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Optimization of nutritional requirements and feeding strategies for clavulanic acid production by *Streptomyces clavuligerus*

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Abstract

The present work reports the nutritional requirements and environmental conditions for submerged culture of *Streptomyces clavuligerus* for clavulanic acid production using orthogonal matrix method (Taguchi L_{16} design) and also fed-batch fermentation for clavulanic acid production by feeding glycerol, arginine and threonoine to the fermentation medium intermittently. Clavulanic acid production was increased by 18% with the span of feeding glycerol and reached a maximum at 1.30 mg/ml with 120 h glycerol feeding as compared to 1.10 mg/ml in the control. The production also increased with the span of feeding amino acids and reached a maximum of 1.31 and 1.86 mg/ml with feeding arginine and threonine, respectively in 120 h. There was an overall increase of 18% and 9% in clavulanic acid production with arginine and threonine feeding as compared to the respective controls (1.10 and 1.70 mg/ml, respectively).

Keywords: Clavulanic acid; Fed-batch fermentation; *Streptomyces clavuligerus*; Threonine; Arginine

1. Introduction

Specific nutritional requirements of microorganisms used in industrial fermentation processes are as complex and varied as the microorganisms in question. Besides the microbial type, the species and strains are very specific as to their requirements for biosynthesis and growth from their environment in a variety of ways. Many investigators have attempted to optimize submerged cultures for antibiotic production from different fungi such as erythromycin from *Saccharospora erythraea* (McDermott et al., 1993; Bhattactacharjee et al., 2002), cephamycin from *Streptomyces clavuligerus* (Park et al., 1994), and clavulanic acid from *S. clavuligerus* (Ives and Bushell, 1997). The productivity of microbial metabolites is closely related to the fermentation process used.

Medium optimization by one-factor-at-time method involves changing one variable (nutrients, pH, temperature, etc.) while fixing the others at a certain arbitrary levels (Xu et al., 2003; Survase et al., 2006). Since most industrial experiments usually involve a significant number of factors, a full factorial design results in a large number of experiments. To reduce the number of experiments to a practical level, only a small set from all the possibilities is selected. Taguchi design involves a special set of general design guidelines for factorial experiments that cover many applications (Xu et al., 2003).

Fed-batch fermentation is an approach aimed at efficiently carrying out fermentation for production of biomolecules. Industrial fermentation of most amino acids is accomplished with this method. Fed-batch culture has been used extensively to increase the productivity of microbial processes such as production of jenseniin G (Ekinci and Barefoot, 2006), ergosterol (Shang et al., 2006), poly-β-hydroxybutyrate (Patnaik, 2006), and chlorophyll (Rangel-Yagui et al., 2004). Ives and Bushell (1997) and Teodoro et al. (2006) applied this approach to clavulanic acid production in *S. clavuligerus*.

Glycerol is one of the best carbon sources for clavulanic acid fermentation (Baggaley et al., 1997; Ives and Bushell,

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1997; Elson and Oliver, 1978). In the absence of glycerol, *S. clavuligerus* does not produce clavulanic acid, but produces cephamycin C, another metabolite of the same organism. Elson and Oliver (1978) used labeled ^{13}C precursor and clearly demonstrated that glycerol fed in the fermentation medium was incorporated into the β-lactam ring of clavulanic acid. Townsend and Ho (1985) suggested L-glycerate to act as an intermediate between glycerol and clavulanic acid. Chen et al. (2002) reported that glycerol at 10–20 g/l increased clavulanic production by *S. clavuligerus* in shake flask cultures. The biosynthesis of clavulanic acid was prolonged with feeding glycerol and the production increased to 0.27 mg/ml as compared to 0.115 mg/ml without feeding. In fermenter batch culture, degradation of clavulanic acid began after 72 h. With glycerol feeding in fed-batch culture, clavulanic acid production was not only increased to about 0.280 mg/ml, but also remained stable up to 130 h. In fed-batch culture, glycerol feeding rather than ornithine feeding has been demonstrated to be the rate limiting for the clavulanic acid synthesis (Chen et al., 2003).

The biosynthesis of clavulanic has been well described (Townsend and Ho, 1985; Valentine et al., 1993 and Stirling and Elson, 1979). It is well documented that pyruvate acts as C3 precursor, whereas arginine acts as the C5 precursor for clavulanic acid production (Ives and Bushell, 1997). Radiolabeled feeding experiments have indicated arginine and ornithine to be efficiently incorporated into clavulanic acid structure (Townsend and Ho, 1985; Romero et al., 1986). Chen et al. (2003) reported no enhancement of clavulanic acid biosynthesis from the arginine feeding, and hence, it was not regarded as a rate-limiting substrate. Romero et al. (1986) reported ornithine to strongly inhibit cephamycin biosynthesis.

The objective of the present study was to study the nutritional requirements and environmental conditions for submerged culture of *S. clavuligerus* for clavulanic acid production using orthogonal matrix method and further study fed-batch fermentation for clavulanic acid production by feeding glycerol, arginine and threonoine to the fermentation medium intermittently.

2. Methods

2.1. Media components

All the media chemicals were purchased from Hi-Media, Mumbai. HPLC solvents were purchased from SD Fine Chemicals Ltd., Mumbai.

2.2. Microbial culture and maintenance

Strain of *S. clavuligerus* MTCC 1142 was procured from MTCC, Chandigarh, India and was maintained on a defined medium containing (%) 0.4 yeast extract, 1 malt extract, 0.4 glucose and 2 agar with a pH adjusted to 7.2 ± 0.2. The slants grown at 25 °C for 4 days were used for inoculation into a seed culture medium (2% glycerol, 1% bacteriological peptone, and 1% malt extract with pH adjusted to 7.0 ± 0.2). For the preliminary studies, 2% of seed culture grown for 48 h in an incubator shaker at 25 °C and 200 rpm was used for inoculation into the production medium.

2.3. Fermentation

The medium designed by Gouveia et al. (1999) was modified and used in the present study. It contained (g/l) 15 glycerol, 20 sucrose, 22.4 proline, 16.8 glutamic acid, 0.4 calcium chloride, 0.1 ferric chloride, 2 potassium dihydrogen phosphate, 5 sodium chloride, 0.1 manganese chloride, 0.05 zinc chloride and 1 magnesium sulphate with a pH adjusted to 7.0 ± 0.2. L16 orthogonal array (Table 1) was used to optimize the concentrations of the media components. The design for the L16-orthogonal array was

Table 1
L16 orthogonal array for clavulanic acid production by *Streptomyces clavuligerus* using MINITAB 13.3

S. no.	A	B	C	D	E	A	B	C	D	E	CA, mg/ml
1	1	1	1	1	1	0.375	0.500	0.560	0.420	0.05	0.114
2	1	2	2	2	2	0.375	1.000	1.120	0.840	0.1	0.373
3	1	3	3	3	3	0.375	2.000	2.240	1.680	0.2	0.317
4	1	4	4	4	4	0.375	3.000	3.360	2.520	0.3	0.354
5	2	1	2	3	4	0.750	0.500	1.120	1.680	0.3	0.328
6	2	2	1	4	3	0.750	1.000	0.560	2.520	0.2	0.530
7	2	3	4	1	2	0.750	2.000	3.360	0.420	0.1	0.306
8	2	4	3	2	1	0.750	3.000	2.240	0.840	0.05	0.290
9	3	1	3	4	2	1.500	0.500	2.240	2.520	0.1	0.328
10	3	2	4	3	1	1.500	1.000	3.360	1.680	0.05	0.269
11	3	3	1	2	4	1.500	2.000	0.560	0.840	0.3	0.274
12	3	4	2	1	3	1.500	3.000	1.120	0.420	0.2	0.218
13	4	1	4	2	3	2.250	0.500	3.360	0.840	0.2	0.218
14	4	2	3	1	4	2.250	1.000	2.240	0.420	0.3	0.226
15	4	3	2	4	1	2.250	2.000	1.120	2.520	0.05	0.463
16	4	4	1	3	2	2.250	3.000	0.560	1.680	0.1	0.475

Where, A is glycerol, B is sucrose, C is proline, D is glutamic acid and E is K_2HPO_4.