [23].

In this study, an efficient acetoin-producing strain was isolated from a solid culture of traditional Chinese vinegar. The strain was further identified as *B. licheniformis* based on its physiological and biochemical characteristics as well as its 16S rDNA sequence. In addition, high yields of acetoin were obtained by optimization of media composition and fermentation conditions.

2. Materials and methods

2.1. Chemicals and medium

Acetoin (98%), 2,3-butanediol (98%), 2,3-diacetyl (98%) and n-butyl alcohol (98%) standards were of chromatographic grade and were purchased from Shanghai Jingchun Reagent Co., Ltd. (Shanghai, China). All other chemicals were of analyti-

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cal grade and were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Luria–Bertani (LB) medium consisted of (g l^{-1}): tryptone 10.00, yeast extract 5.00 and NaCl 10.00 (pH 7.2), with LB medium-added glucose (10.00 g l^{-1}) as screening medium. The initial fermentation medium consisted of (g l^{-1}): glucose 80.00, yeast extract 12.00, peptone 5.00, $(NH_4)_2SO_4$ 5.00, K_2HPO_4 2.00, $MgSO_4$ 1.50, NaCl 0.50, $ZnCl_2$ 0.12, $FeCl_3$ 0.001 and $MnSO_4$ 0.001 (pH at 7.2). Media were autoclaved at 115 °C for 20.0 min.

2.2. Strain isolation and culture conditions

The solid culture from Zhengjiang vinegar factory was inoculated into LB medium and subculture was carried out after 24 h of incubation at 37 °C. The culture was diluted to a million-fold dilution in sterile water and then spread onto LB agar plates. Single colonies were isolated and cultured for 24 h in the screening medium, and the culture was tested by the Voges–Proskauer (VP) reaction [24]. VP-positive microorganisms were inoculated in LB medium and cultured at 37 °C for 12 h, and the cultures were then transferred into initial fermentation medium and cultivated for another 36 h. The production of acetoin was analyzed by gas chromatography (GC).

2.3. Analytical procedures

To monitor the cell growth, cells were separated from the culture broth by centrifugation for 10 min at 13,800 × *g* and 4 °C. Dry cell weight (DCW) was determined by drying washed precipitation at 80 °C to a constant weight.

The concentration of glucose remaining in the culture was measured with a biosensor equipped with a glucoseoxidase electrode (Institute of Biology, Shandong Academy of Sciences SBA-40C (Shandong, China)). Determination of acetoin, diacetyl and 2,3-butanediol was carried out on a Trace GC Ultra gas chromatograph (Thermo Fisher Scientific Co., Ltd.) equipped with a flame ionization detector and TR-WAX capillary column, with N_2 as carrier gas. Analyses of organic acids were performed by high performance liquid chromatography (HPLC) with Agilent 1100 liquid chromatograph (Agilent, USA) [25].

2.4. Identification of *B. licheniformis* MEL09

The morphological and physiological characteristics of the strain MEL09 were identified by a standard test using Bergey's Manual of Systematic Bacteriology [26] and a nucleotide sequence analysis of 16S rDNA as described by Li et al. [27]. Chromosomal DNA was prepared according to the method described by Pospiech and Neumann [28]. The 16S rDNA was amplified using universal primers (16sf95 and 16sr394). The nucleotide was sequenced with an ABI Prism 370 automatic sequencer (Applied Biosystems (Foster City, CA, USA)). An alignment of the MEL09 sequence with sequences from closely related strains collected from the GenBank and Ribosomal Database Project libraries [29] was performed using the CLUSTAL W program and manually edited. A phylogenetic analysis was performed with MEGA 4.0 [30], and the resulting tree was constructed.

2.5. Statistical analyses

Each experiment was carried out in triplicate or more; the mean values are reported. One-way analysis of variance and *t*-test were used to interpret the difference between the means at the 95% confidence level. The orthogonal test was performed with DPS v 7.05. All figures were created using Origin 7.5. Glucose conversion efficiency (GCE) was defined as follows:

$$\mathrm{GCE}\,(\%) = \frac{\text{mass of acetoin produced}}{\text{mass of glucose consumed}} \times 100$$

3. Results and discussion

3.1. Isolation and characterization of acetoin-producing strain

Because all the acetoin-producing strains produced a red color in the VP reaction, these strains were isolated from different samples by VP test. Acetoin production by a strain called MEL09 showed the highest level of red pigment production among the isolates; this maximal level was further confirmed by GC analysis. Relatively high acetoin production (22.32 g l^{-1} with 27.90% GCE) was obtained in batch fermentation by using the initial fermentation medium. Interestingly, MEL09 is capable of growth in a very high concentration of glucose (315 g l^{-1}) and NaCl (100 g l^{-1}), which makes it a good candidate for industrial applications (Fig. 1).

Because MEL09 is gram-positive, motile, rod-shaped, spore-forming, oxidase-positive, and catalase-positive, it was identified as a member of the genus *Bacillus*. The oxidase, catalase, and VP tests disclose very faint differences between MEL09 and *B. licheniformis* WX-02 [31]. Therefore, it was suggested that MEL09 is a strain of *B. licheniformis*.

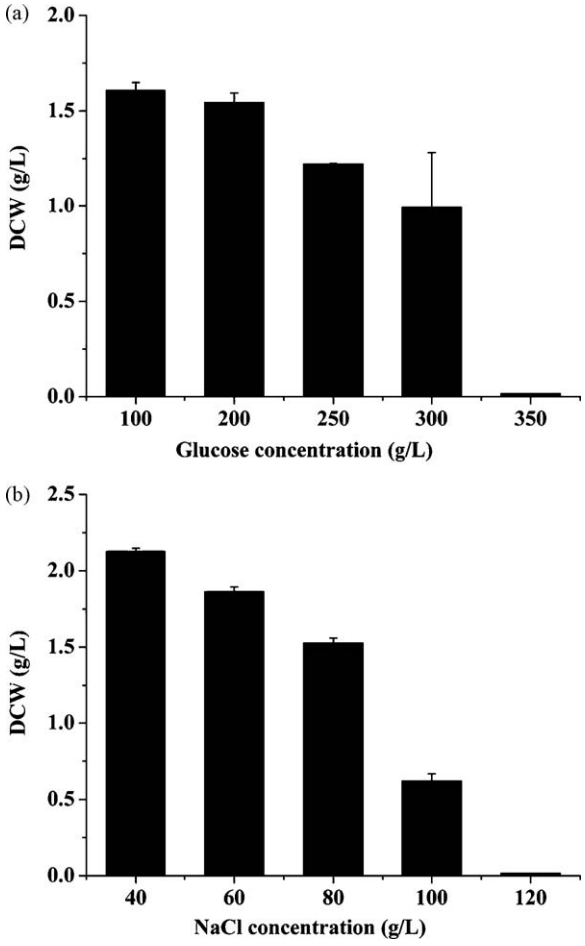

Fig. 1. Tolerance of *B. licheniformis* MEL09 to high concentrations of glucose or NaCl in LB medium.

According to comparisons of the MEL09 16S rDNA gene sequence (GenBank accession no. FJ715927) with other related strains, *B. licheniformis* DSM 13 (X68416) appears most similar (homology, 99%) (Fig. 2). Therefore, the strain was classified as *B. licheniformis* and was deposited as CCTCC M209173 in the China Center for Type Culture Collection.

3.2. Optimization of media components and culture conditions

Several types of carbohydrates were examined as a single carbon substrate for the growth of *B. licheniformis* MEL09 and acetoin production (Table 1). Total biomass was higher when carbohydrates other than glucose were used as the sole substrate than when glucose was used. However, because the highest acetoin production (14.17 g l^{-1}) and yield ($Y_{a/x}$, 3.45 g g^{-1}) were obtained by using glucose, we chose it as the best carbohydrate for acetoin production.

Next, initial glucose concentration, various complex nitrogen sources, and phosphate and metal ions were optimized; one factor was varied at a time. The orthogonal test was applied to obtain the optimal proportion of carbon and complex nitrogen sources. The orthogonal design $L_9(3^4)$ was used to arrange the experiments (Table 2). The order of factor effects on acetoin production was found to be yeast extract > glucose > peptone > $(NH_4)_2SO_4$. The opti-