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L-Lactic acid fermentation by culture of *Rhizopus oryzae* using ammonia as neutralizing agent

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ABSTRACT

In order to avoid the formation of calcium sulfate solid waste in the downstream processing, the traditional neutralizing agent calcium carbonate used in *Rhizopus* lactic acid fermentation was replaced by ammonia water. However, the yield of lactic acid is hampered because of the toxicity of ammonia to the microbial cells. It is imperative to surmount the dilemma for how to maintain the lactic acid production but reduce the damage of cells by ammonia. In this work, ammonium bicarbonate and urea were added to ammonia water to reduce the chemical damage of ammonia on fungal cells. L-Lactic acid production in an air-lift bioreactor by cultures of *R. oryzae* BCRC 33071 and ATCC 9363 were compared. The results showed that the highest productivity (3.01 ± 0.05 g/h l) and yield (85.3 ± 1.3%) was obtained by culture of *R. oryzae* BCRC 33071, using 120 g/l of glucose as substrate and a mixture of ammonium bicarbonate (2%), urea (1%) and ammonia (10%) as neutralizing agent.

1. Introduction

L-Lactic acid is an important material to produce biodegradable and biocompatible polymers, such as poly-L-lactic acid. Synthetic lactic acid made from petrochemical feed-stocks is a racemic mixture, which is optically inactive. Industrial production of lactic acid, pure in L-form, relies predominately on fungal strains such as *Rhizopus* species [1,2]. Although *Lactobacillus debruckii* and other metabolically engineered bacteria or yeast can produce L-lactic acid, too [3–6], the culture medium ingredients, such as yeast extract and peptone for these strains and the downstream steps, such as microfiltration or centrifugation to clarify the culture broth are costly. On the contrary, cheap ammonia salts can serve as nitrogen sources for *R. oryzae* and the filamentous cells can be readily eliminated from culture broth by means of coarse filtration. Traditionally, L-lactic acid is produced through the fermentation of glucose or starch with *Rhizopus* sp. During fermentation $CaCO_3$ was normally used as neutralizing agent [7–13]. In the downstream process for the purification of lactic acid, large amount of $CaSO_4$ (gypsum), a solid waste, is formed [1]. To avoid the formation of calcium sulfate, ammonia water can be used as the neutralizing agent in the submerged fermentation process [14,15]. The main product of fermentation becomes ammonium lactate, wherein ammonia may be recovered by means of ion exchange or electrodialysis [1]. The major drawback of this fermentation process is that NH_4OH, the main component in ammonia water, is caustic and may thereby damage fungal cells, leading to a reduced yield of lactic acid. The submerged culture in a bioreactor includes three phases: gas (air), liquid (fermentation broth) and solid (fungal cells). During the submerged fermentation solutions of strong base such as NaOH or $Ca(OH)_2$ can be fed into the bioreactor by directly dropping onto the culture without significant damage on the living cells. When such strong base is added to the culture broth, OH^- ions readily react with lactic acid before they contact cells. But this is not the case when ammonium water is dropped trough the head-space of the bioreactor. Although ammonia is a weak base, it can move freely in air or in aqueous solution and thus is very penetrative. As shown in Fig. 1, ammonia can move next to the surface of mycelial cells and convert into ammonium hydroxide (NH_4OH). NH_4OH then releases OH^-ion and attack fungal cells in term of chemical burn. Tsujii *et al.* found that ammonia caused gastric mucosal damage in a dose-dependent manner [14]. Both cellular respiration and energy metabolism were impaired by ammonia, which were resulted from the inhibition of oxygen consumption of cells and mitochondria. In our preliminary experiments it was found that when a stream of ammonia water was fed directly into the culture, large mycelial clumps were formed in the region next to the opening of the tube. Fungal cells were damaged and the production of lactic acid was declined. Recently, submerged culture conditions for the mycelial

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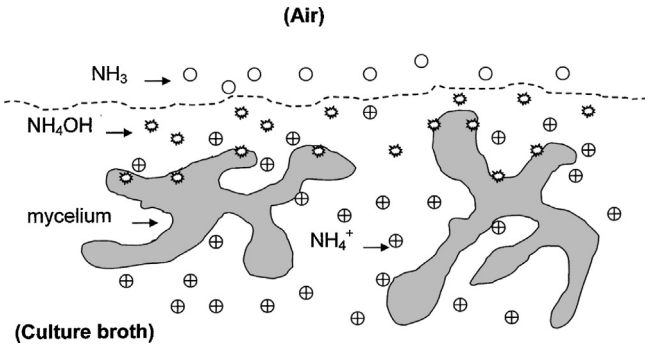

Fig. 1. Attack of NH_4OH on fungal cells.

growth were optimized to enhance the yield of botulin [15]. They suggested that the desired pellet morphology varied from one product to another. Miura *et al.* has used ammonium concentration gradient agar plate to isolate ammonium-tolerant mutants originated from a *Rhizopus* sp. and has compared the efficacy of lactic acid fermentation with these strains using ammonia water as neutralizing agent [16,17]. During the fermentation using ammonia as neutralizing agent the final concentration of lactic acid might be higher than 90 g/l and the concentration of ammonium ion approximate to 1.5 M [16,17].

In the present work, lactic acid fermentation with *R. oryzae* was carried out in an air-lift bioreactor, and a new strategy was adopted to provide ammonia solution as neutralizing agent. Both $(NH_4)HCO_3$ and urea were mixed with ammonia water to reduce the damage of ammonia on fungal cells. Urea was hydrolyzed by the catalysis of urease on the mycelium [18] and the resulted products—NH_3 and CO_2—have similar buffer effect as $(NH_4)HCO_3$. Furthermore, gaseous CO_2 might make mycelia becoming more dispersed than that without urea. This morphology of mycelium is beneficial for the mass transfer of oxygen, nutrients, substrate and product in the culture broth and thus enhances the production of lactic acid. In this work, the ammonia solution was not fed directly to the fermentation broth (Fig. 2(a)). Instead, a porous glass device (Fig. 2(b)) was connected to the end of a tube and through which the ammonia solution was fed into the culture broth by means of diffusion. Most ammonium hydroxide might be neutralized with lactic acid before contacting with mycelium. In this way, the damage of ammonia on fungal cells is expected to be minimized and the production of lactic acid might be enhanced.

2. Materials and methods

2.1. Microorganisms and culture medium

All the chemicals were purchased from commercial sources. *R. oryzae* BCRC 33071 and ATCC 9363 were obtained from the Bioresource Collection and Research Center (BCRC), Taiwan. The originally freeze-dried culture was aseptically ground into powders. Each fermentation process was always started with small amount of culture powders being spread on potato dextrose agar (PDA). The medium for the seed culture were composed of the following ingredients (g/l): glucose, 50; $(NH_4)_2SO_4$, 3; KH_2PO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; $ZnSO_4{\cdot}7H_2O$, 0.04 and $CaCO_3$, 10 (by adding one tenth volume of 10% suspension after 8 h of cultivation). The medium for lactic acid production consisted of the following ingredients (g/l): glucose, 120; $(NH_4)_2SO_4$, 1; KH_2PO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5 and $ZnSO_4{\cdot}7H_2O$, 0.04. The initial pH of all media was adjusted to 6.0 with NaOH. All culture media and the 10% $CaCO_3$ suspension were autoclaved at 121 °C for 15 min. Glucose was autoclaved separately.

2.2. Lactic acid fermentation in an air-lift bioreactor

R. oryzae was grown on PDA, which had been prepared on the bottom of a 500-ml Erlenmeyer flask. After incubation at 30 °C for 7–10 days, the spores formed on the surface of the culture were suspended by adding 50 ml of 0.02% Tween-80 solution to the flask. After a vigorous shaking, the spore suspension was inoculated into a 500-ml Erlenmeyer flask containing 150 ml of seed culture medium at a dose around 1×10^6 spores/ml. After the culture was incubated in a 30 °C shaker at 200 rpm for 8 h, 15 ml of 10% $CaCO_3$ suspension were added to the culture broth. After a successive cultivation for 4 h, the pellet-like culture was formed and this was used as seed culture for the bioreactor [7]. Three hundred milliliters of seed culture were inoculated into an air-lift bioreactor (Fig. 3) containing 3.5 l of fermentation medium. The fermentation was carried out at 35 °C and a superficial velocity of 0.74 cm/s (aeration rate: 1 vvm). During fermentation the pH of the culture broth decreased due to the production of lactic acid. When the pH of the culture broth became lower than a given value, a relay was turned on through the pH controller (Suntex model PC-310) and a peristaltic pump was pulsed to deliver the neutralizing agent into the bioreactor. The culture broth was controlled at a given pH value, in a range

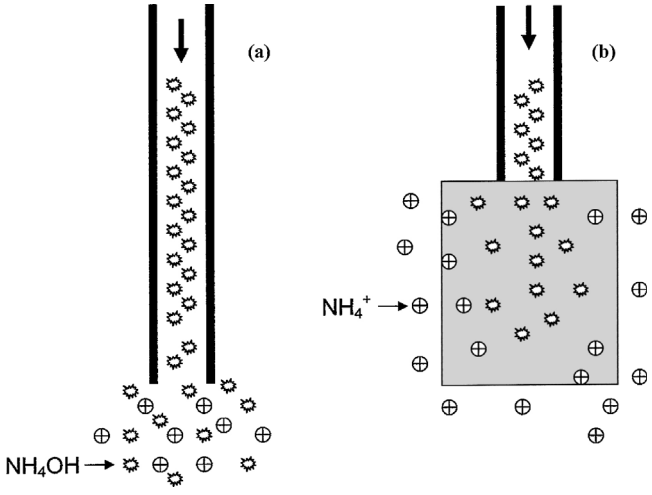

Fig. 2. Feeding of ammonia water via a tube (a) without, or (b) with a diffusion device.

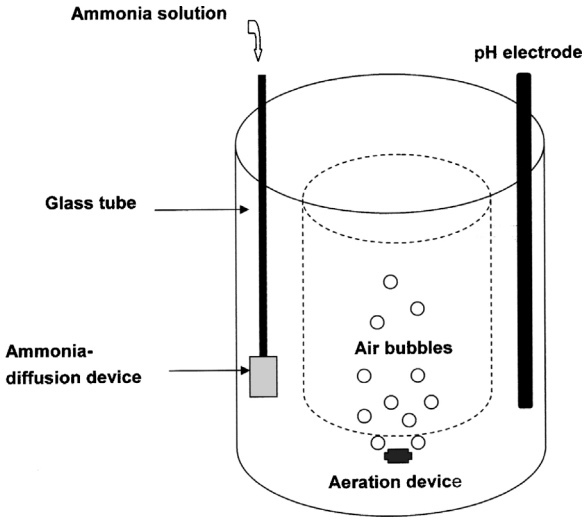

Fig. 3. The air-lift bioreactor.