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3-Phenyllactic acid production by substrate feeding and pH-control in fed-batch fermentation of *Lactobacillus* sp. SK007

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ABSTRACT

3-Phenyllactic acid (PLA), which is produced by some strains of lactic acid bacteria (LAB), is a known antimicrobial agent with a broad spectrum. Batch and fed-batch fermentation by the strain *Lactobacillus* sp. SK007 for PLA production have been reported. With batch fermentation without pH-control, PLA production yield was 2.42 g L^{-1}. When fed-batch fermentation by *Lactobacillus* sp. SK007 was conducted in 3 L initial volume with pH-control at 6.0 and intermittent feeding, which was developed after fermentation for 12 h and every 2 h with 120 mL 100 g L^{-1} PPA phenylpyruvic acid (PPA) and 50 mL 500 g L^{-1} glucose each time, PLA production yield reached 17.38 g L^{-1}. The final conversion ratio of PPA to PLA was 51.1%, and the PLA production rate was 0.241 g L^{-1} h^{-1}. This indicated that PPA was the ideal substrate for PLA fermentation production, and fed-batch fermentation with intermittent PPA feeding and pH-control was an effective approach to improve PLA production yield.

1. Introduction

3-Phenyllactic acid (PLA) is an organic acid produced by some strains of lactic acid bacteria (LAB), which is a novel antimicrobial agent with broad spectrum activity against bacteria and fungal pathogens (Schnürer and Magnusson, 2005; Lavermicocca et al., 2000). Schwenninger et al. (2008) reported PLA could be also produced by a dairy propionic acid bacteria (PAB) that was confirmed as one component of a protective antiyeast *Lactobacillus*/*Propionibacterium* co-culture. PLA has a broad inhibitory activity against both gram-positive and gram-negative bacteira, such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* O157:H7 (Ohhira et al., 2004; Dieuleveux et al., 1998; Dieuleveux and Gueguen, 1998). The inhibitory properties of PLA have also been demonstrated against yeasts (Schwenninger et al., 2008) and a wide range of mould species isolated from bakery products, flour and cereals, including some mycotoxigenic species, namely *Aspergillus ochraceus*, *Penicillium roqueforti*, *Penicillium citrinu* etc. (Lavermicocca et al., 2003). The ability of PLA to act as a fungicide provides new perspectives for the possibility of using this natural antimicrobial compound to control fungal contaminants and extend the shelflife of food and/or feedstuffs (Lavermicocca et al., 2003). Due to its broad inhibitory activity against a variety of food-borne microorganisms, PLA has interesting potential for practical application as an antimicrobial agent in the food industry. Dieuleveux and Gueguen (1998) reported that PLA had a bacteriostatic effect in UHT whole milk, which reduced the population by 4.5 log, to give fewer cells than in the control after 5 days of culture. Lavermicocca et al. (2003) reported that the fungal growth was delayed for 7 days in bread fermented with *Saccharomyces cerevisiae* and the PLA-producing LAB strain, *Lactobacillus plantarum* 21B.

Lavermicocca et al. (2000) reported the production of PLA from *L. plantarum* 21B, which was the first report showing the production of PLA by LAB. Findings from subsequent researches indicated that PLA could be produced by a wide range of LAB species (Ohhira et al., 2004; Li et al., 2007; Dal Bello et al., 2007; Coloretti et al., 2007; Vermeulen et al., 2006; Makras et al., 2006; Armaforte et al., 2006; Valerio et al., 2004; Magnusson et al., 2003; Ström et al., 2002, 2005), but its production was low with levels up to 94 mg L^{-1} when the LAB was cultured in DeMan-Rogosa-Sharpe (MRS) broth (Valerio et al., 2004; Ström et al., 2005). PLA has relatively high MIC values for antiyeast activity (50 to more than 500 mM at pH 4.0–6.0) and the MICs of PLA decreased with decreasing pH (Schwenninger et al., 2008).

It was shown that PLA was a by-product of phenylalanine metabolism in LAB, in which phenylalanine was transaminated to phenylpyruvic acid (PPA) and PPA further reduced to PLA. The transamination process of phenylalanine was the rate-limiting step (Vermeulen et al., 2006; Li et al., 2007). Vermeulen et al. (2006) reported that PLA yield increased from 5 to >30% upon the addition of α-ketoglutarate in *L. plantarum* TMW1.468, due to improving the phenylalanine transamination. Li et al. (2007) reported that PLA content increased 14-fold in *Lactobacillus* sp. SK007 fermentation,

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which reached 1.12 g L^{-1}, when PPA was used to replace phenylalanine as substrate. We optimized the medium components including PPA for PLA production by *Lactobacillus* sp. SK007 using response surface methodology, and the PLA fermentation yield increased to 2.30 g L^{-1} with reduced development time and overall costs (Mu et al., 2009). Using PPA as substrate became an effective approach to PLA production.

Batch fermentation by LAB has been widely studied to produce lactic acid (Xu et al., 2006; Ding and Tan, 2006; Korbekandi et al., 2007), mannitol (Racine and Saha, 2007), xylitol (Nyyssölä et al., 2005) and others. Their production yields could be increased in a large extent by optimal fed-batch fermentation. In this study, PLA was for the first time produced by fed-batch fermentation of *Lactobacillus* sp. SK007, with substrate feeding and pH-control.

2. Methods

2.1. Microorganism and culture media

The PLA-producing strain *Lactobacillus* sp. SK007 (GenBank accession number: DQ534529) was isolated from Chinese traditional pickles and maintained by our laboratory (Li et al., 2007).

The activation medium used for seed activation was DeMan-Rogosa-Sharpe (MRS) medium containing 10 g L^{-1} peptone, 10 g L^{-1} beef extract, 5 g L^{-1} yeast extract, 20 g L^{-1} glucose, 2 g L^{-1} K_2HPO_4, 2 g L^{-1} triammonium citrate, 5 g L^{-1} CH_3COONa, 1 ml L^{-1} Tween-80, 0.58 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.25 g L^{-1} $MnSO_4{\cdot}4H_2O$. The fermentation medium used for PLA production contained 30 g L^{-1} glucose, 5 g L^{-1} phenylpyruvic acid (PPA), 47 g L^{-1} corn steep liquor, 3 g L^{-1} K_2HPO_4, 3 g L^{-1} CH_3COONa, 30 g L^{-1} yeast powder and 3 ml L^{-1} Tween-80 (Mu et al., 2009). Both media were finally corrected to pH 6.20–6.40, and then sterilized at 121 °C for 15 min in an autoclave.

2.2. Reagents

PLA was purchased from Sigma (St. Louis, USA). PPA was obtained from National Engineering Research Center for Biochemistry, Nanjing University of Technology (Nanjing, China). HPLC grade methanol, the reagents for MRS and fermentation medium and other analytical grade chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.3. Preparation of seed culture

To prepare the inoculum for fermentation experiments, one glycerol stock vial was used to inoculate a 250 mL flask containing 200 mL activation medium. The seed activation culture was grown at 30 °C for 24 h. At least two generations of activation cultures were required before fermentation. This culture was used as the seed culture.

2.4. Batch and fed-batch fermentation of Lactobacillus sp. SK007

Batch and fed-batch fermentation were performed in a 5 L fermentor (New Brunswick Scientific (NBS) Co. Inc., USA). Three liters of sterilized fermentation medium was added to the fermentor, and 90 mL seed culture was inoculated. Agitation was applied at a low rate just sufficient to mix the pH-control reagent and feed but minimize mixing air into the medium (100 rpm). No purge air or other gas was applied. The fermentation temperature was adjusted to 30 °C, and the pH was adjusted to 6.0 with 10 mol L^{-1} NaOH, if necessary. During fed-batch fermentation, intermittent feeding scheme was conducted. The feed addition was performed by a peristaltic pump using the sterile and ready-to-use 100 g L^{-1} PPA and 500 g L^{-1} glucose solutions. The PPA and/or glucose were added intermittently at certain period. For the batch fermentation with only substrate feeding, intermittent PPA feeding was developed at fermentation time for 12, 20, and 28 h, with 90 mL 100 g L^{-1} PPA solution each time. For the batch fermentation with both substrate and nutrients, intermittent feeding was developed after fermentation for 12 h and every 2 h with 120 mL 100 g L^{-1} PPA and 50 mL 500 g L^{-1} glucose each time.

2.5. Analytical methods

Cell concentration was measured based on the optical density at 660 nm. To determine cell dry weight (cdw), cells of known optical density were pelleted from a 10 mL sample at 8000 × g for 10 min and washed twice with 10 mL distilled water under the same conditions. The resulting pellets were dried under vacuum at 105 °C to constant weight for 12 h. A conversion factor of 3.1 OD per g cdw was determined and used to estimate the cell mass from optical density at 660 nm. Glucose concentration was analyzed by 3,5-dinitro salicylic acid (DNS) method (Miller, 1959).

For PLA analysis, fermentation broth was centrifuged (10,000*g* for 15 min, 4 °C) and the supernatant was filtered (0.22 μm pore-size membrane) for further analysis. PLA was determined using HPLC equipped with an Agilent Zorbax SB-C_{18} column (4.6 mm × 150 mm, 5 μm). Linear gradient elution was used with methanol/0.05% trifluoroacetic acid (solvent A) and water/0.05% trifluoroacetic acid (solvent B) at 1 mL min^{-1} and A/B ratios of 10: 90, 100: 0, 100: 0, and 10: 90, with run times of 0, 20, 23, and 25 min, respectively. PLA was detected at 210 nm (Li et al., 2007).

3. Results and discussion

3.1. PLA production using batch fermentation

The batch fermentation was carried out using *Lactobacillus* sp. SK007. When *Lactobacillus* sp. SK007 was cultured in the fermentation medium containing 5 g L^{-1} PPA substrate without pH-control, the highest cell dry weight (2.82 g L^{-1}) and PLA yield (2.42 g L^{-1}) were achieved after inoculation and culture for 24 and 32 h, respectively, as shown in Fig. 1, and the final conversion ratio of PPA to PLA and the PLA productivity rate were 48.4% and 0.076 g L^{-1} h^{-1}, respectively. The pH value kept reducing until it

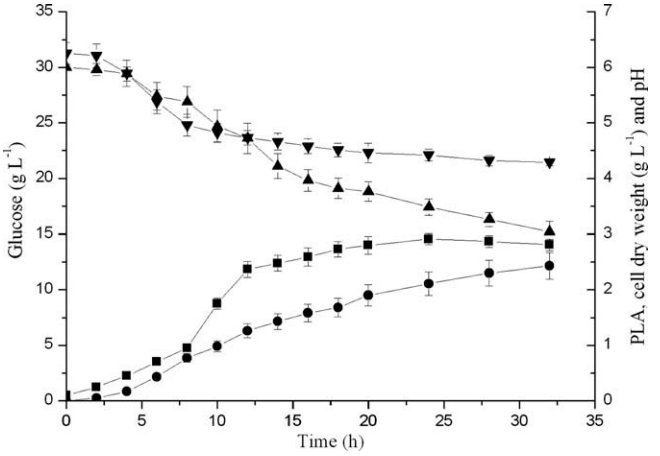

Fig. 1. Time course of PLA production by *Lactobacillus* sp. SK007 in batch fermentation at 30 °C, without pH-control. Symbols: (▲), glucose concentration; (■), cell dry weight; (●), PLA concentration; (▼), pH. Each point represents the mean (n = 3) ± standard deviation.