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Enhancement of lipopeptides production in a two-temperature-stage process under SSF conditions and its bioprocess in the fermenter

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HIGHLIGHTS

- Effects of various temperatures on lipopeptides production was studied in SSF.
- A two temperature stage process was developed.
- The lipopeptides yields were enhancced by 8.40% in flask and 13.11% in fermenter.
- 1000-Fold scale-up fermentation in fermenter was successfully achieved.

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ABSTRACT

A two-temperature-stage process was developed for the production of lipopeptides under SSF conditions. The effects of various temperatures, ranging from 25 to 40 °C, on the bacterial growth during the growth stage and on the production of lipopeptides during the productive stage were investigated. The optimum temperatures were found to be 30 °C for the growth of the strain and 37 °C for the biosynthesis of lipopeptides. The two-stage fermentation temperatures should be 30 °C in the initial 24 h and then 37 °C for the enhanced production of lipopeptides. The bioprocess results obtained in a 50 L fermenter verified the efficacy of this technique, which increased the yield of lipopeptides by 8.40% in flasks and by 13.11% in the fermenter, with a 4 h decrease of fermentation time in the fermenter. The 1000-fold scale-up of fermentation in a fermenter was successfully achieved.

1. Introduction

It was reported recently that soybean flour and rice straw can be utilized for the production of lipopeptides using solid-state fermentation (SSF) (Zhu et al., 2012). In that paper, the researchers evaluated the possibility of using agro-industrial byproducts as substrates for the production of lipopeptides, and optimized the culture conditions to maximize the productivity from SSF. Under the optimized conditions, the yield of lipopeptides reached 50.01 mg/gds. However, these results were obtained in flasks. The conditions in a scale-up bioreactor may be different from those in flasks (Banerjee et al., 2012; Kanda et al., 2010) and require investigation for industrial production.

The fermentation process can be significantly influenced by various physical and chemical parameters (Sharma and Arora, 2010), temperature is one of the most important factors because it can affect the kinetics of the process, which affects the duration and the rate of fermentation as well as the production of fermentative metabolites (Beltran et al., 2008). A higher fermentation temperature might be advisable for faster fermentation, but it might not be beneficial for the yield and quality of the fermentation products (Xu et al., 2010). Generally, a complete fermentation period for lipopeptide production consists of three major stages, i.e., the growth stage, in which microorganisms begin to use the substrates for growth; the productive stationary stage, in which the lipopeptides begin to accumulate rapidly (Wang et al., 2008), and the decline stage, in which both the cell numbers and secondary metabolites yields begin to decline. The optimal temperature for metabolite production may be different than that for cell growth (Raninger and Steiner, 2003; Wei et al., 2003). It has been suggested that changing the temperature may play an important role in the induction of cell synchrony thereby increasing productivity and decreasing the production time (Storms et al., 2012). Whereas previous studies on the fermentation of lipopeptides under SSF conditions have focused mainly on the use of different substrates and the optimization of the media compositions (Al-Ajlani et al., 2007; Mizumoto and Shoda, 2007), the effect of temperature on

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lipopeptides production by SSF in scale-up fermenters has not been investigated. Additionally, the method and the rates of stirring could play important roles in the production of metabolites during fermentation (Mussatto and Roberto, 2003; Zahar et al., 2002). The stirring method would also influence the yield, the oxygen transfer and the energy cost of bioreactors (Zhang et al., 2010); therefore studies of the stirring techniques are essential for scale-up fermentation and should be performed before testing other parameters in the fermenter.

The purpose of this work was to examine the effects of changing the temperature during the productive stage on lipopeptides production and to evaluate the possibility of scaling-up the two-temperature-stage process for SSF from flasks to fermenters. The optimum temperature for fermentation was assessed based on bacterial growth and lipopeptides production. Different stirring methods were investigated in scale-up fermentation to minimize the problems resulting when scaling-up. The soluble protein, soluble sugar and pH were determined to follow the consumption of the substrates and the metabolic course of the fermentation.

2. Methods

2.1. Microorganism

Bacillus amyloliquefaciens XZ-173 (GenBank accession number JF697198), isolated from healthy tomato rhizosphere soil and capable of producing significant amounts of lipopeptide biosurfactants, was used in this study. The strain was taken from −80 °C frozen stock and transferred onto Luria–Bertani (LB) agar plates for pre-culture before use as an inoculum for lipopeptides production under SSF conditions.

2.2. Preparation of the seed culture and solid substrate

A loopful of cells from a fresh LB plate were inoculated into a 250 ml flask containing 50 ml of liquid LB medium and agitated at 170 rpm at 30 °C. After 24 h of growth, the *B. amylolliquefaciens* XZ-173 in the LB medium was used for seed cultures. The agro-industrial byproducts of soybeans (pH 6.58, total C 52.89%, total N 7.63%, and C:N 6.95) and rice straw (pH 7.23, total C 29.35%, total N 0.98%, and C:N 30.35) obtained from a local farm were oven-dried (60 °C for 12 h) and subsequently milled, and the powders were sieved by passing through a 20-mesh screen before use in the solid substrates, which were redried (60 °C for 48 h) to yield the dry substrate.

2.3. Preparation of the bioreactor for SSF

An accumbent 50 L fermenter (Biotech-50SS, Baoxin Bio-Engineering Equipment Co., Ltd, China) was used in this study as the bioreactor for scale-up lipopeptides production using SSF. The fermenter system consisted of two major parts: the fermentation tank and the parameter controller. The solid medium was placed in the tank (diameter 35 cm, depth 60 cm) and mixed by rotating the entire vessel. Sterilization was conducted by running steam into the tank, and aseptic air was forced into the tank to cool the substrates and provide oxygen. The rotation speed, temperature and humidity were regulated by the controller, while the tank pressure and air flux were adjusted manually.

2.4. Solid-state fermentation of lipopeptides

2.4.1. Fermentation in flasks

For flask cultures, 5.58 g of soybean flour and 3.67 g of rice straw supplemented with 1.79% starch, 1.91% yeast extract and 1.0 ml of mineral solution (composition in g L^{-1}: KH_2PO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5; L-phenylalanine, 2×10^{-3}; $MnSO_4$, 5×10^{-3}; $CuSO_4$, 0.16×10^{-3}; and $FeSO_4$, 0.15×10^{-3}) were adjusted to an initial pH of 7.5 and a moisture content of 55% in a 250 ml flask, mixed thoroughly and autoclaved at 115 °C for 30 min. The cooled substrates were inoculated at a 10% inoculum level, mixed carefully under sterile conditions, and then incubated in a chamber with the relative humidity above 80%. In the initial growth stage, the effects of different temperatures (25, 28, 30, 33, 37, or 40 °C) on the bacterial growth were studied. After incubating for a few hours, the productive stage of fermentation began and the temperature was set at a different value (25, 28, 30, 33, 37, or 40 °C) to evaluate the effect of temperature on lipopeptides production.

2.4.2. Fermentation in the fermenter

To investigate the bioprocess of lipopeptides production on a large scale and to verify that the results obtained in flasks could be obtained in the fermenter, 5.58 kg of soybean flour and 3.67 kg of rice straw supplemented with 1.79% starch, 1.91% yeast extract and 1.0 L of mineral solution were adjusted to an initial pH of 7.5 and a moisture content of 55% in a 50 L fermenter, mixed thoroughly and autoclaved at 115 °C for 30 min. After cooling, the substrates were inoculated with 10% inoculum and incubated in the fermenter at the chosen temperature value with the humidity at 80%, the aeration at 0.4 vvm, and the tank pressure at 0.03 Mpa. Different stirring methods, such as continuously stirring at a slow rotational speed or stirring thoroughly for 5 min every 6, 12, 18 or 24 h were studied under isothermal conditions of 30 °C to determine the effect on lipopeptides production in scaled-up fermentation.

2.5. Microbial and productivity analyses

To determine the bacterial biomass during SSF, 5 g of the solid culture was placed in a 250 mL flask containing 45 mL of sterile distilled water. This was mixed thoroughly and shaken at 170 rpm for 20 min at room temperature. Then the mixture was serially diluted (10^{-7}, 10^{-8}, and 10^{-9}) and spread onto LB agar plates. After 24–36 h of incubation at 30 °C, the numbers of colonies were counted and expressed as CFU (colony forming units) per gram of dry substrates (gds) based on the oven-dried weight (CFU/gds).

The isolation and quantification of lipopeptides were performed according to the methods described by Das and Mukherjee (2007) with some modifications. The fermented substrates were mixed with distilled water (1:10, w/v) by stirring for 1 h at room temperature, and then the entire contents were centrifuged at 10,000 rpm for 10 min at 4 °C to remove the insoluble matter. Then 6 mol/L HCl was added to the cell-free supernatant to achieve a final pH of 2.0, and this solution was stored at 4 °C overnight to allow the crude lipopeptides to precipitate. The crude lipopeptides were recovered by centrifugation at 10,000 rpm for 20 min at 4 °C and extracted with dichloromethane. This solution was dried using a rotary vacuum evaporator. The residue was re-suspended in water and neutralized, then lyophilized to powder form. The recovered lipopeptides were expressed as the amount of lipopeptides obtained per gram of dry substrates (gds).

2.6. Chemical analyses

The pH measurements were performed according to the procedure of (Tian et al., 2012), with some modifications. Five grams of the fermented substrates were homogenized with 45 ml of deionized water by stirring for 2 h at room temperature. The mixture was centrifuged at 12,000 rpm for 10 min and filtered using