

Betaine Improves Polymer-Grade D-Lactic Acid Production by *Sporolactobacillus inulinus* Using Ammonia as Green Neutralizer

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Abstract

The traditional CaCO₃-based fermentation process generates huge amount of insoluble waste. To solve this problem, we have developed an efficient and green D-lactic acid fermentation process by using ammonia as neutralizer. The 106.7 g/l of D-lactic acid production and 0.89 g/g of consumed sugar were obtained by *Sporolactobacillus inulinus* CASD with a high optical purity of 99.7% by adding 100 mg/l betaine in the simple batch fermentation. The addition of betaine was experimentally proven to protect cells at high concentration of ammonium ion, increase D-lactate dehydrogenase specific activity and thus promote the production of D-lactic acid.

Key words: *Sporolactobacillus inulinus*, ammonia as neutralizer, betaine as osmoprotectant, D-lactic acid production

Poly-lactic acid (PLA), a biodegradable polymer produced by lactic acid monomer are considered as the most promising substitution for petroleum-derived plastics in future (Zhang and Vadlani, 2013). The application of poly L-lactic acid (PLLA) is limited by its low melting temperature (< 180°C) while this problem can be obviated by blending it with poly D-lactic acid (PDLA). The melting point of the stereocomplex polymer is approximately 50°C higher than that of the respective single polymers (Ikada *et al.*, 1987). This finding has attracted great interest to the production of D-lactic acid. However, the technology for D-lactic acid production is not well established, compared to the L-lactic acid fermentation process (Wang *et al.*, 2012; Li *et al.*, 2013).

In addition, the accumulation of lactic acid causes the pH to continuously decrease and further influences the growth and performance of the strains. Thus, it is necessary to add some neutralization agent to the medium to keep the pH value at a stable level during the process of fermentation. To avoid growth inhibition by lactic acid, CaCO₃ is normally added during fermentation to neutralize lactic acid and maintain the pH within the range of 5.0–6.0. However, a considerable amount of calcium sulfate (gypsum) is produced during the conversion of calcium lactate to free lactic acid, causing extensive environmental burden (Vaidya *et al.*,

2005). Ammonia is a fast and effective neutralizer. It can maintain the pH stability of the broth without producing any calcium sulfate during the process of extraction. Additionally, it can be recovered from the fermentation broth in the process of lactic acid purification, and subsequently recycled for continuous fermentation. Moreover, it can serve as nitrogen source in the fermentation process (Miura *et al.*, 2004). However, the final acid concentration and cell concentration are hampered by the toxicity of ammonia to the microbial cells.

The present paper reports an efficient NH₄⁺-based production of polymer-grade D-lactic acid by *Sporolactobacillus inulinus* CASD [China General Microbiological Culture Collection Center (CGMCC), No 2185] (Wang *et al.*, 2011). The effect of betaine, N,N,N-trimethylglycine, on the activity of D-lactate dehydrogenase (EC 1.1.1.28) and D-lactic acid production under such condition was investigated. The enzyme activity was determined by measuring the initial rate of oxidation of NADH at 340 nm with pyruvate as a substrate. The lactate dehydrogenase specific activity was defined as U/mg total protein. The protein concentration was determined with Bradford reagent (Bradford, 1976).

Both cell growth and lactic acid production would be inhibited by high concentration of the substrates (initial glucose concentration), end-product (fermented lactic

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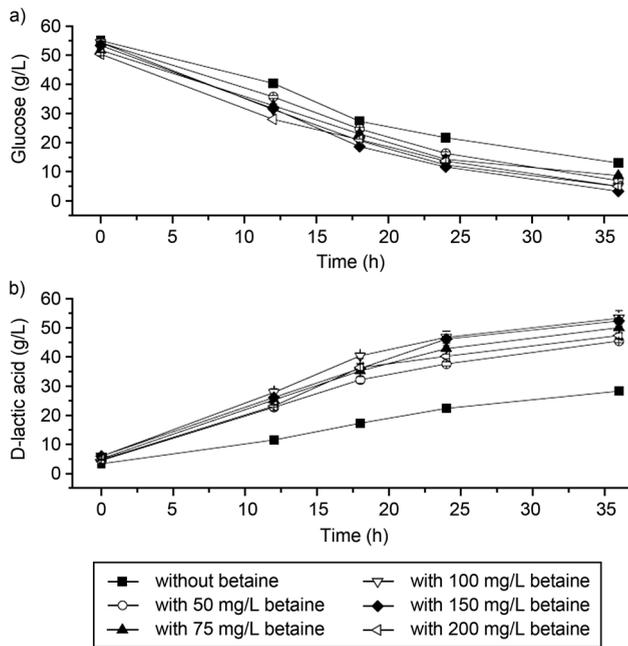


Fig. 1. The effects of betaine concentrations on glucose consumption and D-lactic acid production by strain *S. inulinus* CASD. (a) the glucose consumption and (b) D-lactic acid production. Results are mean \pm SD from three independent experiments.

acid) and ammonia (Ding and Tan, 2006). To relieve the substrate level-osmotic stress, betaine (0–200 mg/l) was added to the medium as osmoprotectant. As shown in Fig. 1, betaine exhibited a positive effect on D-lactic acid fermentation by *S. inulinus* CASD using ammonia as neutralizer. When betaine was added at 100 mg/l, the maximal D-lactic acid concentration was obtained. With further increase of the concentration of betaine, no obvious effect was observed on sugar consumption and lactic acid production. The addition of 100 mg/l betaine could provide enough intracellular accumulation of osmoprotectant to protect cells against the influence of high concentration of ammonium ion in broth, finally resulting in the improvement of D-lactic acid production. Thus, a betaine concentration of 100 mg/l was selected as the optimal concentration and was used in subsequent experiments.

The D-lactate dehydrogenase activity of *S. inulinus* CASD was also positively affected by betaine addition under the ammonium ion stress condition. The specific activity (2.36 U/mg) of lactate dehydrogenase in the crude extract of *S. inulinus* CASD was over two-fold than that without betaine supplementation (1.03 U/mg).

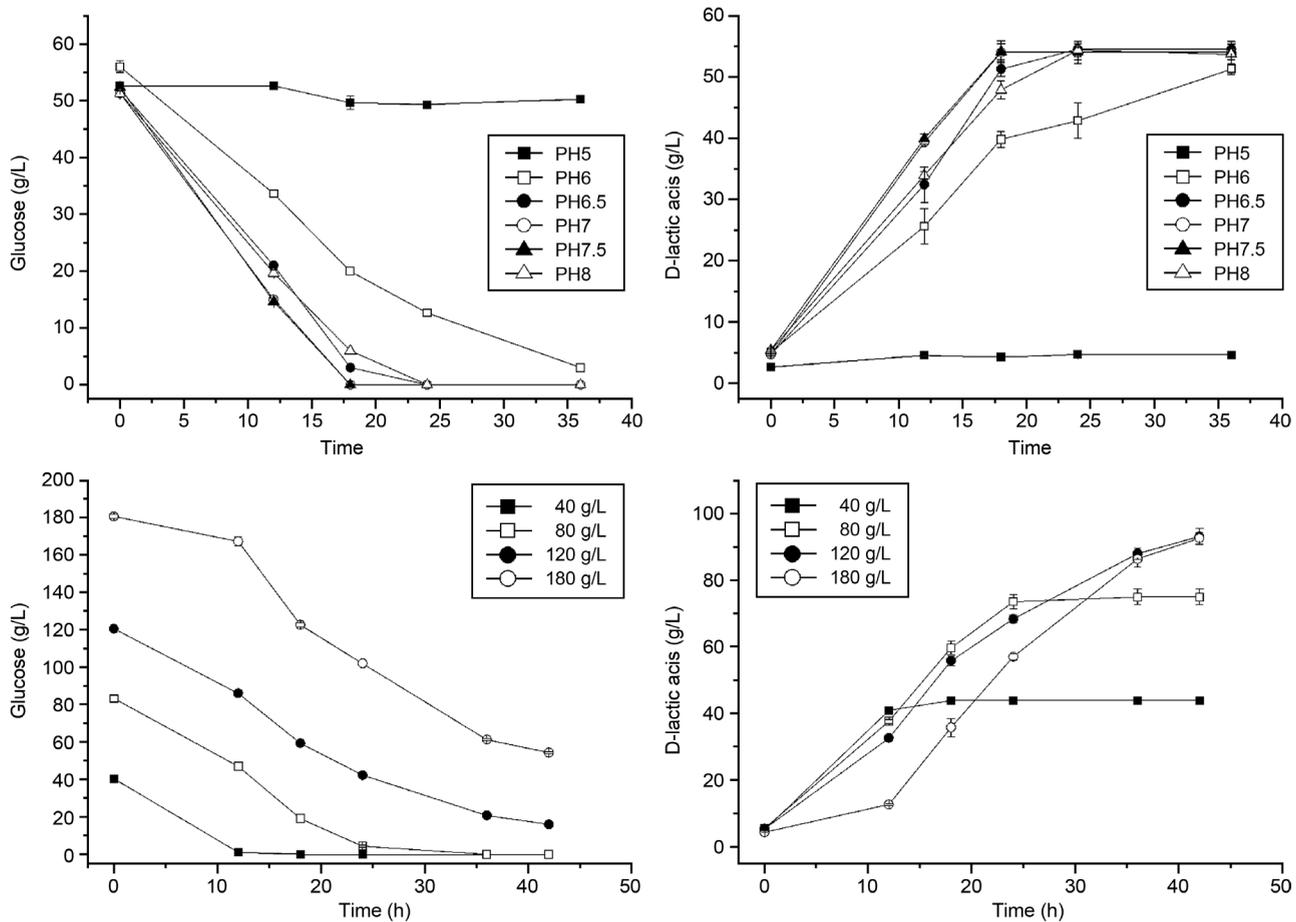


Fig. 2. Optimization of batch fermentation conditions for D-lactic acid production by *S. inulinus* CASD using ammonia as neutralizing agent.

Results are mean \pm SD from three independent experiments. (a) the glucose consumption and (b) D-lactic acid production under different pH conditions; the effects of the initial glucose concentrations on (c) glucose consumption and (d) D-lactic acid production.

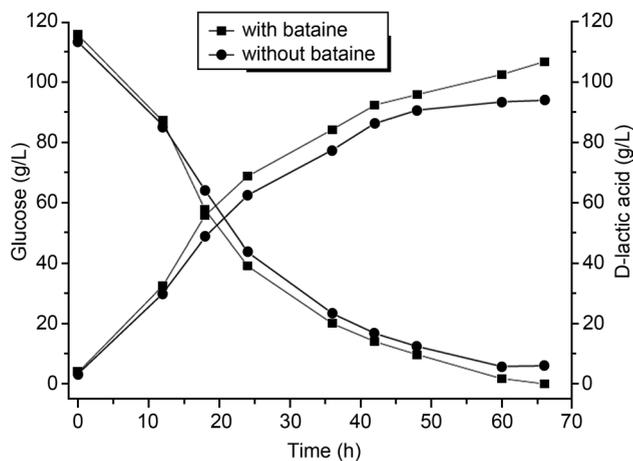


Fig. 3. Batch fermentation of D-lactic acid by *S. inulinus* CASD using ammonia as neutralizing agent with or without betaine.

Additionally, betaine was also found to improve the cell growth under the ammonium ion stress condition. The cell density (OD_{600}) reached 12.56 with betaine addition while it was only 9.42 without betaine supplementation. The increased D-lactate dehydrogenase activity and cell growth clearly indicated that betaine supplementation benefited the D-lactic acid fermentation under the ammonium ion stress conditions.

As ammonia was used as neutralizer, the optimum pH for D-lactic acid production was first investigated. The glucose consumption and lactic acid production were significantly inhibited when the pH of broth was maintained at 5.0 (Fig. 2a-b). The lactic acid concentration increased most rapidly and reached its highest values (54.3 g/l) after 18 h at pH 7.0. Although the concentrations of D-lactic acid reached the same values after 18 h at pH 7.5, pH 7.0 was finally chosen as the optimal pH since less of ammonia was used under such conditions.

To examine the substrate tolerance of strain CASD, different initial glucose concentrations were tested for D-lactic acid production (Fig. 2c-d). When glucose concentration was below 80 g/l, the concentration of D-lactic acid increased as the initial glucose concentration increased. The D-lactic acid concentration increased fast in 24 h with an initial glucose concentration of 80 g/l. However, the D-lactate productivity became much slower than others when the initial glucose concentration was higher than 180 g/l, indicating the inhibition of cell growth and D-lactic acid production by high concentration of glucose (over 180 g/l). As the glucose could almost be consumed and a higher concentration of D-lactic acid obtained (93.2 g/l), 120 g/l was selected as the optimized initial glucose concentration for use in the following batch fermentations.

The fermentations were carried out at 42°C for 66 h in a 5 l bioreactor with working volume of 2 l of optimized medium (glucose 120 g/l, yeast extract 10 g/l

and betaine 100 mg/l or without betaine, pH 7.0) with an agitation speed of 50 r/min without aeration. The glucose in the medium with betaine was completely exhausted and the final D-lactic acid concentration reached 106.7 g/l with a productivity of 1.62 g/l·h (Fig. 3). The fermentation with 100 mg/l betaine could produce 13.6% more lactic acid compared to the fermentation without betaine and raised the yield by 11.4%. Moreover, the optical purity of D-lactic acid was determined to be 99.7%, which meets the requirement for the lactic acid polymerization process.

Using ammonia as neutralizer is a green way to produce lactic acid, while the high concentration of ammonium ion in the broth will significantly affect the cell growth and performance, resulting in a rather low productivity. Okano reported a D-lactic acid fermentation strategy using ammonia solution as pH-controlling agent by a L-lactate dehydrogenase gene deficient *Lactobacillus plantarum* (Okano *et al.*, 2009). However, the final concentration of D-lactic acid was 73.2 g/l and the yield was just 0.85 g/g initial glucose which almost ruled out the possibility of industrialization. Betaine has been applied in many fermentation processes as an efficient osmoprotectant (Sharma and Dubey, 2005; Robert *et al.*, 2000), but its positive effects were not indicated on the protection inhibition caused by ammonium ion. In this study, betaine was proven to have a positive effect on D-lactic acid production when ammonia was used as neutralizer. As a result, an efficient lactic acid production (106.7 g/l), with a small amount addition of betaine (100 mg/l), by the *S. inulinus* strain was developed, providing a good option for polymer-grade D-lactic acid production in a green fermentation process. Thus, an efficient and 'green' D-lactic acid fermentation strategy without producing recalcitrant wastes (e.g., gypsum) for the industrial production of polymer-grade D-lactic acid was established.

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