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STRATEGIES TO INCREASE THE CELL CONCENTRATION OF THE LACTIC ACID BACTERIUM *Pediococcus acidilactici* IN BATCH FERMENTATION

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ABSTRACT

As additives to silage, lactic acid bacteria have been produced for commercial purposes. One of the main challenges is to have a high cell concentrated product during large-scale production of bacterial cells, and the fermentation is a critical step in this process. The objective of this study was first to evaluate the initial cell concentration levels, initial pH and concentration of glucose in de Man-Rogosa-Sharpe (MRS) medium on the growth of a strain of *Pediococcus acidilactici* in microplates. Subsequent experiments analyzed the application of two strategies, feeding medium with pH adjustment or cell recycling after three batch cycles in the number of cells of *P. acidilactici*, under optimized conditions, in shaken-flask scale. The growth of *P. acidilactici* was affected by the initial cell concentration and initial pH rather than the initial glucose concentration in MRS broth. The increase in *P. acidilactici* number using the recycling strategy averaged 2 log cycles relative to the initial cell concentration and was more efficient than the feeding strategy. The conditions for the bacterial cell production was MRS broth with 10 g/L glucose, initial pH 7.0, initial cell concentration 10^8 CFU/mL, shaking 200 rpm, at 35°C.

Keywords: Lactic acid bacteria. Growth optimization. Cell recycle.

1 INTRODUCTION

The demand for lactic acid produced by lactic acid bacteria (LAB) in fermentative process is increasing especially due to the numerous applications of this compound in food, chemical, agricultural, and cosmetic industries. In silage the LAB provide acidity to the environment and prevent the proliferation of mainly mycotoxigenic fungi. Biological additives have been developed and used as silage preservatives, once they increase the organic acid production with fast decrease in the silage pH.[1]. *Pediococcus acidilactici*, characterized as Gram-positive, non-catalase producing, facultative anaerobic, homofermentative, non-motile and non-sporulating bacterium[2], is widely used in fermentation of silage.[3,4]. Additionally, *P. acidilactici* is able to produce pediocin, with high inhibitory effect on pathogenic microorganisms in the intestinal tract, and is considered safe as a probiotic.[5].

A high cell concentrated bacterial product must be obtained for commercial purposes to achieve efficiency in silage fermentation. However, the fermentative process that employs LAB may have end-product inhibition (by lactic acid) reducing the cell growth rate and the metabolite production. An anion-exchange resin added to the fermentation medium increased the growth of *P. acidilactici* because the concentration of lactic acid was reduced.[6]. The conditions in which fermentation takes place may reduce the substrate-level inhibition caused by lactic acid produced by this bacterium.[3]. The objective of this study was first to evaluate the initial cell concentration levels, initial pH and concentration of glucose in de Man-Rogosa-Sharpe (MRS) medium on the growth of a strain of *P. acidilactici*. Subsequent experiments analyzed the application of two strategies, feeding medium with pH adjustment or cell recycling after three batch cycles in the number of cells of *P. acidilactici*, under optimized conditions.

2 MATERIAL & METHODS

A strain of *P. acidilactici* ATCC8042 (CCT0585) was utilized in the experiments. The strain was maintained in MRS Agar slants at 4°C and frequently transferred to fresh medium for the assays.

First experiment: The bacterium was grown in MRS broth overnight at 35°C and the optical density was adjusted to ~0.8 (equivalent to ~10^9 CFU/mL). The assays were performed in Corning ® Costar 96-well sterile polystyrene plates with transparent flat bottoms. Each well contained 180 µL of sterile MRS medium supplemented either with 10 g/L or 20 g/L of glucose, initial pH values of 5.0, 6.0 and 7.0, and three initial cell concentration levels: 10^6, 10^7, and 10^8 CFU/mL of *P. acidilactici* (20 µL of inoculum volume), in a full factorial design. The assays were carried out in triplicates. The microplate was incubated at 35°C with shaking, for 24 h. The growth of the culture was monitored by measuring the absorbance at 600 nm every 15 min, using a microplate reader (Tecan Infinite M200, Mannedorf, Switzerland).

Second experiment: Firstly, the bacterium was grown in 2 L-Erlenmeyer flasks containing 1 L of MRS broth (10 g/L glucose), initial pH 7.0, initial cell concentration 1-3 x 10^8 CFU/mL, at 35°C, shaking 200 rpm. Samples were taken each 1 h to determine the optical density at 600 nm. Subsequently, two strategies were utilized: 1) Feeding: after an initial growth phase during 7 h in a Erlenmeyer containing 0.9 L of MRS broth, as described above, the pH was adjusted to approximately 7.0 with NaOH, and a volume of 100 mL of a sterile nutritive solution consisting of 100 g/L glucose and 100 g/L bacteriological peptone (pH 7.0), was added to the flask. Samples were taken each 1 h until stabilization in the values. 2) Recycling: after an initial growth phase during 7 h in an Erlenmeyer containing 1 L of MRS broth (1st cycle), as described above, the fermented broth was centrifuged at

8,000 rpm for 10 minutes, at 4°C. The cell mass was resuspended in fresh MRS broth and incubated in the same conditions (2nd cycle). Samples were taken each 1 h until stabilization in the values. The procedure was repeated once again (3rd cycle) and Samples were taken each 1 h until stabilization in the values. All experiments were conducted in duplicates. The samples taken during the cultivation time or at the end of each strategy phase were plated onto MRS Agar. After centrifugation of the samples, the total reducing sugar concentration and pH were determined by the 3,5-dinitrosalycilic acid[7], and in a digital pH-meter, respectively.

3 RESULTS & DISCUSSION

The results regarding the growth of *P. acidilactici* in microplate under varying initial cell concentration, initial pH and glucose concentration showed that the initial pH had an effect on growth, with increased optical density values and growth rate at initial pH 7.0 compared to pH 6.0 or 5.0, regardless the glucose or initial cell concentrations. The maximal specific growth rate and maximal growth of *P. acidilactici* were achieved at pH 6.5 in MRS broth.[8]. With initial cell concentration of 10^8 CFU/mL, increased optical density values and growth rate were observed regardless the glucose concentration or initial pH. No substantial effect was observed in the optical density and growth rate when the concentrations of 10 and 20 g/L of glucose were compared, in all initial pH and cell concentration values (Figure 1a to 1f). Othman et al.[3] did not observe increase in the number of *P. acidilactici* with the increment of glucose concentration from 10 to 20 g/L. The results here indicate that the initial cell concentration and initial pH of the medium are the most relevant parameters to be considered in the growth of *P. acidilactici*. For the second set of experiments, initial pH 7.0, initial cell concentration 10^8 CFU/mL and 10 g/L glucose in MRS broth were chosen to verify the effects of two strategies to enhance the growth of this LAB in flask-scale.
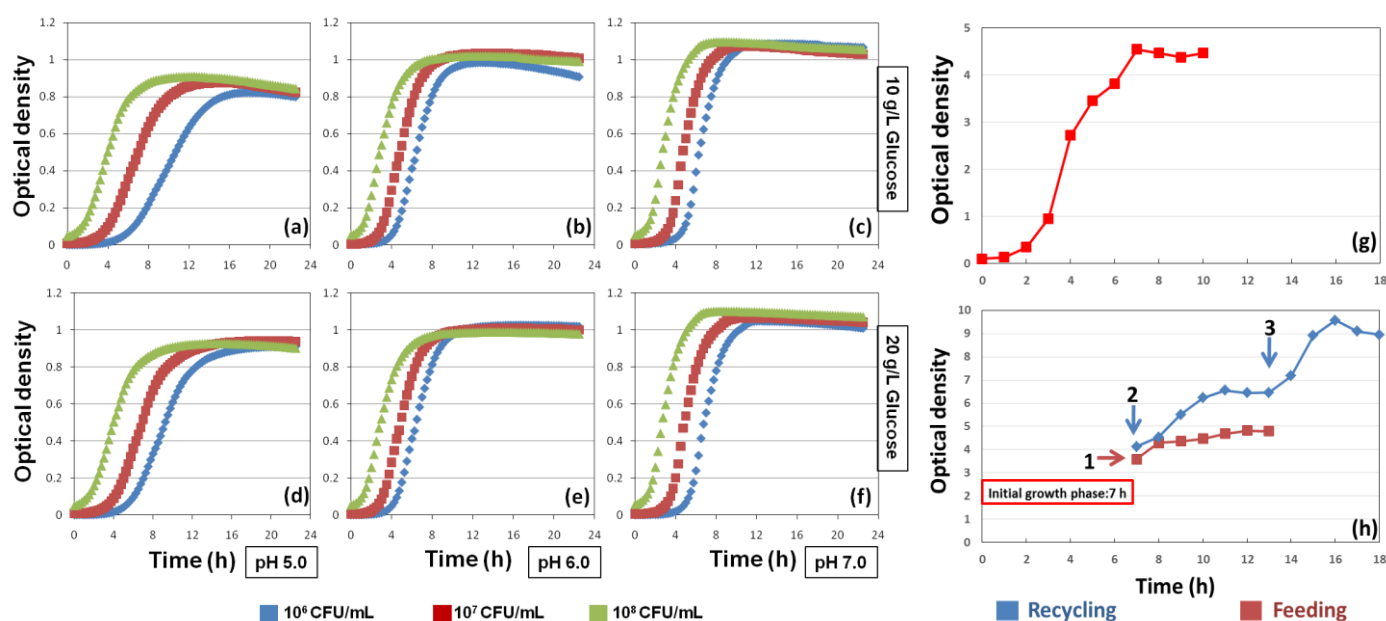

Figure 1 Growth of *P. acidilactici* em MRS broth, at 35°C, 200 rpm. (a) to (f): growth in microplates with MRS containing 10 or 20 g/L glucose, at initial pH of 5.0, 6.0 and 7.0, and initial cell concentration of 10^6, 10^7 and 10^8 CFU/mL. (g) and (h): growth in Erlenmeyer flasks with MRS broth (10 g/L), initial pH 7.0, initial cell concentration 10^8 CFU/mL. In (h), after a initial growth phase during 7 h, the culture medium had the pH adjusted to 7.0 and a nutritive solution consisting of glucose and peptone was added (1, feeding), or the culture medium was centrifuged and the cell mass was added to fresh MRS broth, in two consecutive cycles (2, cycle 2; 3, cycle 3, recycling).

As in Figure 1g, the bacterium reached the growth stationary phase at 7 hours with optical density around 4.5. When the culture had the pH adjusted to 7.0 and was fed with a nutritive solution to reconstitute the initial glucose and peptone concentrations after 7 h, the increase in growth was not significant (final optical density 5.0 after 13 h), as demonstrated in Figure 1h (red line). However, with recycling strategy, i.e., the cell mass was recovered from the fermented broth and inoculated into fresh medium, the optical density increased from around 4.0 to 6.5 after 6 h (total time of 13 h), when a new cycle was initiated. After 3-5 h of growth (total time 16-18 h), the optical density oscillated between 9.0-9.5 (Figure 1h, blue line).

The addition of NaOH to control the medium pH did not reduce the end-product inhibition of *P. acidilactici* growth[3], as it was observed here in the feeding strategy, because it did not result in enhanced growth. When the fermented broth was replaced by fresh medium, in the recycling strategy, a higher bacterial count was observed (Table 1).

The consumption of sugar after 7 h, when growth was peaked, was 71%, with a low pH, and a final cell concentration of 7.3 x 10^9 CFU/mL. When the strategy of recycling is considered, a more remarkable increase in bacterial number is observed, and the cell concentration enhanced almost 2 log cycles (after the 3rd cycle) in relation to the initial cell concentration (Table 1).

In this work a batch fermentation was utilized to produce a concentrated suspension of *P. acidilactici*. Maybe a fed-batch system would result in higher bacterial cell numbers as demonstrated by Othman et al.[3], who showed numbers as high as 10^{14} CFU/mL after optimizing feeding rate, agitation speed, aeration, pH, and resin addition to MRS broth. In conclusion, the fermentation process is the critical factor in large-scale production of bacterial cells for commercial purposes. The strategies to enhance cell yield should combine adequate supply of nutrients while removing/extracting/neutralizing the end-product that impairs the cell production.

Table 1 Number of bacteria, total reducing sugars and pH in the fermented broth after each period of time in each strategy. The initial cell concentration was 1-3 x 10^8 CFU/mL, initial pH 7.0, 10 g/L glucose in MRS broth, at 35ºC, 200 rpm.

Time - Strategy	CFU/mL	Total reducing sugars (g/L)	pH
After 7 h	7.3 x 10^9	2.9	4.6
After 13 h - feeding	7.7 x 10^9	4.3	4.7
After 13 h – recycling (2nd cycle)	9.0 x 10^9	1.6	6.3
After 18 h – recycling (3rd cycle)	1.6 x 10^{10}	n.d.[1]	n.d.[1]

[1] n.d.= not determined

4 CONCLUSION

The growth of *P. acidilactici* was affected by the initial cell concentration and initial pH rather than the initial glucose concentration in MRS broth. The increase in *P. acidilactici* number using the recycling strategy averaged 2 log cycles relative to the initial cell concentration and was more efficient than the feeding strategy. The conditions for the bacterial cell production was MRS broth with 10 g/L glucose, initial pH 7.0, initial cell concentration 10^8 CFU/mL, shaking 200 rpm, at 35ºC.

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