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Enhanced effectiveness of *Rhizopus oryzae* by immobilization in a static bed fermentor for L-lactic acid production

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ABSTRACT

The static bed fermentor was successfully employed for cell immobilization in this study. By complete immobilization of *Rhizopus oryzae* on a fibrous matrix, cell-free fermentation broth resulted in improved mass transfer and easy operation compared with free cell fermentation in a typical stirred fermentor. Environmental conditions generated in this fermentor configuration favoring the immobilized *R. oryzae* to grow and produce lactic acid without substrate repression at high initial glucose concentration during batch cultivation. Approximately 67% of the theoretical lactate yield (0.50 g/g) with the productivity of 1.05 g/L h and the final titer of 75.28 g/L was obtained from fermentation with high glucose concentration (150 g/L). Complete cell immobilization supported the continuous operation of lactic acid production by *R. oryzae* since cell wash out was diminished. When operating the continuous culture at an appropriate dilution rate, a sufficiently high concentration of lactic acid (72.32 g/L) with a small amount of remaining glucose (<5 g/L) was obtained. Impurities remaining in the hydrolysates were generally considered toxic to microbial conversion; nevertheless, immobilized cells in the static bed fermentor exerted high tolerance to the salt impurities (Na^+, Cl^-) remaining in the cassava pulp hydrolysates to be used in lactate fermentation medium.

1. Introduction

Lactic acid is recognised as a versatile chemical with multiple applications in food, pharmaceuticals, cosmetics, and household products [1]. The commercial production of lactic acid has long relied on bacterial fermentation because of its high final titer, yield, and productivity [2]. The potential application of lactic acid as the major building block in compostable plastic, polylactic acid, has recently driven market demand, while the product specifications for polymer grade lactic acid are of prime consideration [3,4]. Alternatively, lactic acid obtained from fungal fermentation holds great promise in producing polymer grade product because *Rhizopus oryzae* conveys only the *l-ldh* gene; therefore, optically pure L-lactic acid is obtained [5,6]. Lactic acid fermentation by *R. oryzae* is heterofermentative; therefore, ethanol is typically found to be the major byproduct, resulting in low lactate yield and productivity. To limit ethanol production, oxygen supply is necessary for growth and lactate production in fermentation by *R. oryzae*; therefore, sufficient mixing and aeration are mandatory.

Morphological diversity is a challenge in the submerged cultivation of *R. oryzae* for lactic acid. Change in morphology during cultivation is commonly observed as the effect of medium composition, pH, temperature, agitation, and so on [7]. It has been claimed that small pellets are preferable in cultivating *R. oryzae* for lactic acid as they enhance oxygen transfer in the fermentation broth and could be utilized for repeated batch operation. Nonetheless, increase in pellet size and density has typically been observed during fermentation.

Several techniques have been used to immobilize *R. oryzae* for proper morphology, good mass transfer, and homogeneity during fermentation in order to increase lactic acid production [8,9]. These techniques, for example, include entrapment in the polymeric matrices [10], mechanical containment in the microporous membrane filters [11], biofilm attachment on the support [12], and

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adsorption onto the fibrous matrices [13,14]. Several fermentors have been used for the immobilized cells of *R. oryzae* including air-lift fermentor, drum contactor, reciprocating jet fermentor, tower fermentor, and hollow fiber fermentor [15–18]. Generally, low lactic acid yields of approximately 0.65–0.78 g/g glucose with the final titer of 40–73 g/L were obtained in most immobilization studies [15,19–23]. Nonetheless, fibrous bed fermentors have been reported to enhance lactic acid production by fungal immobilization in the fibrous matrices, e.g., cotton cloth, affixed in the fermentors in batch, repeated batch, and fed-batch operations [13,14]. Through cell attachment on the flat sheet surface provided in this fermentor configuration, it was claimed that oxygen transfer was significantly improved, and therefore the lactic acid production increased.

A static bed fermentor, a type of fibrous bed fermentor, was investigated for its effectiveness in controlling the morphology of *R. oryzae* throughout fermentation. In typical batch fermentation, substrate repression was often troublesome when operating at high initial concentration. With proper morphological control in this fermentor configuration, it was believed that cultivation under this environmental stress was plausible. Long-term cultivation and the consumption of low cost substrates with acceptable fermentor productivity were desirable in fermentation operation especially at industrial level because of lowered operation costs. In this study, the immobilized *R. oryzae* on the cotton cloth in the static bed fermentor was tested according to three different aspects: susceptibility to grow and produce lactic acid at high initial glucose concentration; long-term stability during continuous culture; and tolerance to toxic impurities contained in the cellulosic hydrolysates to be used as the carbon source in fermentation.

2. Materials and methods

2.1. Culture, inoculum preparation, and media

The fungus *Rhizopus oryzae* NRRL395 used in this work was kindly provided by the Northern Regional Research Center, Peoria, IL, USA. The stock culture was maintained on potato dextrose agar (PDA) plates and subcultured every month to maintain viability and activity.

The sporangiospores were collected from the 7-day culture on PDA plates by shaving and extracting with sterile deionized water. The spore suspension was diluted to 10^6 spores/mL with sterile deionized water. The diluted spore suspension (10 mL) was inoculated into the fermentor containing the sterile growth medium of 4 L initial volume.

Unless otherwise noted, glucose was used as the sole carbon source in both growth and production media. A growth medium containing (per liter) 50 g glucose and 5 g yeast extract was used for germinating spores and initiating hyphal growth. The production medium for enhancing lactic acid production while maintaining metabolic activities with minimal cell growth of *R. oryzae* was composed of (per liter) 70 g glucose, 0.6 g KH_2PO_4, 0.25 $MgSO_4$, 0.088 g $ZnSO_4$, and 0.3 g urea [13,14]. The feeding medium used in continuous operation consisted solely of glucose at the concentration of 150 g/L while urea was intermittently added every 24 h at the concentration of 0.15 g/L.

To investigate the efficacy of immobilized *R. oryzae* to utilize cellulosic material for lactate production, cassava pulp hydrolysate was also prepared for use as the carbon source in both growth and production media. Fresh cassava pulp was pretreated by direct steam or NaOH explosion at 121 °C, 15 psig for 60 min. After that, the mixture was subjected to hydrolysis using enzymes (cellulase and amylases) or 1 M HCl. The detailed procedure can be found in Thongchul et al. (2010) [24]. Similar to the glucose based media previously mentioned, the initial total sugars in the hydrolysates were adjusted to approximately 50 g/L for growth medium and 70 g/L for production medium.

2.2. Fermentor design

Free cell fermentation was carried out in a 5 L stirred fermentor (Biostat B Plus, Sartorius, Germany) while immobilized cell fermentation was conducted in a static bed bioreactor modified from the 5 L stirred fermentor (Biostat B Plus, Sartorius, Germany). The static bed bioreactor was constructed with a perforated stainless steel matrix mounted with a 100% cotton cloth affixed to the top plate of the fermentor. A detailed configuration can be found elsewhere [13].

2.3. Fermentor set up and operation

The fermentor containing the 4 L growth medium was autoclaved at 121 °C for 30 min. After sterilization and cooling down, a dissolved oxygen (DO) probe was calibrated with sterile nitrogen and air. The fermentor was controlled at 30 °C with specified agitation and aeration rates. The fermentor was inoculated with 10 mL spore suspension (10^6/mL). The growth phase took approximately 48 h for spore germination and initial cell growth before the growth medium was discarded and the fermentor was filled with 4 L sterile production medium. During the production phase, the pH was automatically controlled at 6.0 by 5 M NaOH. During operation, antifoam was intermittently added to prevent foaming. For batch cultivation, fermentation finished when glucose depletion or lactate production stopped. In continuous culture, fermentation finished when lactate production ceased or glucose concentration exceeded 10 g/L. Fermentation broth samples were taken approximately every 6 h. In continuous operation, after changing the medium to production medium, batch operation was initiated until the remaining glucose in the fermentation reached 10–20 g/L when continuous operation started. The feed solution was continuously transferred into the static bed fermentor while the broth was taken out at the same volumetric flow rate at the specified dilution rate. Fermentation broth samples were also taken approximately every 6 h.

Batch operation was first conducted to reiterate the efficacy of immobilized *R. oryzae* in the fibrous bed fermentor over free cell culture in the stirred fermentor previously as claimed by Chotisubha-anandha et al. (2011) and Tay and Yang (2002) [13,14]. Additionally, the key factors that controlled lactate production by *R. oryzae* were described during batch operation in the static bed fermentor to further determine the operating conditions during long-term cultivation. Those included agitation, aeration, and initial glucose concentration. Later, the robustness of immobilized *R. oryzae* was tested during long-term cultivation under continuous operation. The effect of feed medium compositions on the fermentation kinetics was observed during the cultivation. Also, cultivation of the immobilized *R. oryzae* in the medium containing high salt content like those obtained from the hydrolysis products of cellulosic materials was investigated for its ability to grow and produce lactate compared to those in the base case cultivation in glucose medium.

2.4. Sample analyses

The amount of total sugars in cassava pulp hydrolysates was determined by completely hydrolyzing the hydrolysate sample (1 mL) with 1 M HCl (9 mL) for 1 h at 121 °C, 15 psig. Monosaccharides released in the samples (mainly glucose in all hydrolysates with trace amounts of xylose (less than 4 g/L) in the case of

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