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ORIGINAL ARTICLE

Improvement of clavulanic acid production by *Streptomyces clavuligerus* with peanut derivatives

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Abstract Clavulanic acid (CA) production by *Streptomyces clavuligerus* was compared in fermentation media containing various nitrogen sources, including soybean meal, peptone, corn steep powder, cotton seed meal, ammonium sulfate, and ammonium nitrate, respectively. The basal fermentation medium was also supplemented with various concentrations of peanut derivatives. The highest CA concentration was obtained in medium containing soybean meal supplemented with peanut protein. The production of N^2-(2-carboxyethyl)-L-arginine (CEA) by a wild-type and a mutant strain of *Streptomyces clavuligerus* [in which the gene encoding β-lactam synthetase (*orf3*) was inactivated] in fermentation broth was compared. The assay of CA and CEA in the peanut protein-containing fermentation broth of the *orf3* blocked mutant of *S. clavuligerus* and the analysis of amino acids and fatty acids of peanut seed flour showed that peanut protein can provide the aspartate amino acids family, arginine, and glycerol for the production of CA. Based on these results, we conclude that peanut seed flour and its derivatives can be used for CA production on an industrial scale as a supplementary nitrogen and carbon source.

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Introduction

Clavulanic acid (CA) is a commercially important broad-spectrum β-lactamase inhibitor produced by *Streptomyces clavuligerus*. The wide clinical use of commercial formulations of CA, such as Augmentin (amoxicillin and CA) and Timentin (ticarcillin and CA) has made CA a product valued in excess of a billion dollars/annum (Saudagar et al. 2008) and created a powerful incentive to optimize the fermentation process of this compound.

The biosynthesis pathway of CA has only recently been elucidated. D-glyceraldehyde-3-phosphate (Khaleeli et al. 1999) and arginine (Valentine et al. 1993) are the precursors of CA production. The first step in the biosynthetic pathway is the condensation of two primary metabolites, D-glyceraldehyde 3-phosphate and L-arginine, to give N^2-(2-carboxyethyl)-L-arginine (CEA) (Caines et al. 2004). Romero et al (1986) observed that the addition of arginine to the fermentation medium of *S. clavuligerus* can lead to the intracellular accumulation of glutamate, which negatively affects the production of CA. Chen et al. (2003) observed that in the absence of glycerol, supplementation with ornithine or arginine alone did not enhance CA production, but the addition of ornithine and arginine (particularly ornithine) to a medium containing glycerol resulted in increased CA production. Garcia-Dominguesa et al. (2010) reported that the optimum molar ratio of glycerol to ornithine to reach the highest concentration of CA was 40:1.

Various carbon and nitrogen sources have been studied in the search for the optimal production protocol for CA

production. These studies have been reviewed by Saudagar et al. (2008). Peanut (*Arachis hypogaea* L.), with a 24% protein content, is a major source of plant protein in most tropical and subtropical regions of the world. Peanut is also one of the most important oil seed crop and has about a 50% oil content (Salunkhe et al. 1992). Peanut meal has been used as a major or auxiliary ingredient of fermentation medium to produce various antibiotics, including oxytetracycline (Yang 1996), fungichromin (Zang et al. 2010), rifamycin B (Venkateswarlu, et al. 2000), penicillin G, penicillin V, and cephalosporin (Schugerl and Seidel 1998). However, to data, little research has been done peanut in association with CA fermentation (Nabais and Cardoso 1995).

The major aim of the study reported here was to compare the effect of peanut seed and its fractions on the growth of *S. clavuligerus* and on CA production with that of other important industrial nitrogen sources, including soybean meal, peptone, corn steep powder, cotton seed meal, ammonium sulfate, and ammonium nitrate. In addition, we studied the effect of peanut protein on CEA production using *S. clavuligerus* RFL35, which is a mutant strain of *S. clavuligerus* in which the gene encoding β-lactamase enzyme (*orf3*) is inactivated, resulting in the complete loss of CA production and the accumulation of CEA in the fermentation broth (Bachmann et al. 1998).

Materials and methods

Bacterial strains, media, and culture methods

Streptomyces clavuligerus DSM738 (obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen) and *S. clavuligerus* RFL35 (a gift from Professor Townsend, Johns Hopkins University, Baltimore, MD, USA) were inoculated on sporulation medium (Shirling and Gottlieb 1966) [(g/l): glucose, 4; malt, 10; yeast extract, 4; $CaCO_3$, 2; agar, 20] and incubated at 28°C for 14 days. A 1-ml aliquot of spore suspension (approx. 10^7–10^8 spores/ml) was then inoculated into 1,000-ml Erlenmeyer flasks containing 250 ml of seed medium (Cole 1977) ([(g/l): peptone, 10 g; glycerol, 20 g; malt, 10; pH 7.0]. The flasks were incubated at 28°C on a rotary shaker at 220 rpm for 20–22 h. The seed culture was then inoculated into 250-ml Erlenmeyer flasks (5% seed culture, v/v) containing 50 ml fermentation medium and incubated at 28°C for 96 h at 220 rpm. The cultures of *S. clavuligerus* RFL35 and *S. clavuligerus* DSM738 in subsequent studies were grown in fermentation media. The composition of the basal fermentation medium was ([(g/l): dextrin, 20; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot 1H_2O$, 0.01]) (Taghavimehr 2008). The control fermentation medium (1) comprised basal fermentation medium supplemented with 5 g/l soybean meal, and control fermentation medium (2) was composed of control fermentation medium (1) plus 5 g/l glycerol. The pH of all fermentation media was adjusted to 6.8±0.1 with KOH.

Preparation of peanut seed fractions

Peanut seeds (*Arachis hypogea* L. cv. Astaneh) were milled carefully and used as peanut seed flour. The peanut seed flour was mixed well with chloroform:methanol:water (34:16:50, v/v/v). The solid phase was then separated and, after drying, was used as peanut meal. The liquid phases (organic and aqueous phases) were separated. After evaporation of the solvent of the organic phase under low pressure, the remaining organic phase was used as peanut oil (Hamilton and Hamilton 1992). Peanut protein was prepared by mixing peanut meal with an aqueous alkaline solution and removing the insoluble material by centrifugation. The pH of the supernatant was adjusted to 4.0±0.1. The precipitate was collected by centrifugation and used as peanut protein after drying (Desai 2000).

Peanut amino acids analysis

The amino acid composition and content of peanut were determined as described by Nemati et al. (2004). The sample was first subjected to complete acid hydrolysis to release their amino acid residues. Subsequent separation and determination of amino acids was achieved by reversed-phase high-performance liquid chromatography (HPLC) following pre-column derivatization. Orthophtaldialdehyde (OPA) was used as the derivatization reagent. The derivatized amino acids were analyzed using a Knauer HPLC system (Knauer, Berlin, Germany) consisting of a Knauer controller Quaternary pump, a Spark Triathlon autosampler, and a fluorescence detector (RF-10x1; Knauer) operating with Eurochrom 2000 software (ver. 1.2, 1992–1996; Knauer). Separations were achieved using a 250×4-mm, reversed-phase column (OPA special; Knauer). The mobile phase consisted of two components: eluent (A), methanol–sodium phosphate (pH 6.5, 12.5 mM) (10:90, v/v); eluent (B), methanol–tetrahydrofuran (97:3, v/v). The gradient separation conditions were as follows: 15–20% B in 5 min, 20–32% B in 12 min, 32–60% B in 10 min, 60–90% B in 3 min, and 90–15% B in 2 min. The mobile phase flow rate was 1.0 ml/min. Amino acid standards were provided by Sigma (St. Louis, MO).

Peanut oil fatty acid analysis

The kinds and frequencies of fatty acids in the peanut oil sample were determined by gas–liquid chromatography. Fatty

acids of the oil were prepared in an alcoholic potassium hydroxide solution, and the ester derivatives were prepared using a sulfuric acid:methanol:toluene reagent (Hamilton and Hamilton 1992). Fatty acid methyl esters were analyzed on a gas chromatograph (model GC-1A; Shimadzu Scientific Instruments, Kyoto, Japan) equipped with a capillary column (length 50 m i.d. 0.25 mm; model OV17; Shimadzu) using N_2 (2 ml/min flow rate) and a flame ionization detector. The operating temperature was 210°C. Fatty acid standards were provided by Sigma.

Determination of dry cell concentration

The samples were centrifuged at 2,600*g* for 20 min, the pellet was suspended in distilled water and was re-centrifuged and was dried at 104°C for 14 h. The biomass

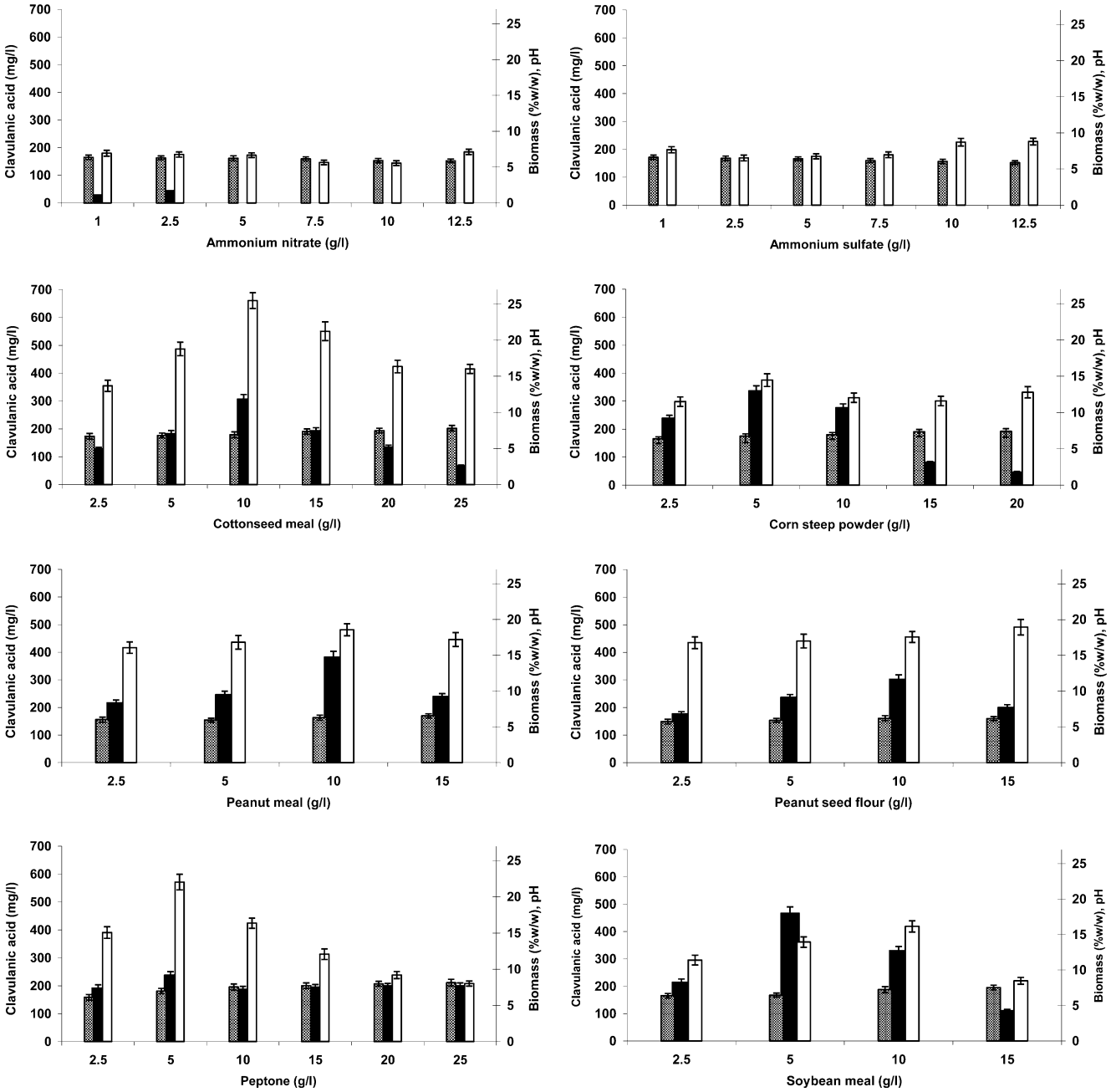

Fig. 1 Effect of various nitrogen sources on clavulanic acid (CA) production by *Streptomyces clavuligerus* DSM 738. *Bars*: *Gray* pH, *white* biomass, *black* CA concentration. All experiments were performed in triplicate in three batches in the basal fermentation medium. Variance of the results is shown as *error bars*

was determined by measurement of the ratio of the dry cell weight to the dry weight of the culture (O'Cleirin et al. 2005).

Assay of CA

The concentration of CA in the fermentation broth was determined by HPLC, as described by Foulstone and Reading (1982). This method is based on measuring the absorbance of imidazole-derived products of CA at 311 nm. The derivatized CA was analyzed on a HPLC system (Adept 4900; Cecil Instruments, Cambridge, UK) equipped with a UV detector (CE4200; Cecil Instruments, UK) at 420 nm. A C18 (250×4.6 mm; Hichrom, Theale, Berkshire, UK) column was used. The mobile phase consisted of methanol (30%) and 50 mM phosphate buffer (70%), and the total flow rate 0.5 ml/min. The column temperature was 27°C, and the sample injection volume was 20 µl. A potassium clavulanate standard was provided by Kosar Pharmaceutical Co., Tehran, Iran.

Assay of N^2-(2-carboxyethyl)-L-arginine

The analysis of CEA as a guanidine compound was done using reverse-phase HPLC following pre-column derivatization with benzoin and fluorescence detection Kai et al. (1983). The fermentation broth was centrifuged for 30 min at 2600*g* and 4°C to remove the biomass and insoluble components of the medium. The supernatant was separated from the solid components and the pH adjusted to 2.8±0.1 by drop-wise addition of HCl (5 N). The supernatant was then centrifuged for 30 min at 2600*g* and 4°C to precipitate

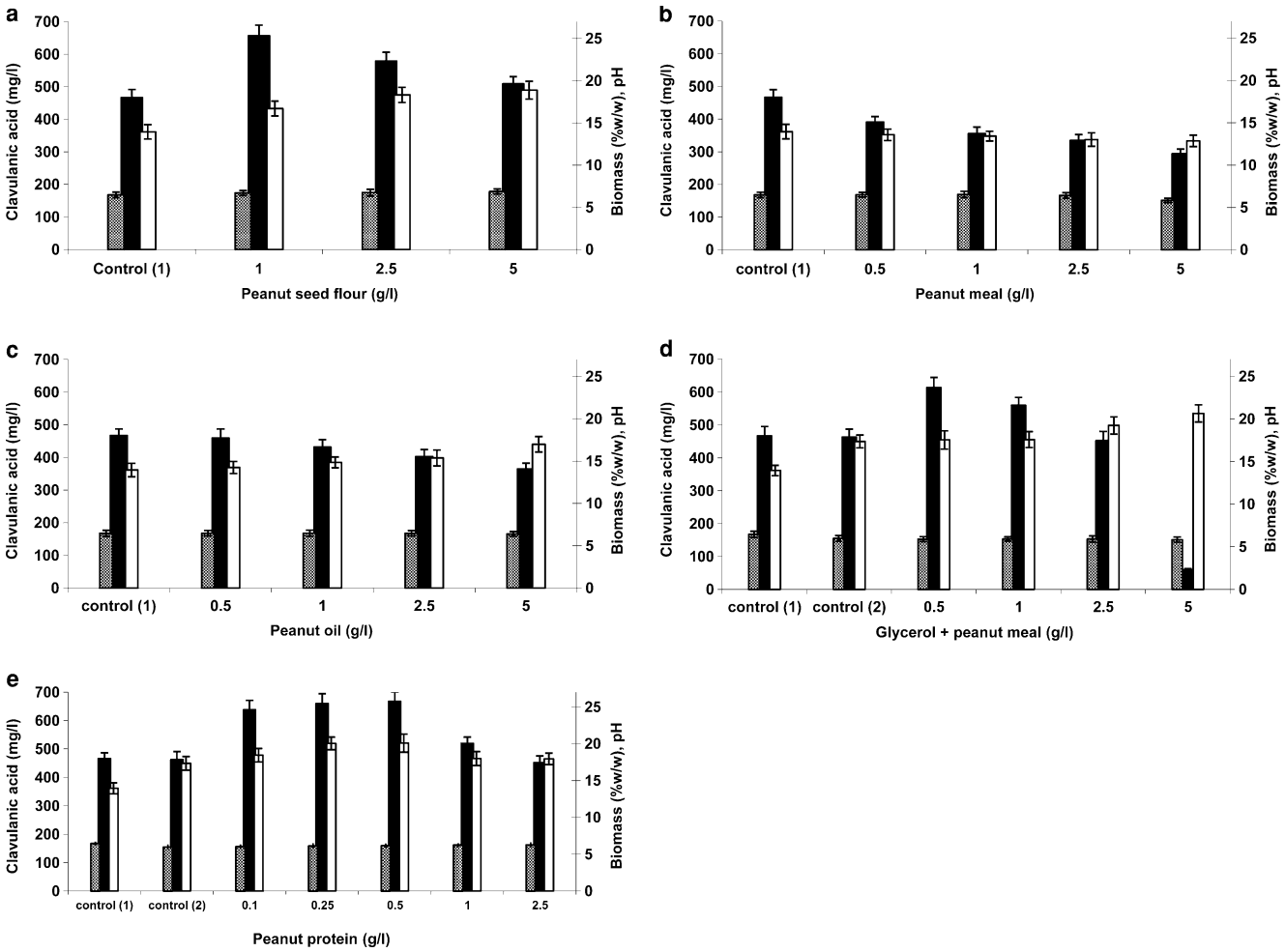

Fig. 2 Effect of peanut seed fractions on CA production by *Streptomyces clavuligerus* DSM 738. *Control (1)* basal fermentation medium containing 5 g soybean meal/l, *Control (2)* basal fermentation supplemented with 5 g soybean meal/l and 5 g glycerol/l. *Bars*: *Gray* pH, *white* biomass, *black* CA. All experiments were performed in triplicate in three batches, and variance of the results is shown as *error bars*. Control medium (1) and control medium (2) were used as the basal medium in **a–c** and **d–e**, respectively

proteins. Once again, the supernatant was separated from the solid components and its pH adjusted to 6.5–7 by the drop-wise addition of KOH (5 M). The diluted samples were derivatized by benzoin as described by Kai et al. (1983). The derivatized CEA was analyzed using a HPLC system consisting of a Wellchrom K-1001 HPLC pump and a Knauer RF-10AXL HPLC fluorescence spectrophotometer detector with a xenon lamp. The pH-resistant Shodex C18 column (D18-613; Showa Denko K.K., Tokyo, Japan) was used. The mobile phase consisted of a methanol: water:0.5 M Tris–hydrochloric acid buffer (pH 8.5) solution (55:30:15) at a total flow rate 0.8 ml/min. Fluorescence detection at λ_{em}=425 nm with λ_{ex}=325 nm was used. The column temperature was 27°C and the sample injection volume was 100 μl.

Data analysis

One-way analysis of variance (ANOVA) and the Tukey HSD test were performed in SPSS ver. 10 software (SPSS, Chicago, IL).

Results

Effect of various nitrogen sources on CA production

The effects of various industrial nitrogen sources on the growth of *S. clavuligerus* DSM 738 and CA production are shown in Fig. 1. A comparison of highest reached concentration of CA and corresponding nitrogen sources revealed that the maximum concentration of CA (467 mg/l) was obtained in the medium containing 5 g/l soybean meal. The concentration of CA in this medium was 1.22-, 1.39-, 1.52-, 1.55-, 1.96-, and 10.38-fold higher than that in media containing 10 g/l peanut meal, 5 g/l corn steep powder, 10 g/l cotton seed meal, 10 g/l peanut seed flour, 5 g/l peptone, and 2.5 g/l ammonium nitrate, respectively. As seen in the Fig. 1, no CA was produced in the media containing ammonium sulfate. *S. clavuligerus* DSM 738 was characterized by short hyphae when grown in media with inorganic nitrogen sources, whereas the hyphae were long and not fragmented in the other media. Maximum growth of the strain was obtained in the media containing cotton seed meal or peptone. Although these results showed that soybean meal was the best nitrogen source for CA production, they also suggested that the productivity of CA in the soybean meal-containing media could possibly be enhanced by other supplementations. Therefore, peanut-derived compounds were added to the basal fermentation medium supplemented with 5 g/l soybean, i.e., control fermentation medium (1), and their effects on growth of *S. clavuligerus* DSM738 and CA production were studied (Fig. 2a–c).

Effect of peanut derivatives as fermentation medium supplements

The effect of various concentrations of peanut seed flour on CA production is shown in Fig. 2a. Supplementation of control medium (1) with all of the different concentrations of peanut seed flour tested increased both CA production and *S. clavuligerus* DSM 738 growth. The highest concentration of CA (657 mg/l) was obtained in the medium containing 1 g peanut seed flour/l [40% more than that of control (1) medium].

The peanut seed used for the study had 51% oil, with oleic acid (56%), linoleic acid (23%), palmitic acid (9%), stearic acid (3%), and arachidic acid (3%) as the major fatty acids. The effect of various concentrations of peanut meal and peanut oil is shown in Fig. 2b, c, respectively. All of the concentrations these supplements that were studied had the effect of decreasing CA production, with the effect of peanut oil less than that of peanut meal. This effect could possibly be due to a synergistic effect of the two fractions of peanut seed, including oil and meal. The addition of peanut meal decreased the growth of *S. clavuligerus* DSM 738, but supplementation of the fermentation medium with peanut seed oil increased biomass production.

Cumulative effects of peanut meal and glycerol on CA production

The addition of 5 g glycerol/l to control medium (1) did not increase CA production ($P > 0.05$). However, the growth of *S. clavuligerus* DSM 738 was stimulated by glycerol, and the biomass production in the glycerol-containing medium was 24.4% higher than that in control medium (1) (Fig. 2d). CA production increased when various concentrations of peanut meal were added to the control fermentation

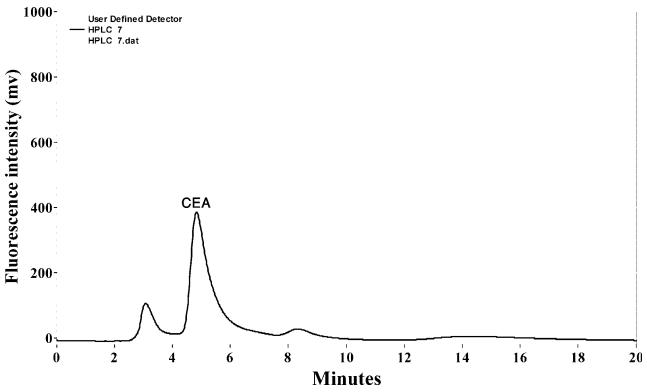

Fig. 3 Analysis of N^2-(2-carboxyethyl)-L-arginine (*CEA*) in the fermentation broth of *S. clavuligerus* RFL35 using high-performance liquid chromatography. The CEA chromatogram appears at a retention time of 4.7 min

Table 1 Comparison of clavulanic acid and biomass production in various fermentation media at the optimum concentration of each medium component

Component	Main nitrogen source	Auxiliary nitrogen source	Auxiliary carbon source	CA (mg/l)	Biomass (%)
1	5 g soybean meal/l	-	-	467	13.95
2	10 g peanut seed flour /l	-	-	302	17.58
3	10 g peanut seed meal /l	-	-	382	18.57
4	5 g soybean meal/l	-	5 g glycerol/l	463	17.36
5	5 g soybean meal/l	1 g peanut seed flour/l	-	657	16.70
6	5 g soybean meal/l	0.5 g peanut meal/l	-	391	13.95
7	5 g soybean meal/l	-	0.5 g peanut seed oil/l	459	14.25
8	5 g soybean meal/l	1 g peanut seed meal/l	5 g glycerol/l	614	17.54
9	5 g soybean meal/l	0.5 g peanut seed protein/l	5 g glycerol/l	668	20.09

All media contained 20 g dextrin/l as the main carbon source. Throughout the study, formulations (1) and (4) were denoted as control medium (1) and control medium (2), respectively

medium (2) (Fig. 2d), with the maximum increasing effect observed in the medium containing 0.5 g peanut meal/l; the CA concentration in this medium was 32% higher than that of control medium (1) and control medium (2).

Effect of peanut protein on CA production

The effect of various concentrations of peanut protein on the growth of *S. clavuligerus* DSM 738 and CA production is shown in Fig. 2e. These experiments were performed in control medium (2). The addition of peanut protein increased both CA production and *S. clavuligerus* DSM 738 growth, with the highest concentration of CA measured in the medium containing 0.5 g peanut protein/l; this concentration surpassed that of control medium (1) and control medium (2) by 43%.

Amino acids of peanut protein

The peanut seed used for the study had 25% protein and its amino acids composition (%) is aspartic acid (11.33), glutamic acid (22.99), serine (6.28), histidine (2.64), glycine (2.96), arginine (18.1), tyrosine (3.91), methionine (2.31), valine (3.76), phenylalanine (5.49), isoleucine (3.59), leucine (8.5), lysine (4.39), and alanine (3.75). Based on this analysis, peanut seed contains a high percentage of arginine, glutamic acid, and aspartic acid.

Effect of peanut protein on CEA production

The addition of peanut protein (0.5 mg) to control medium (2) increased the concentration of CA by 43%, possibly by increasing the arginine concentration and enhancing CEA synthase activity. To test this hypothesis, *S. clavuligerus* RFL35 was inoculated in control fermentation medium (2) containing 0.5 g peanut protein/l. The fermentation broths analyzed for the production of CEA by HPLC and the results are shown in Fig. 3. The addition of peanut protein increased CEA production and the yield of CEA/biomass. CEA concentration, growth of *S. clavuligerus* RFL35, and yield (CEA/biomass) in control medium (2) plus 0.5 g/l peanut protein were higher than those in control medium (2) by 40, 11, and 27%, respectively.

Discussion

Medium optimization is a powerful tool to enhance the yield of biotechnological processes, including antibiotic production. Each component of a medium should be adjusted to its optimum concentration as increased or decreased amounts will reduce the yield of the product and productivity of the culture. The production of CA by *S. clavuligerus* is markedly influenced by carbon and nitrogen sources (Saudagar et al. 2008). Supplementation with amino acids (asparagine, aspartate, and threonine) (via urea

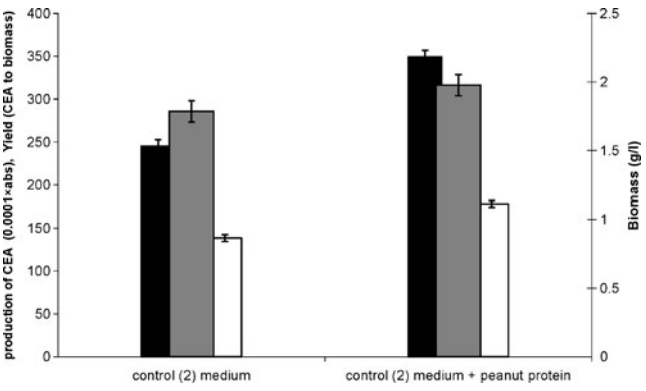

Fig. 4 Effect of peanut protein on the amount of CEA produced by *Streptomyces clavuligerus* RFL35. *Black bar* CEA concentration (0.0001×absorbance at λ_{em}=425 nm; λ_{ex}=325 nm), *gray bar* biomass (g/l), *white bar* yield of CEA [concentration to biomass (0.0001× absorbance /l)]

cycle) makes more C_5 precursors (arginine) available for the biosynthesis of CA (Bushell et al. 2006).

As seen in Fig. 2a, the optimum concentration of peanut seed flour for CA production is 1 g/l. The lower amount of CA in media containing more than 1 g peanut seed flour/l can be attributed to nitrogen catabolite repression. In a similar study, Omura et al. (1980) reported that ammonium at certain concentrations stimulated leucomycin production by *Streptomyces kitasatoensis*. However, ammonium ions at high concentrations inhibited leucomycin production due to nitrogen catabolite repression. As seen in Fig. 2c, good growth of *S. clavuligerus* was seen in those media containing peanut oil, but thee was no enhancing effect on CA production; however, the addition of peanut seed flour decreased biomass production (Fig 2b). This result may be due to the preference of *S. clavuligerus* for oil in terms of volume compared to carbohydrates or proteins. It has been shown that it would take 1.24 l of soybean oil to add 10 kcal of energy to a fermenter, where as it would take 5 l of glucose or sucrose to add same amount of energy, assuming that they are being added as 50% w/w (Saudagar et al. (2008). *S. clavuligerus* has good lipase activity (Large et al. 1999) and grows well on oil-containing media (Maranesi et al. 2005).

The results obtained in our study (Fig. 1) and those of Wang et al. (2005), Ortiz et al (2007), and Saudagar et al. (2008) demonstrate that soybean meal is a good nitrogen source for CA production and *S. clavuligerus* growth. Our results also show that peanut is not the ideal main nitrogen source for CA production, possibly because of the relatively poorer growth of *S. clavuligerus* DSM738 in the peanut meal-containing medium (Fig. 1, Table 1). We therefore propose that peanut seed flour, at its optimum concentration, could be used as an auxiliary carbon and nitrogen source for CA production to provide a sufficient concentration of glycerol, AFAA (arginine family of amino acids), and arginine. The flour of peanut (*Arachis hypogea* L. cv. Astaneh) also has 51% oil. *S. clavuligerus* can produce lipase (Large et al. 1999), and peanut oil is converted slowly to glycerol and fatty acids as a source of D-glyceraldehyde-3-phosphate. However, the addition of high concentrations of peanut seed flour or its derivatives did not significantly increase CA levels.

The concentration of CA and biomass production in at the optimal concentrations of the additives studied is shown in Table (1).

As seen in Table 1, there is no significant difference between CA concentration in the medium containing peanut seed flour and that containing peanut protein as the auxiliary nitrogen source. However, the use of peanut protein over peanut see flour, apart from the extraction cost, has some benefit. Membrane filtration, such as ultrafilteration and diafilteration, is used for the separation of CA (Nabais and Cardoso 1995). Insoluble medium ingredients which remain in the fermentation broth may result in the fouling of membranes. The extraction of protein from peanut seed removes the insoluble and indigestible materials, including oil and fibers, and results in decreasing the costs of down-stream processes without any effect on the yield of CA. Peanut oil can be obtained during the peanut protein extraction processes. Peanut oil is a valuable oil that is commonly used as frying oil and in cosmetics (Yoneyama et al. 1994). It can also be used as a source of fuel for the diesel engine (Encinar et al. 2005; Davis et al. 2009).

Taking into account the results of the CEA analysis, the growth of *S. clavuligerus* RFL35, the $Y_{CEA/biomass}$ in soymeal- and glycerol-containing medium (control 2) plus 0.5/l peanut protein, the $Y_{CEA/biomass}$ in control medium (2) (Fig. 4), and analysis of peanut protein amino acids, we conclude that the addition of peanut protein to the fermentation medium enhanced CA production by *S. clavuligerus* by enhancing CEA synthase activity.

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