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Online recovery of nisin during fermentation coupling with foam fractionation

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ABSTRACT

Online foam fractionation was proposed for recovering nisin during the fermentation of *Lactococcus lactis* subsp. *Lactis* ATCC 11454. Two strategies of fermentation coupling with foam fractionation were designed. In each strategy, sterile air and nitrogen were individually bubbled into the broth. The results showed that the activity of nisin in both continued to increase after the completion of the foam fractionation process. The maximum total nisin activity (4657 IU/mL) was achieved when online foam fractionation was carried out using sterile air of 30 mL/min between 6 h and 12 h, which was improved by 36.2% compared with that of the traditional batch fermentation (3420 IU/mL) without online separation. This improvement is mainly attributed to the relief of product inhibition through online recovery of nisin, which demonstrates a promising process of industrial nisin production.

1. Introduction

Nisin is an antimicrobial peptide produced by some strains of *Lactococcus lactis* (Cao et al., 2007; Trmčić et al., 2011). It has strong antibacterial activity against a wide range of Gram-positive bacteria (Lv et al., 2004), with characteristics of non-pollution, easily decomposition, and negligible toxicity for humans (Delves-Broughton et al., 1996). In the view of these advantages, nisin has been approved by the World Health Organization as a food preservative for the use in food industry (Delves-Broughton et al., 1996; Sonomoto et al., 2000). Commercial nisin is mainly produced by liquid fermentation. However the high cost compared with other chemical preservatives during the process has limited the commercial utilization of nisin in food industry. Therefore, it is necessary to improve the nisin production using a relatively cheap separation method.

Foam fractionation is a promising method for recovery and concentration of surface-active biological compounds because of its low cost, high effectiveness, environmental friendliness, and simple operation (Burghoff, 2012; Wang et al., 2012; Yan et al., 2012; Lu et al., 2013). What's more, it has already been successfully applied in the waste treatment processes (Li et al., 2012; Liu et al., 2013; Wang et al., 2013; Li et al., 2014), the producing of many biosurfactants, such as surfactin and cellulose (Davis et al., 2001; Chen et al., 2006; Zhang et al., 2007, 2011), and also the strategy for online recovery natural products (Taura et al., 2013). Nisin has certain surface activity due to the marked hydrophobic nature of nisin as an antimicrobial peptide (Liu et al., 2010), which therefore can be online recovered by foam fractionation during fermentation. However, different nisin producing strains have different tolerance to oxygen (Amiali et al., 1998; Desjardins et al., 2001). Hence, in order to exam the effects of oxygen on the producing strains used in this study, it is necessary to consider that which gas (sterile air or nitrogen) should be used in foam fractionation process. Before this study, it has been tested that there is only little detrimental to the *L. lactis* growth and nisin production at the gas flow used in this paper. So the flowing research was carried out on the premise of this fact.

In this paper, foam fractionation was used as a method for *in situ* recovery of nisin from the fermentation broth, and the objective was to improve nisin production by decreasing nisin inhibition. Firstly, the batch fermentation without online recovery (control group) was carried out. Under the same conditions, two strategies of fermentation coupling with foam fractionation experiments were performed as follows: in the first strategy, a gas was bubbled into the fermentation broth at 60 mL/min between 8 h and 11 h during fermentation, while in the second strategy, a gas was bubbled into the fermentation broth at 30 mL/min between 6 h and 12 h during fermentation. In each strategy, air and nitrogen as the experimental gas were individually bubbled into the broth for comparison. Finally, the effects of online recovery on cell growth and nisin activity were evaluated.

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2. Materials and methods

2.1. Microorganisms and medium

The nisin-producing strain used in this study was *L. lactis* subsp. *Lactis* (ATCC 11454), which was grown on medium containing 40 g of sucrose, 10 g of KH_2PO_4, 15 g of peptone, 15 g of yeast extract, 2 g of NaCl, 0.2 g of $MgSO_4{\cdot}7H_2O$ per liter of distilled water with initial pH of 7.6. The formula used for cultivating comes from Tianjin Kangyi Biotechnology Company. *Micrococcus luteus* (NCIB 8166), purchased from China General Microbiological Collection Center, was used as the indicator strain in the nisin bioactivity assay and grow on the SI medium (initial pH 7.2) containing 5 g of glucose, 2 g of Na_2HPO_4, 8 g of tryptone, 3 g of yeast extract powder, 5 g of NaCl, 5 mL of Tween 20, 10 g of agar (if necessary) per liter of distilled water. All media were autoclaved at 121 °C for 20 min and stored at 4 °C before use.

2.2. Analytical methods

Samples were taken from the fermenter every hour during fermentation. Biomass was estimated by optical density at 600 nm (OD_{600}). Obtained values were converted to the cell dry weight (CDW) according to a standard curve ($CDW = 6.7547 \times OD_{600} - 0.0247$).

Nisin activity was measured by a modified dual-dosage agar diffusion assay (Wu et al., 2009). Briefly, 23 μL suspension of the indicator strain, which was cultured overnight at temperature of 35 °C before use, was diluted in 6 mL of normal saline solution. The SI medium (initial pH of 7.2) containing agar was autoclaved at 121 °C for 20 min and cooled to 50 °C. The diluted indicator strain suspension was then thoroughly mixed with 27 mL of the sterilized SI medium containing agar and poured into sterile Petri plates to solidify for 2 h at room temperature. These plates were then inverted and stored at 4 °C until use. The inoculated plates were used within 12 h in order to ensure sterility.

During the measurement, a sample taken from the fermentation process was immediately adjusted to pH of 2 with concentrated HCl. Then the sample was heated for 5 min in boiling water bath before cooled to room temperature. The sample was then centrifuged at 2000*g*, 4 °C for 10 min, after that the supernatant was collected and stored at 4 °C until analyzed. A stock standard nisin solution (2000 IU/mL) was prepared by adding 0.01 g of commercial nisin (10^6 IU/g, Sigma Chemical) into 5 mL of 0.02 M HCl. Three 300-fold and 600-fold dilutions of the nisin supernatant, as well as the standard nisin solution, were prepared using 0.02 M HCl.

Eight holes were bored into each agar plate using a sterilized stainless steel borer (outer diameter = 7 mm) during the measurement of the nisin activity. In each agar plate, 138 μL of the 300-fold and the 600-fold standard nisin solutions were added into separated holes respectively. The rest of the holes were injected with aliquots of the samples. The plate was then pre-diffused at 4 °C for 1 h and incubated at 35 °C for another 24 h. Diameters of the inhibition zones were measured, and the nisin activity expressed as international unit (IU) per milliliter was calculated by the following equation:

$$lg\frac{C_x}{C_s} = \frac{(\Phi_{xh} + \Phi_{xl}) - (\Phi_{sh} + \Phi_{sl})}{(\Phi_{xh} + \Phi_{sh}) - (\Phi_{xl} + \Phi_{sl})} \cdot lg2 \quad (1)$$

where C_x is the activity of the sample in question (IU/mL) while C_s is that of the standard solution (2000 IU/mL), Φ_{xh} is the inhibition zone diameter generated by the 300-fold dilution of the sample (mm) while Φ_{xl} is that caused by the 600-fold dilution (mm). Similarly, Φ_{sh} is the inhibition zone diameter generated by the 300-fold standard nisin solution in the same plate (mm) while Φ_{sl} is that caused by the 600-fold dilution (mm). The measurement of each sample was performed in triplicate using three different plates.

2.3. Experimental design

Batch fermentation was conducted in a 1.2 L bioreactor with working volume of 600 mL at temperature of 30 °C. The fermentation broth was inoculated with 6% (v/v) of *L. lactis* which has been cultured for 12 h. The pH of fermentation broth was maintained at 6.0 adjusted by 6 mol/L NaOH when it decreased to 6.0 from the initial pH of 7.6. The agitation of 150 rpm was used to keep the broth homogeneous. Samples were withdrawn at regular intervals and the measurement of each sample was performed in triplicate.

The apparatus used for online recovery of nisin by foam fractionation during fermentation is presented in Fig. 1. A foaming column with a swollen ball, which has an internal diameter of 17 mm and a height of 18 cm, was integrated with the fermentation reactor. The swollen ball is in favor of drainage of ascending foam. The bioreactor is customized. It was made of glass, the height of which is 20 cm (without the swollen ball) or 40 cm (with the swollen ball). The inner diameter of the bioreactor is 9 cm. To generate foams, the sterile gas was supplied to the bottom of the reactor via compressed air pump when foam fractionation was applied to recover nisin in the fermentation broth. Foaming was pressed out of the bioreactor via the foam fractionation column due to the continuous aeration, which was then collected in a separate receptacle. The collected foam ultimately collapsed due to the addition of antifoam agent (polyether defoamer).

In order to make foam fractionation coupling with fermentation on nisin production more effective, two strategies were designed as follows:

Strategy 1: A gas was bubbled into the fermentation broth at 60 mL/min between 8 h and 11 h during fermentation.
Strategy 2: A gas was bubbled into the fermentation broth at 30 mL/min between 6 h and 12 h during fermentation.

In the two strategies, sterile air and nitrogen were used as experimental gas respectively.

Foam fractionation performance was measured in terms of enrichment (E) and recovery (R) in the end of fermentation, which were defined as follows:

$$E = \frac{C_f}{C_T} \quad (2)$$

$$C_T = \frac{C_f V_f + C_t V_t}{600} \quad (3)$$

$$R = \frac{C_f V_f}{C_f V_f + C_t V_t} \times 100\% \quad (4)$$

where C_f is the nisin activity in the foamate, C_t is the nisin activity of the remaining in the fermentation broth, V_f is the volume of foamate collected, V_t is the volume of remaining fermentation broth, and C_T is the total nisin activity.

The carryover of cells in the foam can be quantified by the cell enrichment defined by the equation below:

$$\text{Cell enrichment} = \frac{C_{f\ cell}}{C_{t\ cell}} \quad (5)$$

where $C_{f\ cell}$ is the concentration of cells in the foamate, $C_{t\ cell}$ is the concentration of cells in the remaining fermentation broth.