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Correlative effect of dissolved oxygen and key enzyme inhibitors responsible for L-lactate production by immobilized *Rhizopus oryzae* NRRL395 cultivated in a static bed bioreactor

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ABSTRACT

In typical fermentation by *Rhizopus oryzae*, ethanol was found as the major byproduct. The simplest way to limit ethanol was to supply high dissolved oxygen (DO) during fermentation. Our previous work showed metabolic fluxes shifted toward lactate production with lower ethanol formation in the fermentation containing enzyme inhibitors including 1,2-diazole, 2,2,2-trifluoroethanol, 4-methylpyrazole, and 3-hydroxypyruvate. In this study, we further investigated the dynamic responses of the living *R. oryzae* cultivated in the static bed bioreactor to the effects of enzyme inhibitors and DO levels together. Either increasing DO level or adding inhibitor alone limited ethanol and promoted lactate production while cell biomass remained unchanged. Lactate yield was increased by 24% with the increasing DO from 40% to 80% during the fermentation. Among 4 inhibitors studied, 2,2,2-trifluoroethanol gave the maximum improvement in fermentation efficiency. Approximately, 32% increasing lactate yield was obtained in the fermentation at 40% DO with 0.1 mM 2,2,2-trifluoroethanol. Together, inhibitor and high DO level better controlled metabolic flux toward lactate production. Dramatic increasing lactate yield of 54% was obtained in the fermentation containing 2,2,2-trifluoroethanol at 80% DO control with 100% improved productivity. Findings from this work exhibited the simple yet effective way to improve lactate fermentation by *R. oryzae*.

1. Introduction

Lactic acid has long been widely used in food, pharmaceutical, cosmetic, and chemical industries. Lactic acid production is currently attracting a great deal of research and development. Nowadays, lactic acid consumption is increased as the consequence of an emerging market of polylactic acid, a renewable, compostable, and biocompatible polymer, which provides an environmental friendly alternative to biodegradable plastics derived from petrochemicals [1–3]. Naturally, lactic acid can be found in 2 optical isomers, i.e., D(−) and L(+)-lactic acids. However, the existing commercial applications in food and health cares are mostly limited to L-lactic acid due to the toxicity of D-lactate to living cells [4].

The optically pure L-lactate can be obtained via microbial fermentation process. *Rhizopus oryzae* only possesses L-lactate dehydrogenase and lacks racemase; thus, being considered as one promising strain for L-lactate fermentation with low nutrient requirements [5–14]. Nonetheless, lactate fermentation by *R. oryzae* yields lower production comparable to typical bacterial fermentation process. While the theoretical lactate yield from glucose by bacterial fermentation was about 0.85 g/g, lactate yield from glucose fermentation by *R. oryzae* was only 0.6–0.8 g/g [15]. Recently, L-lactate fermentation by *R. oryzae* was extensively studied for improved yield and productivity. Submerged fermentation in several bioreactor designs was conducted. Stirred fermentor, a typical bioreactor widely used in submerged microbial fermentation, provided high oxygen supply so that limiting ethanol byproduct;

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however, generating high shear stress that caused damage to fungi and reduced lactate production [16,17]. In addition, morphological changes were commonly observed during fungal fermentation. This led to changes of nutrient supply including oxygen along the cultivation and subsequently difficulty in operational control to achieve stable run and high production rate [16,18–20].

Cell immobilization was introduced in lactate fermentation by *R. oryzae*. Via immobilization using several techniques, lactate production was successfully improved as the results of better mass transport especially oxygen and high cell density fermentation [7–10,12,16]. Not only improving lactate production, immobilization could also provide ease of operation and possibility of long-term operation [21–24]. A static bed bioreactor is one of the immobilized cell bioreactors developed for lactate fermentation by *R. oryzae*. Remarkably improved lactate yield approaching the theoretical value was obtained in this bioreactor; nonetheless, trace amount of ethanol still existed in this immobilized culture system [24].

The results obtained from our previous study in a shaken flask culture of *R. oryzae* proved that ethanol production could be controlled by adding the inhibitors that regulated the alcohol fermentative pathway [25–27]. This subsequently gave the increasing lactate yield and productivity. It was observed that introducing substrate/cofactor analogs of alcohol dehydrogenase (ADH) such as 1,2-diazole and 2,2,2-trifluoroethanol as well as those analogs of pyruvate decarboxylase (PDC) including 4-methylpyrazole and 3-hydroxypyruvate into the production medium during cultivation of *R. oryzae* led to changes of metabolic fluxes and eventually showed the positive impact on lactate production [25–27].

In this study, further improved lactate yield and productivity by using both enzyme inhibitors and high DO level in lactate fermentation was attempted. The static bed bioreactor was applied for cell immobilization to enhance oxygen transfer under varied DO levels while ADH and PDC inhibitors were added during the production phase in order to inhibit ethanol formation. By these combinatorial effects, glucose flux would be driven toward lactate resulting in the increase in yield and productivity. The fermentation kinetics and the specific activities of key enzymes responsible for lactate and ethanol production were described. The fermentation efficiency was investigated.

2. Materials and methods

ADH and PDC inhibitors were tested for their effects on metabolic responses of immobilized *R. oryzae* cultivated in the static bed bioreactor under different dissolved oxygen (DO) levels. Changes of end product yield and productivity were investigated. The dynamic responses of the living culture to both changes in DO level and inhibitor were described and compared.

2.1. Microorganism, inoculum preparation, and medium compositions

R. oryzae NRRL395 was obtained from Agricultural Research Service Culture Collection, US Department of Agriculture, Peoria, IL, USA. Spore suspension was prepared from the 7-day sporagiospores harvested from potato dextrose agar plate suspended in sterile DI water. The spore concentration was adjusted to 10^6 spores/mL before inoculation.

Lactic acid fermentation by *R. oryzae* consisted of 2 phases. Growth medium was used for spore germination and initial growth during the growth phase. Growth medium contained (per liter) 50 g glucose and 5 g yeast extract. After growth was successively initiated, the production medium was used during lactate production phase. The production medium consisted of (per liter) 70 g glucose, 0.6 g KH_2PO_4, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.088 g $ZnSO_4{\cdot}7H_2O$, and 0.3 g urea [21,24].

2.2. Bioreactor construction

As shown in Fig. 1, a static bed bioreactor was modified from a 5-L stirred fermentor by mounting a perforated stainless steel covered by a cotton towel at the top plate of the bioreactor [24]. The bioreactor was equipped with the automatic control of temperature, pH, agitation, and aeration. The air fed into the bioreactor was sterilized by passing through a membrane filter (0.2 μm).

2.3. Bioreactor start up and fermentation operation

The fermentor containing 3 L growth medium was autoclaved at 121 °C, 15 psig for 30 min. After the fermentor was cool down, the temperature was set at 30 °C and DO probe was calibrated with nitrogen and air. The fermentor was then inoculated with 10 mL spore suspension (10^6 spores/mL). The agitation and aeration rates were set at 700 rpm and 1.0 vvm air, respectively. Spores were allowed to germinate and immobilize onto the cotton cloth for 48 h. The growth medium was withdrawn at the end of the growth phase. The fermentor was filled up with 3 L sterile production medium. The bioreactor was controlled at the same temperature (30 °C) and aeration rate (1.0 vvm). The pH was controlled at 6.0 by 5 M NaOH. To control the DO levels at 40%, 60%, and 80%, cascade control was applied by varying the agitation speed between 100 rpm and 300 rpm and volumetric flow ratio of oxygen to air. During the production phase, enzyme inhibitors regulating alcohol fermentative pathway including 1 mM 1,2-diazole, 1 mM 2,2,2-trifluoroethanol, 0.1 mM 4-methylpyrazole, and 1 μM 3-hydroxypyruvate were added into the medium. It should be noted that the concentrations of the selected inhibitors were previously optimized elsewhere [25,26]. Both DO level and enzyme inhibitor were observed for their effects on fungal metabolism. Growth reported as cell dry weight, glucose consumption, end product formation as well as enzyme activity were investigated. During the production phase, sample was collected every 6 h for analyzing remaining glucose and end products. After 72 h, cell biomass was harvested from the fibrous bed for cell biomass determination and enzyme assays.

2.4. Analysis of remaining glucose and end products

High performance liquid chromatography was used to analyze the fermentation broth sample for the remaining glucose, lactate, and ethanol. Before analyzing, the broth sample was filtered through cellulose acetate membrane and diluted with double distilled water. Diluted particle-free sample (15 μL) was injected into an organic acid analysis column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300 mm × 7.8 mm) maintained at 45 °C in a column oven. 0.005 M H_2SO_4 was used as an eluant at the flowrate of 0.6 mL/min. A refractive index detector was used to detect the organic compounds. Standards containing glucose, lactate, and ethanol at different concentrations in between 0 and 2 g/L were injected as the references. The peak area was used for the comparison basis.

2.5. Cell biomass

At the end of fermentation, cell biomass immobilized on the cotton towel was collected from the perforated stainless steel. The