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Optimisation of the glycerol-to-ornithine molar ratio in the feed medium for the continuous production of clavulanic acid by *Streptomyces clavuligerus*

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ABSTRACT

Amino acids are well metabolized by *Streptomyces clavuligerus* during the production of clavulanic acid using glycerol as main carbon and energy source. However, only a few amino acids such as arginine and ornithine are favorable for CA biosynthesis. The aim of this work was to optimize the glycerol:ornithine molar ratio in the feed medium containing only these compounds to maximize CA production in continuous cultivation. A minimum number of experiments were performed by means of a simple two-level full-factorial central composite design to investigate the combined effect of glycerol and ornithine feeding on the CA concentration during the intermittent and continuous process in shake-flasks. Statistical analysis of the experimental data using the response surface methodology showed that a glycerol-to-ornithine molar ratio of approximately 40:1 in the feed medium resulted in the highest CA concentration when fermentation was stopped. Under these optimized conditions, in bench-scale fermentor runs, the CA concentration reached more than double the concentration obtained in shake-flasks runs.

1. Introduction

Clavulanic acid (CA) is commercially produced by *S. clavuligerus* in media containing glycerol as the main carbon and energy source, and complex nitrogen sources such as soybean flour or soybean cake, which produces high CA yields. These complex nitrogen sources, however, usually also contain unknown substances that interfere with the microbial metabolism. Thus, amino acids and soluble components such as protein extracts and hydrolizates produce less viscous and more homogeneous broths, enhancing mass and heat transfer, facilitating the monitoring and control of process variables, and simplifying the separation and purification steps [1,2]. Although most amino acids are well metabolized by the microorganism, only some of them are favorable for CA biosynthesis. Such is the case of arginine and ornithine, precursors of the CA molecule [3]. However, Romero et al. [3,4] observed that supplementary arginine in the medium may lead to intracellular accumulation of glutamate, which negatively affects the production of CA. Ives and Bushell [5] fed a continuous culture with single amino acids belonging to the main carbon flux of the biosynthetic pathway of CA. These authors observed that the addition of leucine, isoleucine, serine or valine, but not arginine, increased the production rate. In another recent work, Bushell et al. [6] evaluated carbon fluxes, working with media limited by phosphate, nitrogen or carbon and using metabolic flux analysis techniques. The researchers found no significant correlations between reactions whose fluxes are linked directly to arginine. This finding is consistent with the work of Kirk et al. [7], suggesting that the biosynthesis of this amino acid is saturated in the CA production process under phosphate-limited conditions. Chen et al. [8] observed that feeding ornithine in fed-batch culture not only provided an adequate supply of arginine for CA production, but also inhibited the glycerol-using cephamycin biosynthesis. These results made it clear that ornithine, rather than arginine, effectively enhances CA production provided there is a sufficient amount of C3 precursor (glycerol). In this work, a simple two-level full-factorial central composite design [9,10] was applied to investigate the combined effects of glycerol and ornithine concentrations in feed medium on CA production in intermittent and continuous cultivation of *S. clavuligerus* in shake-flasks. Response surface methodology was used to determine statistically the optimum glycerol-to-ornithine molar ratio in order to obtain the maximum concentration of CA.

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Table 1
Range and levels of the independent variables (glycerol and ornithine concentrations, in the flask at the moment of each addition), in coded and original units, according to the two-level full-factorial central composite design, and CA concentration when fermentation was stopped (response variable), in mg/L.

Run	Variables				Response
	Coded units		Original units		CA (when fermentation was stopped) (mg/L)
	Glycerol (x_1)	Ornithine (x_2)	Glycerol (mM)	Ornithine (mM)	
1	−1	−1	70.7	0.62	249.3
2	+1	−1	103.3	0.62	174.0
3	−1	+1	70.7	3.62	261.3
4	+1	+1	103.3	3.62	212.3
5	$-\sqrt{2}$	0	64.1	2.12	228.7
6	$+\sqrt{2}$	0	109.8	2.12	236.0
7	0	$-\sqrt{2}$	87.0	0	214.4
8	0	$+\sqrt{2}$	87.0	4.24	189.0
9	0	0	87.0	2.12	339.5
10	0	0	87.0	2.12	311.1
11	0	0	87.0	2.12	350.3
12	0	0	87.0	2.12	332.5

2. Materials and methods

S. clavuligerus ATCC 27064 was stored in the form of vegetative cells (8.0 g L^{-1} dry weight) at −70 °C in cryotubes, utilizing glycerol 20% (w/v). Inoculum medium was composed of (g/L) glycerol (15.0), soybean protein isolate (Soytone) (15.5), yeast extract (1.0), malt extract (10.0), K_2HPO_4 (0.8), $MgSO_4{\cdot}7H_2O$ (0.75), 1.0 mL/L of salt solution containing: (in g/L) $MnCl_2{\cdot}4H_2O$ (1.0), $FeSO_4{\cdot}7H_2O$ (1.0) and $ZnSO_4{\cdot}7H_2O$ (1.0). The composition of the production medium was the same, excluding malt extract. The media were supplied with 21 g/L of the buffering agent 3-(N-morpholino) propanesulfonic acid (MOPS), and the initial pH was adjusted to 6.8.

Intermittent and continuous fermentation was carried out in 500 mL shake-flasks (250 rpm, 28 °C, 42 ± 1 mL working volume) and in a bench scale fermentor (News Brunswick Bioflo 2000, 5 L working volume) with automatic control of pH (6.8 ± 0.2), temperature (28 °C) and DO level (approximately 40% saturation by varying the agitation speed), at an aeration rate of 1 vvm. After 48 h of cultivation in both shake-flasks and fermentor, broth withdrawal and fresh medium feeding were done at 24 h and 12 h intervals, respectively, in the shake-flasks and the fermentor runs, maintaining a mean dilution rate of $D = 0.005\ h^{-1}$, constant volume. Feed medium was prepared with distilled (95%) and tap (5%) water to avoid mineral deficiency, and contained MOPS (21 g/L) and several combinations of glycerol and ornithine concentrations. The same medium and dilution rate were used in a 5 L bioreactor run. CA was determined by the method described by Bird et al. [11], cell mass was determined as dry weight, and glycerol by the method described by Lambert and Neish [12].

A two-level full-factorial central design with additional star points to set a second-order design (composite) [9,10] was applied to investigate the combined effects of glycerol and ornithine concentrations in feed medium on CA production. The concentration range of the independent variables – glycerol (x_1) and ornithine (x_2)

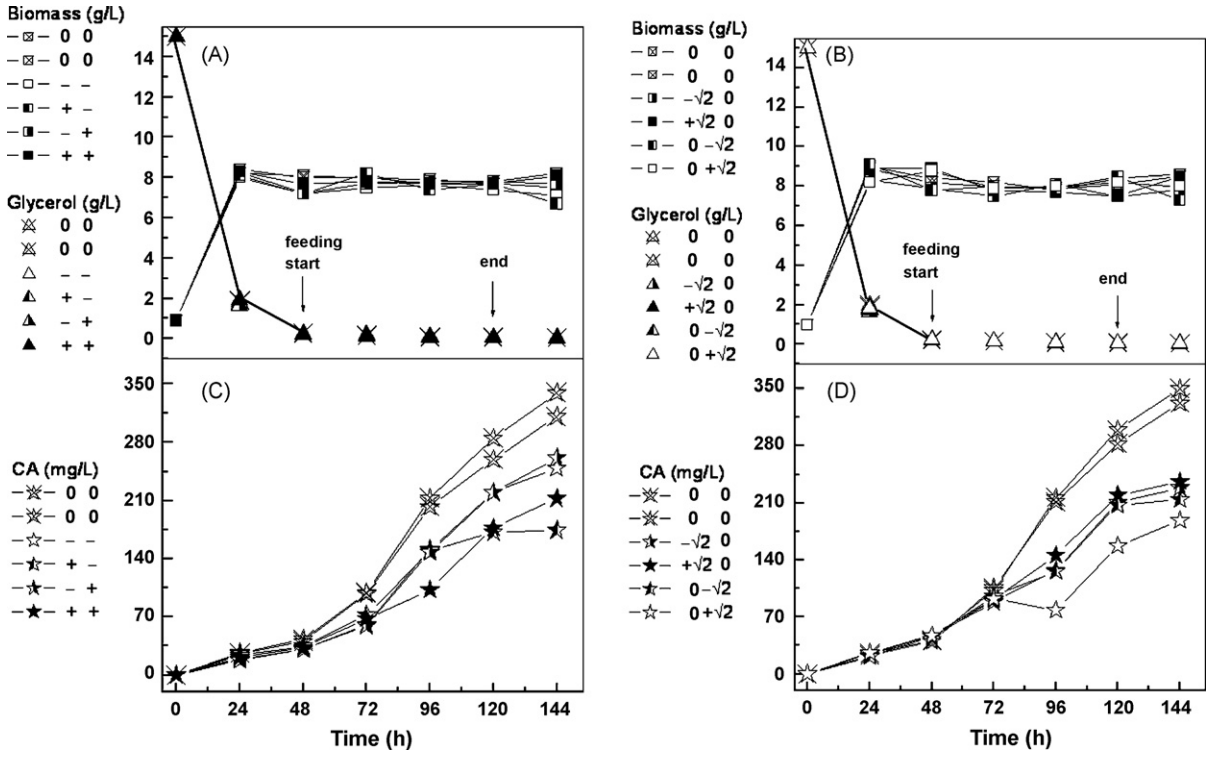

Fig. 1. Data from central factorial design with additional star points to set a second-order design (composite) – time course of biomass and glycerol (A and B), and CA concentration (C and D) during the intermittent and continuous process in shake-flasks.