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Application of a pH Feedback-Controlled Substrate Feeding Method in Lactic Acid Production

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Abstract Substrate concentration in lactic acid fermentation broth could not be controlled well by traditional feeding methods, including constant, intermittent, and exponential feeding methods, in fed-batch experiments. A simple feedback feeding method based on pH was proposed to control pH and substrate concentration synchronously to enhance lactic acid production in fed-batch culture. As the linear relationship between the consumption amounts of alkali and that of substrate was concluded during lactic acid fermentation, the alkali and substrate in the feeding broth were mixed together proportionally. Thus, the concentration of substrate could be controlled through the adjustment of pH automatically. In the fed-batch lactic acid fermentation with *Lactobacillus lactis*-11 by this method, the residual glucose concentration in fermentation broth was controlled between 4.1 and 4.9 g L^{-1}, and the highest concentration of lactic acid, maximum cell dry weight, volumetric productivity of lactic acid, and yield were 96.3 g L^{-1}, 4.7 g L^{-1}, 1.9 g L^{-1} h^{-1}, and 0.99 g lactic acid per gram of glucose, respectively, compared to 82.7 g L^{-1}, 3.31 g L^{-1}, 1.7 g L^{-1} h^{-1}, and 0.92 g lactic acid per gram of glucose in batch culture. This feeding method was simple and easily operated and could be feasible for industrial lactic acid production in the future.

Keywords Lactic acid · Fed-batch · *Lactobacillus lactis*-11 · Ammonium hydroxide · pH feedback

Introduction

Lactic acid is a widely used industrial product in numerous fields such as food preservation, pharmaceutical, leather, and textile. Poly-lactic acid is also a promising environment-

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friendly feedstock for biodegradable plastics [1, 2]. So far, the lactic acid produced was mainly neutralized by calcium carbonate, by which excessive calcium sulfate is a serious challenge to the environment, which could be solved by substitution of calcium carbonate with ammonium hydroxide. Neutralization of lactic acid by ammonium hydroxide will result in ammonium lactate which is a nutrient that can be assimilated by rumen bacteria and therefore can be used as animal feed [3]. What is more is that ammonium lactate could be converted to lactic acid in other separation processes, such as ion exchange resin [4] and electrodialysis [5], which would not only change the lactic acid production process but also alleviate environment pressure caused by gypsum [6].

Lactic acid production would be depressed due to the inhibition caused by high glucose concentration [1]. To avoid this problem, different measures were taken by researchers. First, in batch cultures, enzymes were added into fermentation broth so that the glucose release from starch and conversion to lactic acid could proceed simultaneously [7–9]. Second, engineered lactic acid bacteria that can degrade starch to glucose were also used to achieve the same goal in batch experiments [10–14]. Last but not the least, fed-batch with different feeding methods was also a good option to solve this problem.

The commonly feeding methods are mainly constant, intermittent, and exponential feeding methods. It was found that when the concentration of residual glucose was controlled between 5 and 10 g L^{-1} through constant feeding strategy in fed-batch culture, the lactic acid production was improved remarkably [15]. Mu et al. [16] used intermittent phenylpyruvic acid feeding and traditional pH control method to improve 3-phenyllactic acid production yield effectively. To maintain the residual glucose concentration relatively stable, several feeding methods were investigated and lactic acid production was enhanced when exponential feeding method was employed [17]. There are also some other feeding methods proposed recently. Mass et al. [18] reported a novel process that the alkaline substrate was automatically added into fermentation broth to adjust the pH value during lactic acid production with lime-treated wheat straw. Based on pH increasing rate, Tsuge et al. [19] proposed a computer-controlled pH-stat substrate feeding method for poly-D-3-hydroxybutyrate production. However, in those studies, glucose concentration could not be controlled accurately because of the shortcomings of those feeding methods themselves. As the glucose concentration cannot be monitored online, its exact concentration was difficult to be controlled by simple feeding strategy in fed-batch fermentation.

We have proposed a feeding method based on pH in which the carbon source and alkali were mixed together. Hence, the concentration of carbon source could be controlled at an expected level through the adjustment of pH value, which had been applied in nisin production successfully in our laboratory [20, 21]. In this paper, the pH feedback-controlled feeding method was introduced to control the glucose concentration in lactic acid fermentation broth, and the advantage of this method was investigated to increase lactic acid production.

Materials and Methods

Microorganism and Media

Lactobacillus lactis-11 (provided by Shan Dong University, China), a lactic acid-producing strain, was grown on deMan Rogosa Sharpe (MRS) [22] medium. The inoculum was grown in MRS broth at 42 °C with shaking at 100 rpm for 12 h, and the inoculation size was 10% (*v*/*v*) for all experiments. The fermentation broth consisted of (per liter of distilled water): 5 g yeast, 10 g peptone, 10 g beef extract, 10 g NaCl, 5 g sodium acetate, 2 g triammonium

citrate, 0.4 g $MgSO_4$, 0.01 g $MnSO_4$, and with different glucose concentrations in different experiments.

Batch Culture

The batch cultures were carried out in a 5-L stirred fermentor (BaoXing, ShangHai, China). No air was introduced and 100 rpm agitating speed was employed to keep fermentation broth homogenous. The culture temperature and pH were set at 42±0.1 °C and 6.0±0.02, respectively. The pH was controlled by automatically adding 6 M ammonium hydroxide.

pH Feedback-Controlled Fed-Batch Culture

Except the substrate fed in the fed-batch culture, the other cultivation conditions were the same as the batch experiments. As the pH limits were set at 5.98 and 6.02, the peristaltic pump coupled to pH controller would be activated when the pH was below the lower limit. Therefore, the glucose and ammonium hydroxide could be added to the fermentation broth proportionally at the same time with one peristaltic pump.

Analytical Methods

The biomass concentration was expressed as cell dry weight, which was determined by measuring the optical density (OD) of broth at 620 nm. The optical density was proportional to cell dry weight. One OD unit corresponds to 0.53 g L^{-1} of biomass. Glucose was determined by SB-40C biosensor analyzer (Institute of biology, Shandong Province Academy of Sciences, P.R. China). Lactic acid was measured by HPLC [23].

Results and Discussion

Batch Culture

The changes of three parameters (cell dry weight, lactic acid, and glucose) were shown in Fig. 1 in the course of lactic acid batch fermentation with initial glucose concentration of

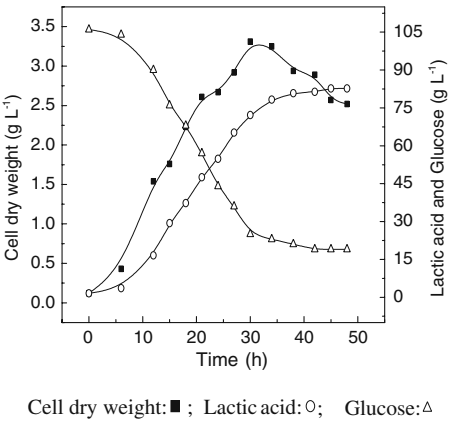


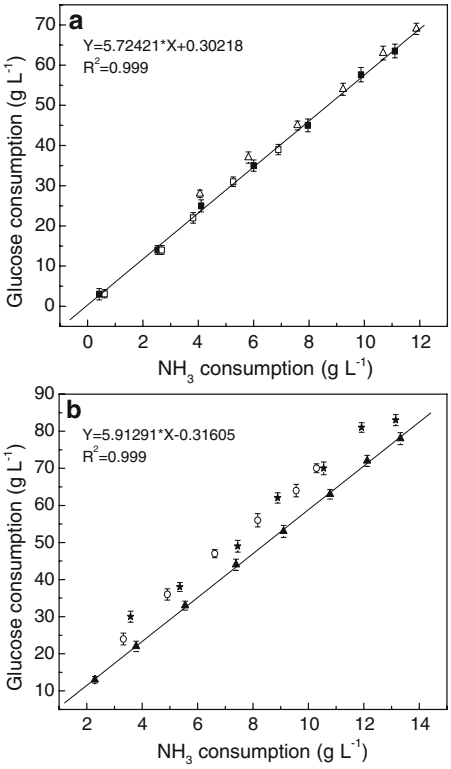
Fig. 1 Lactic acid production by *Lactobacillus lactis*-11 at controlled pH 6.0 under anaerobic condition in batch culture with initial glucose 106 g L^{-1}, T=42 °C. Cell dry weight (*square*); Lactic acid (*circle*); Glucose: (*triangle*)

106 g L^{-1} at controlled pH 6.0. The maximum cell dry weight was 3.31 g L^{-1} at 30 h; after that, the cell concentration decreased rapidly due to the inhibition caused by high concentration of residual glucose and lactic acid accumulation, which would be demonstrated further in the section of fed-batch culture. The lactic acid concentration increased with cell growth and reached a relative steady level after the fermentation for 30 h; moreover, the decrease of glucose became slow obviously. The volumetric productivity, maximum concentration of lactic acid, and yield were 1.7 g L^{-1} h^{-1}, 82.7 g L^{-1}, and 0.92 g lactic acid per gram of glucose, respectively.

Determination of Linear Relationship Between Glucose and Ammonium Hydroxide

Since glucose was mainly converted to lactic acid by homofermentative bacterium, the amount of alkali consumed for neutralization should be proportional to the amount of glucose converted to lactic acid [20]. Based on the relationship between the consumption amounts of alkali and glucose, the concentrated glucose and alkali (ammonium hydroxide) could be mixed together in fed-batch culture. The ratio of glucose to ammonium hydroxide could be calculated according to the batch experiments

As shown in Fig. 2a, the linear relationship between the consumption amounts of glucose and that of ammonium hydroxide was obtained in three batches of lactic acid fermentation with different initial glucose concentrations (38, 64, and 80 g L^{-1}). The slope



(a) 38 g L^{-1} :□; 64 g L^{-1}:■; 80 g L^{-1}:△;
(b) 89 g L^{-1}:▲; 106 g L^{-1}:★; 122 g L^{-1}:○

Fig. 2 Relationship between glucose and ammonium hydroxide consumption in lactic acid batch fermentation under anaerobic condition by *Lactobacillus lactis*-11 with initial glucose 38, 64, and 80 g L^{-1} (**a**) and 89, 106, and 122 g L^{-1}, respectively (**b**). **a** 38 g L^{-1} (*empty square*); 64 g L^{-1} (*filled square*); 80 g L^{-1} (empty triangle). **b** 89 g L^{-1} (*filled triangle*); 106 g L^{-1} (*star*); 122 g L^{-1} (*circle*). Each *point* represents the mean (n=3)±standard deviation

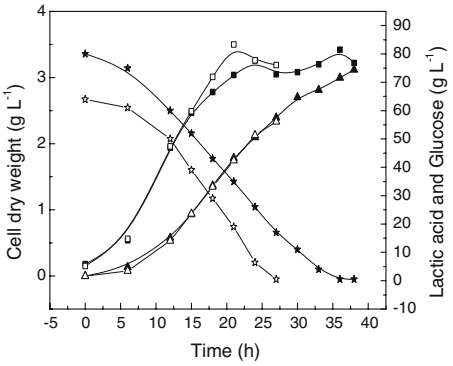
indicated the ratio of glucose to ammonium hydroxide (5.7 g g^{-1}, R^2=0.999), which would be used in the pH feedback-controlled fed-batch fermentation. It could be seen that the slope of the fitting line at initial glucose concentration of 80 g L^{-1} slightly deviated from the line obtained at the initial glucose concentrations of 64 and 38 g L^{-1}, which could be explained by the glucose inhibition. Moreover, this phenomenon was more obvious at a higher initial glucose concentration. As seen in Fig. 2b, when the initial glucose concentrations were 89, 106, and 122 g L^{-1}, the slopes increased to 5.9, 6.1, and 6.4, respectively. Hence, the slope would increase with the initial concentration of glucose when the initial glucose concentration was higher than 80 g L^{-1}.

Compared with Fig. 1, the results shown in Fig. 3 indicated that the inhibition due to glucose and lactic acid accumulation at the initial glucose concentrations of 64 and 80 g L^{-1} was not obvious. The corresponding volumetric productivity and maximum concentration of lactic acid were 2.1 and 1.96 g L^{-1} h^{-1} and 56.1 and 74.4 g L^{-1}, respectively. The concentration of lactic acid increased with biomass until glucose was used up and both the entire fermentation processes were similar. When the initial glucose concentration was lower than 80 g L^{-1}, the substrate inhibition was negligible. Therefore, the relationship between the consumptions of glucose and ammonium hydroxide based on the two batches of lactic acid fermentation could reflect the real situation in fed-batch culture without substrate inhibition.

pH Feedback-Controlled Fed-Batch Culture

During the whole process of lactic acid fermentation, the concentration of glucose should be controlled at low level [15] to alleviate the inhibition resulted from high concentration of substrate. In order to eliminate substrate inhibition, glucose was added into the fermentation broth in pH feedback-controlled manner. Feeding solution was the mixture of glucose and ammonium hydroxide. According to the ratio of glucose to ammonium hydroxide (5.7 g g^{-1}) from the batch cultures, the concentrations of glucose and ammonium hydroxide in feeding solution were 385 and 67 g L^{-1}, respectively.

Figure 4 showed the course of pH feedback-controlled fed-batch lactic acid fermentation. The initial glucose concentration was 38 g L^{-1} and feeding was started from 18.5 h and ended at 46 h. During the feeding course, the concentration of residual glucose was maintained in the range of 4.1–4.9 g L^{-1}, which demonstrated that the ratio of glucose



Cell dry weight: ▪(▫ 64 g L^{-1}); Lactic acid : ▲ (△ 64 g L^{-1}); Glucose: ★(☆ 64 g L^{-1})

Fig. 3 Lactic acid production by *Lactobacillus lactis*-11 at controlled pH 6.0 under anaerobic condition in batch culture with initial glucose 80 and 64 g L^{-1}, respectively, T=42 °C. *Filled square* Cell dry weight (*empty square*, 64 g L^{-1}); *filled triangle* Lactic acid (*empty triangle*, 64 g L^{-1}); *filled star* Glucose (*empty star*, 64 g L^{-1})

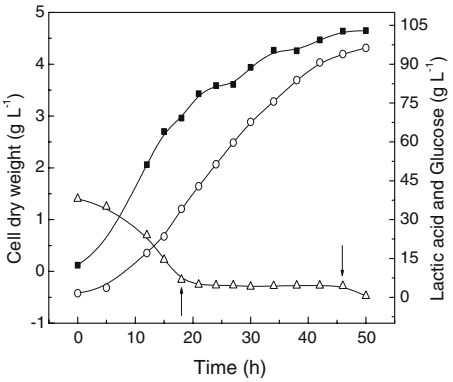


Fig. 4 pH-based feedback-controlled fed-batch lactic acid production by *Lactobacillus lactis*-11 under anaerobic condition with initial glucose 38 g L^{-1}, T= 42 °C. The *up arrow* indicates the start (18.5 h) of glucose addition and *down arrow* stands for the end (46 h) of glucose feeding. Cell dry weight (*square*); Glucose: (*triangle*); Lactic acid (*circle*)

to ammonium hydroxide in the feeding solution was appropriate to keep the glucose concentration at a relative steady level in the fermentation broth. Before 20 h, the cell growth increased rapidly; after that, the biomass growth slowed and reached a steady state at the end of fermentation. Unlike Fig. 1, the lactic acid production rate did not decrease until 40 h. The final concentration of lactic acid, maximum cell dry weight, lactic acid volumetric productivity, and yield were 96.3 g L^{-1}, 4.7 g L^{-1}, 1.9 g L^{-1} h^{-1}, and 0.99 g lactic acid per gram of glucose, respectively, whereas in the case of batch culture with initial glucose concentration of 106 g L^{-1}, the corresponding results were 82.7 g L^{-1}, 3.31 g L^{-1}, 1.7 g L^{-1} h^{-1}, and 0.92 g lactic acid per gram of glucose, respectively (shown in Fig. 1).

The effect of residual glucose concentration on lactic acid production was also investigated. When the residual glucose concentration was around 5 g L^{-1}, the maximum lactic acid production was obtained. When the residual glucose concentration was above 10 g L^{-1}, the inhibition of residual glucose would be observed and lactic acid production would decrease obviously, which was consistent with the results reported [15]. This conclusion could also be drawn from the comparison between Figs. 1 and 4. The lactic acid concentration in Fig. 1 was lower than that in Fig. 4 after 30 h, whereas the residual glucose concentration in Fig. 1 (25–19 g L^{-1}) was higher than that in Fig. 4 (4.1–0.5 g L^{-1}). Accordingly, instead of the lactic acid accumulation, the high concentration of residual glucose was the major factor that resulted in the decrease of cell concentration and final lactic acid production. Therefore, lactic acid production could be enhanced in pH feedback-controlled fed-batch fermentation.

Comparison of Different Feeding Methods

Compared with other feeding methods, the main advantage of pH feedback-controlled feeding method was in controlling residual glucose concentration in an expected range which was useful for lactic acid production. Based on pH increasing rate, Tsuge et al. [19] proposed a computer-controlled pH-stat substrate feeding method. When the pH increase rate decreased to 5% of the maximum value, the substrate would be added into the fermentation broth by feeding pump. However, this would result in the pH value varying between 7.0 and 8.3, which was unfavorable for some bacteria sensitive to pH environment. Mass et al. [18] reported a novel process using alkaline substrate to adjust the pH value

during lactic acid fermentation. This method was aimed to reuse the part of lime that used to pretreat the wheat straw. The concentration of substrate cannot be maintained at an expected level by this method.

Although constant rate feeding method was commonly used in various fed-batch fermentations, with the fermentation proceeding, the physiology status of biomass was changing and the substrate consumption rate would also alter with it. Therefore, this method was hard to fit for the whole fermentation process and keep the residual glucose concentration at a relative steady level [24]. Exponential feeding mathematical model was concluded from many experiments. As microorganism growth process was complex, a little change of cell conditions would lead to a significant deviation of residual glucose concentration from the expected situation [17]. In contrast, pH feedback-controlled feeding method was drawn from the relationship between glucose and ammonium hydroxide consumption, which would not be affected by the growth changes of biomass. Hence, the concentration of glucose and pH of the fermentation broth could be controlled synchronously at an expected level by this method during the whole lactic acid fermentation process.

Conclusions

During the process of lactic acid fermentation, the linear relationship between the consumption amounts of glucose and ammonium hydroxide was obtained, which was the theory basis of pH feedback-controlled feeding method. By using this method, the residual glucose in lactic acid fermentation broth could be controlled at an expected level accurately; therefore, the substrate inhibition could be eliminated. Compared with other reported lactic acid hyper-producing bacterium, the final concentration of lactic acid was not outstanding because of the disadvantage of the *Lactobacillus lactis*-11, but this feeding method, which was simple and easily operated, could benefit for lactic acid production and may be applied for other organic acid fermentations. It would be feasible for industrial lactic acid production to be employed in the future.

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