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Utilization of soybean derivatives on clavulanic acid production by *Streptomyces clavuligerus*

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Abstract

The influence of the type of soybean derivatives as nitrogen sources, as well as the simultaneous influence of the concentrations of nitrogen and carbon sources in the production of clavulanic acid (CA), by *Streptomyces clavuligerus*, were investigated. Firstly, two runs in shake flasks were performed utilizing soybean flour (SF) and soybean protein isolated (SPI) in the culture medium with concentration of 1.6 g L^{-1} total nitrogen (TN). The CA production in the culture medium with SF was much higher, about double the production obtained with SPI. SF was utilized in the additional experiments to study the quantitative influences of the concentrations of SF as nitrogen source and soybean oil (SO) as carbon source, on CA production. Six runs were performed in a bench scale bioreactor. The experiments had the following concentrations: TN (1.6, 2.4 and 3.2 g L^{-1}) and SO (16.0 and 23.0 g L^{-1}), respectively. The cellular growth, evaluated in terms of rheological parameter consistency index (K) of the broths, reached a maximum value (K_{max}) proportional to the initial concentration of total nitrogen, but K_{max} was not influenced by the initial concentration of soybean oil (C_{SO}). In general, it has been observed that the consistency index (K) decreased rapidly due to the cell fragmentation caused by high volumetric power input. In the range studied, CA production increased with the decrease of C_{SO} and the increase of C_{SF}. The batch cultivation, utilizing lipid as supplemental substrate, simulated a fed-batch cultivation, in which the glycerol feeding rate is defined by the lipid hydrolysis rate, and CA production was favored by a slower supply of glycerol and fatty acids. Maximum CA production of 906 mg L^{-1} was obtained with $C_{SF} = 40.0$ g L^{-1} and $C_{SO} = 16.0$ g L^{-1}, respectively. So far, this is the highest CA production ever found in the literature using wild strain of *S. clavuligerus* in batch cultivations. An empirical relationship was proposed to correlate maximum CA concentration with C_{SF} and C_{SO}. The correlation obtained fitted the experimental data well, allowing the prediction of CA production for different experimental conditions and further process optimization.

Keywords: Clavulanic acid; *Streptomyces clavuligerus*; Soybean flour; Soybean protein isolate; Soybean oil; Lipid

1. Introduction

Clavulanic acid (CA) is a β-lactam compound produced by *Streptomyces clavuligerus*. It is structurally related to penicillin and capable of inhibiting a wide variety of β-lactamases commonly found in microorganisms resistant to penicillin and cephalosporins [1]. *S. clavuligerus* produces at least 21 secondary metabolites, including several β-lactam antibiotics [2].

Defining the culture medium composition is a primary stage of fundamental importance in the development of fermentation bioprocesses. The culture medium should provide energy, carbon and nitrogen sources, and minerals for cellular growth and product biosynthesis. It should contain nutrients easily available in the market and, if possible, of low cost.

The literature reports several different culture media for CA production by *S. clavuligerus*. As nitrogen source for CA production, apart from works studying the effect of free amino acids on CA production [3–5], protein from soybean derivatives is extensively utilized in the fermentation process [6–13]. In these studies, soybean protein, in the form of extract or a commercialized protein isolate produced by the soybean industry, is used. In their work, Mayer and Deckwer [6] observed that when soybean flour is utilized in place of its extract, the CA production was higher. However, there are no reports in the literature comparing the use of soybean flour and soybean protein isolate in the CA production process. The amount of nitrogen in the culture medium should be sufficient to assure good cell growth; however, the presence of NH_4^+ ions in excess, product of protein catabolism, can inhibit the CA biosynthesis [3].

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In regards to energy and carbon sources, due to the inability of *S. clavuligerus* to utilize simple carbohydrates such as glucose [14], alternative substrates such as dextrin, starch, and mainly glycerol have been extensively utilized in this process [1,2,6,9,10,12,13]. Romero et al. [3] studied the role of glycerol in the biosynthesis of CA by *S. clavuligerus* suggesting that glycerol, converted in glyceraldehyde 3P, is a precursor of C-3 unit of CA molecule. Indeed, high concentrations of glycerol (2 at 3%, w/v) have been reported to suppress antibiotic production in *S. clavuligerus* [15]. Controlled levels of glycerol in the fermentation broth can be maintained in fed-batch cultivations with glycerol or complete medium feed, considered as a means of improving CA production [6,9,16]. An alternative means to maintain a certain level of glycerol for long periods is the use of lipids instead of glycerol, as suggested by Butterworth [17]. According to Large et al. [18], "lipids and oils are considered essential medium components in the antibiotic industry because they possess natural antifoam properties, are a cheaper alternative carbon source when compared with carbohydrates and may increase secondary metabolite titres". Several examples of the successful use of vegetable oils as carbon source for antibiotics production are found in the literature [19–22]. So far, very few works in the literature deal with the use of lipids in the CA production process by *S. clavuligerus*. Lee and Ho [23] utilized various carbon sources, including fatty acids and palm oil, and obtained the highest CA production (ca. 5 mg L^{-1}) with palm oil. Large et al. [7] reported CA production in relation to the viscosity of the medium. Their results suggest CA production of approximately 80 mg L^{-1}, with a culture medium containing starch and an unspecified vegetable oil (23 g L^{-1}). Maranesi et al. [11] evaluated CA production in shake-flask culture with media containing different types and concentrations of edible vegetable oils. The highest CA titre, 722 mg L^{-1} in 120 h, was obtained with a culture medium with soybean flour, as nitrogen source, containing glycerol (10 g L^{-1}) and soybean oil (23 g L^{-1}), as carbon and energy sources. Also, the substitution of corn and sunflower seeds for edible oils produced similarly good results in terms of CA titre and productivity.

As seen above, there are some works in the literature reporting the use of soybean derivatives, as nitrogen source, and lipids, as carbon source, in the CA production bioprocess. However, there are no works that evaluate simultaneously and quantitatively the influences of these nutrients on CA production in bioreactors. Therefore, the objective of the present work was to study of the influence of specific types of soybean derivatives (soybean flour and soybean protein isolate) as nitrogen sources, as well as the simultaneous influence of the concentrations of nitrogen and carbon sources (soybean oil) in CA production by *S. clavuligerus*, in a bench scale bioreactor.

2. Materials and methods

2.1. Microorganism

Vegetative cells of *S. clavuligerus* ATCC 27064 (5.0 g L^{-1} dry weight), stored in cryotubes (glycerol 10%, v/v) at −70 °C, were used throughout the present work.

2.2. Culture media

The seed medium used presented the following composition (in g L^{-1} distilled water): glycerol, 15.0; bacto peptone, 10.0; malt extract, 10.0; yeast extract, 1.0; K_2HPO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 0.75; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001; MOPS buffer, 21 (100 mM). The medium was adjusted to pH 6.8 with NaOH 5 M solution prior to being autoclaved at 121 °C for 15 min.

The medium used in the inoculum cultivation was equivalent in composition to the corresponding production culture medium, except for those in the bioreactor experiments from which the MOPS buffer was withdrawn since the pH was controlled automatically and silicone antifoam was added.

The production culture medium used in the present work was based on that proposed by Maranesi et al. [11], which presents the following composition (in g L^{-1} distilled water): glycerol, 10.0; soybean flour (SF), 20.0; soybean oil, 23.0; K_2HPO_4, 1.2; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001, pH 6.8. As the nitrogen present in the medium originates from the soybean flour (SF), which contains around 50% of protein, the concentration of total nitrogen in the medium (TN) was 1.6 g L^{-1}.

Firstly, the influence of the type of nitrogen source in the production culture medium was evaluated in two runs (E1-1 and E1-2) performed in orbital shaker. In experiment E1-1, the main culture medium presented the same composition as that proposed by Maranesi et al. [11]. In the experiment E1-2, soybean flour (SF) was substituted for soybean protein isolate (SPI), in order to maintain the same concentration of TN. Since the SPI contains around 92% of protein, its concentration was 10.9 g L^{-1}.

In the second stage of this work, the influence of the total nitrogen (TN) and soybean oil (SO) concentrations in the production of CA was examined. The experiments, performed in a bench scale fermentor, presented the following TN and SO concentrations (in g L^{-1}), respectively: E2-1 (1.6 and 16.0); E2-2 (2.4 and 16.0); E2-3 (3.2 and 16.0); E2-4 (1.6 and 23.0); E2-5 (2.4 and 23.0); E2-6 (3.2 and 23.0).

2.3. Culture conditions

The seed culture was obtained by adding the content of a cryotube (3.5 mL of cell suspension) to 50 mL seed medium in a 500 mL Erlenmeyer flask and incubated in a rotary shaker (New Brunswick Sci., model G-25) at 28 °C, 250 rpm, for 24 h. For the experiments in shaker, two Erlenmeyer flasks of 500 mL with 45 mL of inoculum medium were inoculated with 5 mL of seed culture and incubated in a rotary shaker at 28 °C and 250 rpm for 24 h. The culture obtained (100 mL) was inoculated into a 3 L flask containing 900 mL of production medium; then, 50 mL aliquots of this inoculated medium were transferred to each one of the 500 mL Erlenmeyer flasks. Cultivations were performed in an orbital shaker (New Brunswick Sci. Inc.) at 28 °C, 250 rpm, for 120–160 h, and one flask was removed every 12 h for the determination of cell growth, glycerol, lipid, and product concentrations. For the bench scale bioreactor cultivations, eight Erlenmeyer flasks of 500 mL with 45 mL inoculum medium were inoculated with 5 mL of the seed culture and incubated in a rotary shaker at 28 °C and 250 rpm for 24 h. The whole content of the flasks (400 mL) was transferred to the bioreactor; model Bioflo III (New Brunswick Sci.), with 3.6 L of production medium, making up 4 L of fermentation broth. All cultivations were conducted batchwise at 28 °C, 800 rpm and 0.5 vvm, and the pH was automatically controlled at 6.8 ± 0.1 by adding 2 M HCl or 2 M NaOH solution. Dissolved oxygen concentration was monitored by a sterilized galvanic electrode (Mettler-Toledo InPro6000 Series). Samples of 25 mL were withdrawn every 6 h, approximately. An aliquot was utilized to determine cell growth and another one was centrifuged at 3720 × *g* and 5 °C for 15 min to obtain a clear supernatant to determine total carbohydrates, glycerol, total lipids, and CA concentrations.

2.4. Analytical methods

On account of the complex nature of the media and hyphal morphology of the microorganisms, the cell growth was determined indirectly by measuring the broth rheological parameter *K* (consistency index) using a Brookfield concentric-cylinders rheometer. The carbohydrate content in the supernatants was estimated by phenol–sulfuric acid reaction [24].