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Production of Clavulanic Acid by Streptomyces clavuligerus in Batch Cultures with Using Wheat Bran as the Source of Carbon

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Sanaz Hasani Khayat, Saeid Akbarzadeh Kolahi*

[1]*Herbal Medicines Research Center, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran, Iran (HMRC).*

[2]*Department of Biotechnology, Faculty of Advanced Sciences &Technology, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran, Iran (IAUPS).*

[3]*Department of Pharmacology &Toxicology, Faculty of Pharmacy, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran, Iran (IAUPS).*

Abstract

Nowadays Microbial biotechnology is considered as one of the most important and profitable branches of industry. Through this approach, we were able to produce biotechnological products with higher efficiency using the generator microorganisms. Clavulanic acid has been used in combination with commonly used beta-lactam antibiotics in order to fight against bacterial infection resistant to such antibiotics and this product plays a major role in pharmaceutical industry especially in the production of Co-Amoxiclav. The fermentation stage plays a major role in producing secondary products. The compositions of fermentation media play an important role in the titer and productivity of secondary metabolites and the cost of raw materials. Carbon substrate is one of the most important and expensive components of media in this stage. The present research was carried out to sought the effect of various densities of wheat bran as the natural source of carbon and the cost of clavulanic acid production. In this research, we used wheat bran, instead of corn oil, as the suitable carbon and energy resource in the formulation of the fermentation process. The final product was cultivated for a nine-day period at 28 °C. The pH value, biomass, clavulanic acid concentrations (by HPLC and Spectrophotometry) and morphology of the strain had been studied. The results showed that the clavulanic acid production increased by 12 percent, approximately, compared to the control medium. Clavulanic acid production was obtained in the fermentation medium containing 17 g/L wheat bran which was 180 mg/L.

Keywords: Clavulanic acid, Wheat bran, Carbon source, Streptomyces clavuligerus, Microbial fermentation.

***Corresponding author**: Saeid akbarzadeh kolahi, Assistant Prof. of Toxicology and Pharmacology Department of Biotechnology, Faculty of Advanced Sciences &Technology, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran, Iran (IAUPS).
Email address: Saeidakbarzadeh4@gmail.com

INTRODUCTION

the antibiotics are used to treat severe human infections caused by microorganisms. Antimicrobial resistance is recognized as one of the greatest threats to human health worldwide. Recently, infection by resistant bacteria has become too common and some pathogens have even become resistant to multiple types or classes of antibiotics (Boucher et al. 2009). The production of beta-lactamase enzymes is the most common mechanism of bacterial resistance to beta-lactam antibiotics such as penicillins and cephalosporins. These enzymes are secreted by a wide range of important pathogenic Gram-positive and Gram-negative bacteria. Beta-lactamases catalyze the hydrolysis of the beta-lactam ring, splitting the amide bond. As a result, the antibiotics become ineffective against bacterial growth (Bush et al. 1995). Although a wide variety of microorganisms synthesize antibiotics, the majority of the clinically useful compounds are produced by actinomycetes (Khetan et al. 1999). Among these organisms, members of the genus Streptomyces are responsible for the production of about 80% of the known secondary metabolites, especially antibiotics (Challis et al. 2003). Streptomyces clavuligerus is an actinomycete that produces clinically useful beta-lactam compounds, such as cephamycin C and clavulanic acid (Paradkar et al. 1996).

Clavulanic acid is a secondary beta-lactam metabolite produced by Streptomyces clavuligerus that has a potent beta-lactamase inhibitory activity (Roubos et al. 2002). Despite sharing the beta-lactam ring typical of penicillins, clavulanic acid has low intrinsic antimicrobial activity. However, such a similarity in chemical structure allows its action as a competitive inhibitor of beta-lactamases secreted by certain bacteria to confer resistance to beta-lactam antibiotics (Saudagar et al. 2008). Clavulanic acid has been used clinically in conjunction with beta-lactamase sensitive and beta-lactam antibiotics to treat diseases caused by several pathogenic bacteria. The combined action as beta-lactamase inhibitor and antibacterial agent makes clavulanic acid very important both clinically and economically (Lynch et al. 2004).

The pharmacokinetic characteristics of clavulanic acid supported the development of combined therapy regimens with amoxicillin and ticarcillin, and the therapeutic success of these combination drugs is well recognized. Clavulanic acid formulations have been used widely and effectively in the treatment of a broad range of clinical infections for nearly 20 years (Finlay et al. 2003). Augmentin, a brand name, containing a combination of amoxicillin and potassium clavulanate, is one of the best-selling antibiotics (Rolinson, 1994). Considering the application of clavulanic acid in the pharmaceutical industry, the cheap production of this material can be a great aid to our economy and pharmaceutical industry. 30 to 40 percent of production cost is spent on the appropriate cultivation medium, thus selecting an appropriate medium is of great significance since it results in maximum output for each gram of substrate and a higher rate of production and cheaper and much more easier affordability (Lancini & Lorenzetti, 1993). Clavulanic acid can be produced industrially by the fermentation of Streptomyces clavuligerus PTCC 1705 strain, which requires a source of carbon, nitrogen, and energy for the biosynthesis of cellular matter and products during normal cell operation, maintenance and production (Saudagar et al. 2008). Optimization of the composition of the medium plays a major role in increasing the production rate of biological products, and so does carbon resource. (Clerck et al. 1995). Therefore, the selection of the most suitable medium composition is of primary importance to increase the productivity and decrease the cost of any bioprocess (Ortiz et al. 2007). It has the appropriate chemical compounds and is cheap rendering its utilization in the industry of metabolite production quite affordable (Hamedi et al. 2006). As the main source of carbon, wheat bran has properties such as abundant production and availability. This research attempted to study the effect of utilizing wheat bran as the source of carbon in the fermentation medium of the bacteria generating clavulanic acid. The results of this study should help develop novel strategies for improving the use of wheat bran-containing medium to enhance clavulanic acid production. The utilization of wheat bran is important for the microbial production of secondary metabolites due to their stimulation of bacterial growth and product synthesis.

MATERIALS AND METHODS

Microorganism

The strain used in this work was Streptomyces clavuligerus PTCC 1705. Streptomyces clavuligerus strain was purchased from Iranian Industrial Bacteria and Fungus Collection center.

Culture media

Streptomyces clavuligerus were inoculated on sporulation medium (GYM Streptomyces Medium), containing (in g/L distilled water): malt extract, 10; yeast extract, 4; glucose, 4; calcium carbonate, 2; agar, 12. The medium was adjusted to pH 7 and sterilized at 121 ºC for 20 min by autoclaving. This bacteria was cultivated on an exclusive medium for 14 days at a temperature of 28 °C. 1 ml aliquot of spore suspension (approx. 107–108 spores/ml) was then inoculated into 500-ml Erlenmeyer flasks containing 100 ml of seed medium. The seed medium used presented the following ingredients (in g/L distilled water): bacteriological peptone, 10; glycerol, 20; malt extract, 10; with a pH adjusted to 7.0 ± 0.2 (Cole, 1977). The flasks were incubated at 28 °C on a rotary shaker at 200 rpm for 48 h.

Investigating and selecting the best medium containing Mycelium as the inoculants liquid

To select the best flask for seeding, 5-milliliter sample was collected from every flask and the samples were studied in terms of the growth morphology of bacteria, the absence of microbial pollution and the biomass formed. After preparing bacterial development on the slides, warm coloring was conducted in accordance with the standard protocol and its pollution and morphology were checked. To calculate the biomass formed, the following formula was used:
B – A = 1 milliliter of the seeding medium weight
C – A = sediment weight

$$biomass\ percentage = 100 \times \frac{sediment\ weight}{1\ milliliter\ of\ the\ seeding\ medium\ weight}$$

A represents the pure weight of microtube, B represents the weight of microtube containing 1 milliliter of the sample, and C represents the dry weight of mycelium after sedimentation.

Producing clavulanic acid

The production culture medium was based on that described by Maranesi et al. (2005) which present the following composition (in g/L distilled water): Starch, 10; soybean flour, 20; corn oil, 23; $Ca_3(Po_4)_2$, 1.2; $ZnSO_4.H_2O$,0.001; $FeSo_4 7H_2O$,0.001; $MnCl_2.4H_2O$, 0.001. The medium was adjusted to pH 7 ± 0.2 with 1 N NaOH solution prior to autoclaving at 121 °C for 20 min. To produce clavulanic by Streptomyces clavuligerus bacteria, the exclusive medium to produce clavulanic acid with a concentration of 13.6, 17 and 20.4 g/L of wheat bran was used as the source of carbon. The composition of the medium is represented in the table below (Table 1). The control medium contained corn oil 23 g/L as the source of carbon.

Table 1: The medium used to produce Clavulanic acid

Material used	Amount (g/L)
starch	10
wheat bran	13.6 – 17 - 20.4
Soybean	20
Ca3(Po4)2	1/2
ZnSO4.H2O	0.001
FeSO4.7H2O	0.001
MnCl2.4H2O	0.001

The exclusive cultivation medium was prepared in flasks with a capacity of 500 milliliters and 5 to 10 percent of the volume of the medium was filled with the inoculant. The flasks were then cultivated in incubator's shaker at a temperature of 28 °C with a rate of 200 rpm for 9 days. Some 5-milliliter samples were collected every day from the 3rd to the 9th day. The samples were studied for their pH and formation of biomass. The samples were then centrifuged with a rate of 4000 rpm for 20 minutes and the upper solution was transferred to another vial. To exclude the intervening proteins, pH of the fermentation liquid was set to 3± 0.2 using hydrochloric acid, and the resulting solution was centrifuged for 20 minutes at a rate of 4000 rpm. The supernatants were filtered through a Millipore membrane (pore diameter 0.22 μm). The upper fluid was used to measure clavulanic acid produced.

Clavulanic acid measurement

The clavulanic acid concentration in the fermented broth was determined by measuring a derivative spectrophotometrically, obtained by the reaction of the clavulanic acid with imidazole, as proposed by Bird et al.1982 (PG T80 Series UV/Vis Double Beam Spectrophotometer). Spectrophotometer and liquid chromatography were used to measure the amount of clavulanic acid produced. To measure the amount of clavulanic acid produced through a spectrophotometer, first, the standard curve was drawn using potassium clavulanate provided by Dana Pharmaceutical Company. The assay results were checked by a high performance liquid chromatography (HPLC Younglin YL9100) method, as described by Foulstone & Reading (1982).

RESULTS AND DISCUSSION

In this research, the effect of different wheat bran concentrations as the carbon source on Streptomyces clavuligerus growth and the production of clavulanic acid has been examined in batch cultures. The objective of this research is the optimization of culture medium composition.

The capability of Streptomyces clavuligerus PTCC 1705 strain to produce clavulanic acid in media containing wheat bran as carbon source was investigated in exploratory shake-flask cultures and compared with corn oil containing a medium. In the batch cultivation, production of clavulanic acid started approximately between 24 and 30 h of cultivation(still during the growth phase). However, most of the production occurred later, after the wheat bran had been consumed. This fact indicates that the production of clavulanic acid is not associated with microorganism growth, in agreement with the results obtained by Baptista Neto et al. (2000). The results obtained in this work showed that clavulanic acid was partially associated with growth. However, most of the production phase occurs with no association with the growth phase. From the data obtained in these experiments, it could be concluded that media containing 20.4 g/L wheat bran produced the highest clavulanic acid. In the other hand, comparing the HPLC results obtained from the culture medium, containing wheat bran in a medium containing corn oil as a carbon source, at the eighth day of fermentation showed that the production of clavulanic acid increased by 20 mg/L. In the other words, the clavulanic acid production increased by 12 percent, compared to the control medium. Therefore, the use of wheat bran as a sole source of carbon in the production of clavulanic acid as an alternative of wheat bran had a positive effect and was proven to be a promising choice. The possible reason for the observed results could be explained with respect to the increased utilization of wheat bran with feeding strategies. In the control culture with feeding corn oil, clavulanic acid production reached a maximum of 160 mg/L.

Mounir et al. (2010) checked out improvement and enhancement of clavulanic acid production in Streptomyces clavuligerus using vegetable oils and they concluded that using olive oil as a sole source of carbon and energy for cultivation of Streptomyces clavuligerus was a promising strategy for clavulanic acid production, also using corn oil as a sole source of carbon is suitable. The results observed in the present study were in accordance to choosing corn oil as a source of carbon in a fermentation medium. Chen et al. (2002) reported that glycerol at 10–15 mg/ml increased clavulanic production by S. clavuligerus in shake flask cultures, while Romero et al. (1984) reported glycerol above 15 mg/ml to inhibit clavulanic acid biosynthesis.

In Figure 1, it can be observed that the third-day fermentation of Streptomyces clavuligerus mycelium is low density. Figure 2 shows that on

the seventh-day fermentation of Streptomyces clavuligerus mycelium are densely populated and are intertwined. As it is seen in Figure 3, on the ninth day fermentation of Streptomyces clavuligerus mycelium fragmentation marking the end of fermentation.

The investigation on biomass weight percentage in evaluating the culture medium indicated that the fermentation of the biomass has increased on a daily basis up to the 8th day, but the reduction of biomass and death occurred the following days (Figure 4).

Examining the effect of different wheat bran concentrations on the clavulanic acid production and its relation with pH during different days of fermentation showed that the levels of pH had dropped in the initial days of production and had increased from the 8th day onward (Figure 5).

Examining the effect of different wheat bran concentrations on the clavulanic acid production indicated that the amount of clavulanic acid production increased by increasing the wheat bran quantity. The influence of wheat bran on the production rate of clavulanic acid was studied using spectrophotometer and HPLC. Figure 6 represents the spectrophotometer of the 8th day of fermentation with all the three concentrations (13.6, 17, 20.4 g/L) of wheat bran.

As it is seen in Figure 7, higher concentrations of wheat bran indicated that the amount of clavulanic acid production increased by increasing the wheat bran quantity. On the other hand, the production rate of clavulanic acid with various concentrations of wheat bran at different days had been analyzed using a spectrophotometer. Clavulanic acid concentration reached a maximum of 246 mg/L according to spectrophotometry results when 20.4 g/L of wheat bran was used (Figure 7).

The production rate of clavulanic acid in various days with a wheat bran concentration of 17 g/L as the source of carbon was measured using HPLC method. Figure 8 shows the results of production of clavulanic acid by using HPLC analysis. According to Figure 8, the highest rate of production has been recorded on the 8th day.

The production rates in both the production culture medium and control medium on differents days were checked by one another using HPLC technique. The standard curve was drawn for clavulanic acid (Figure 9). The production rates in both the production and control mediums on the fifth, seventh and eighth days could be seen using HPLC technique (Figure 10, 11, 12, 13, 14, 15).

The comparison of HPLC results of production culture medium and control medium expressed the fact that not only the wheat bran was the appropriate substitute for carbon source (corn oil) but the clavulanic acid producing amount had been increased compared to the control medium.

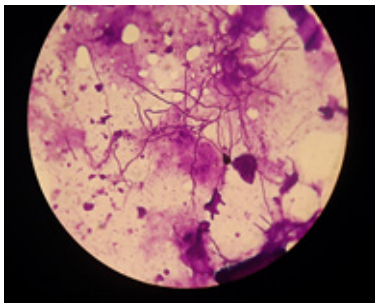

Figure 1: The mycelium in the third-day fermentation of Streptomyces clavuligerus.

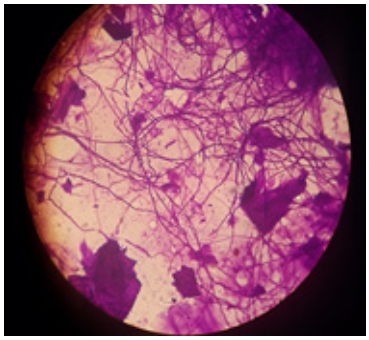

Figure 2: The mycelium in the seventh-day fermentation of Streptomyces clavuligerus.

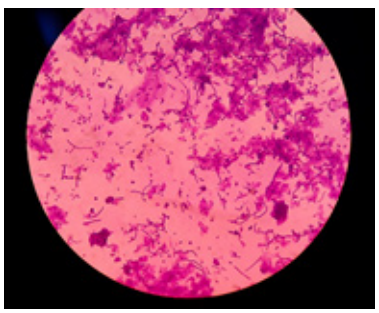

Figure 3: The mycelium in the ninth day fermentation of Streptomyces clavuligerus.

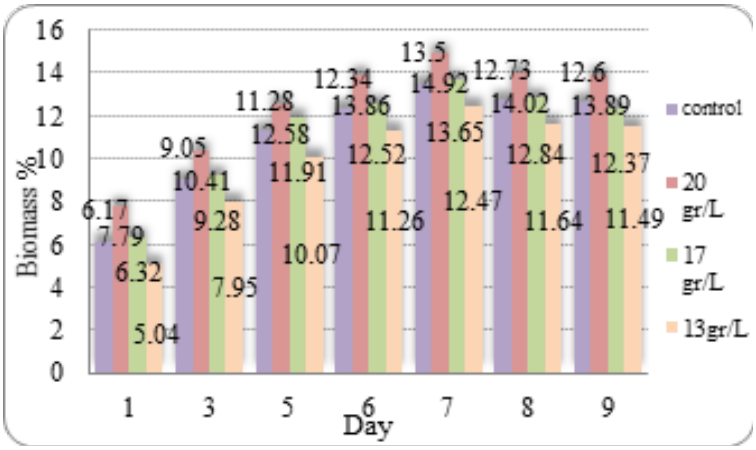

Figure 4: Biomass fluctuations.

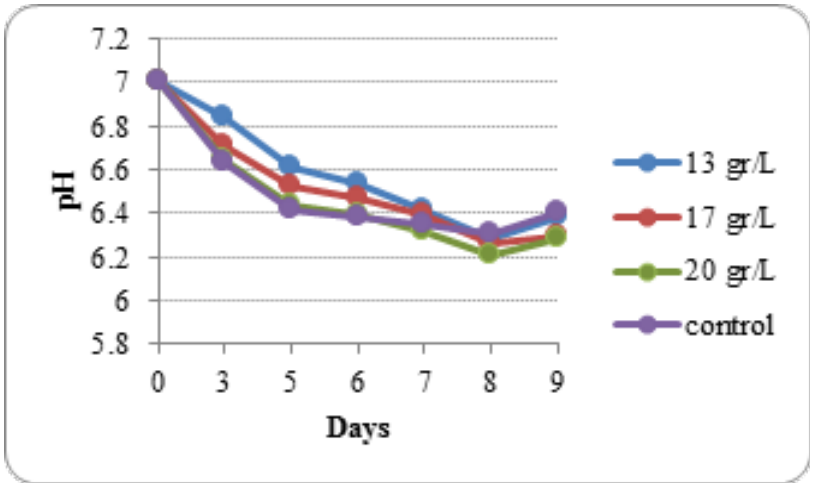

Figure 5: Fluctuations of the acidity of the production medium in various concentrations of wheat bran. 13.6 (less), 17 (equal) and 20.4 (more) g/L.

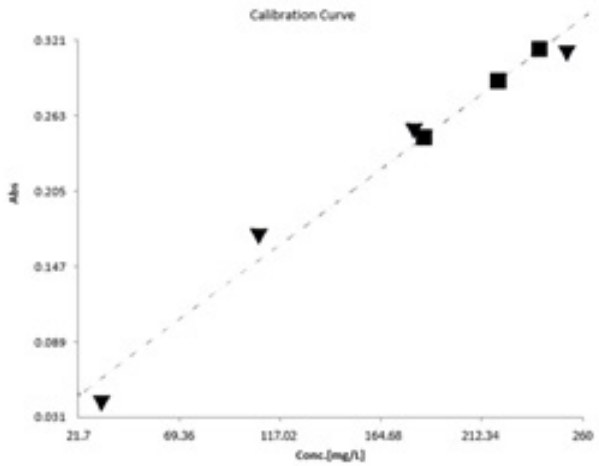

Figure 6: The spectrophotometer of the 8th day of fermentation with three concentrations of 13.6, 17 and 20.4 g/L.

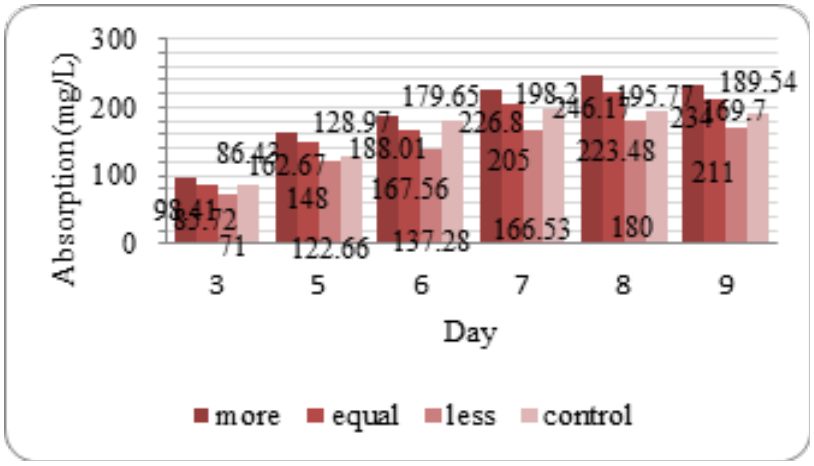

Figure 7: The spectrophotometer results of clavulanic acid levels on different days and with various concentrations of wheat bran of 13.6 (less), 17 (equal) and 20.4 (more) g/L.

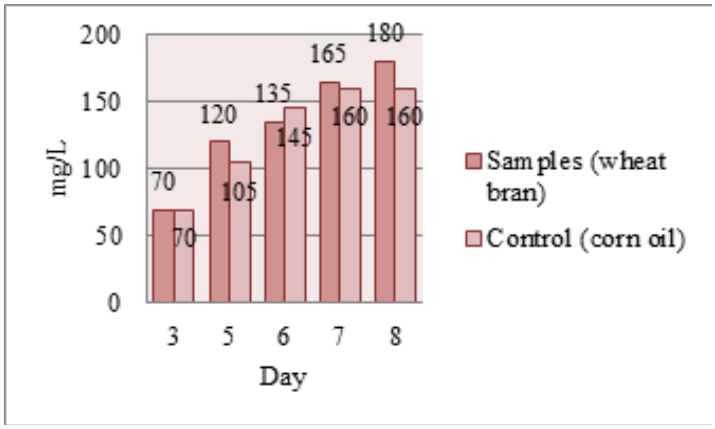

Figure 8: The comparison of HPLC results of production culture medium with a wheat bran concentration of 17 g/L as the source of carbon and control medium with a corn oil in different days.

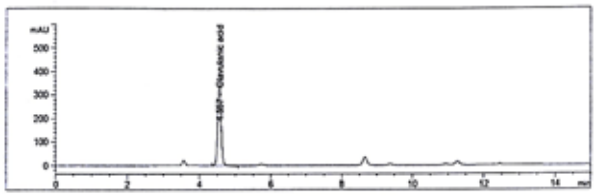

Figure 9: The standard curve of clavulanic acid (100.5 mg/L).

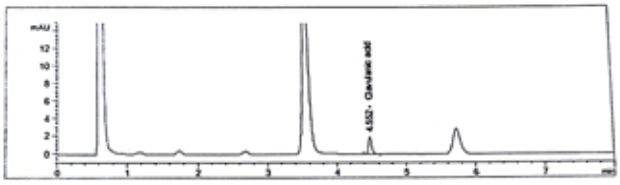

Figure 10. HPLC curve of clavulanic acid production culture medium with a wheat bran concentration of 17 g/L on 5th days (120 mg/L).

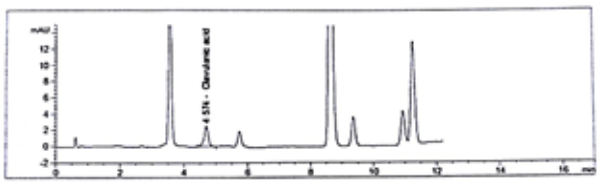

Figure 11: HPLC curve of clavulanic acid produced on 5th days using the control cultivation medium (105 mg/L).

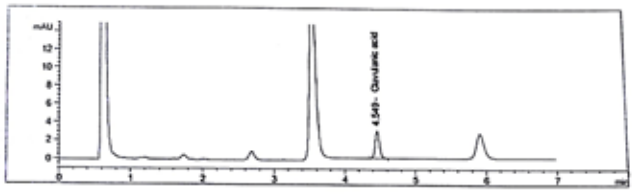

Figure 12: HPLC curve of clavulanic acid production culture medium with a wheat bran concentration of 17 g/L on 7th days (165 mg/L).

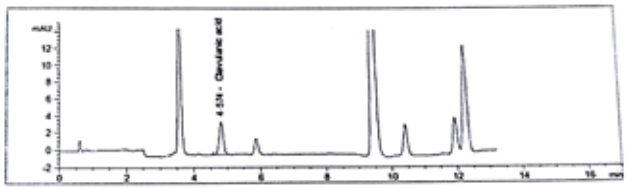

Figure 13: HPLC curve of clavulanic acid produced on 7th days using the control cultivation medium (160 mg/L).

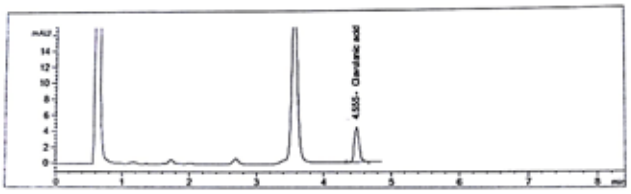

Figure 14: HPLC curve of clavulanic acid production culture medium with a wheat bran concentration of 17 g/L on 8th days (180 mg/L).

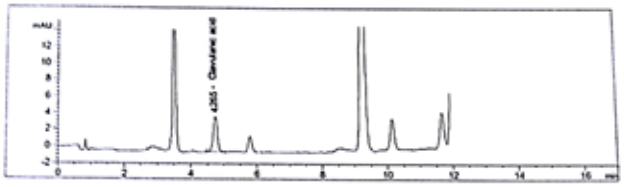

Figure 15: HPLC curve of clavulanic acid produced on 8th days using the control cultivation medium (160 mg/L).

Medium optimization is a powerful tool to enhance the yield of biotechnological processes, including pharmaceutical products production. The overall aim of this work was to expand our knowledge of the regulation of optimization of culture medium composition of Streptomyces clavuligerus. Based on this research, wheat bran appeared to be the most favorable for clavulanic acid production. Product yield is considered as one of the most important parameters to assess the suitability of a culture medium used as a source of carbon because it depends on the metabolic pathways involved in the conversion of substrate into the product (Efthimiou et al., 2008). The increase of producing a product and the decrease of production costs are the important competition aspects in the global markets for biotechnology products' sale. One of the important methods related to optimization is the medium conditions and other work procedure on producing a strain that in the present study the main focus is on the optimization of medium combinations. Fermentation stage is the major focus of production operation costs of medicinal byproducts. Nowadays, in the industrial fermentation, the price reduction is the important and basic factor. One way to decrease the costs is to substitute the culture medium raw material with cheaper and more accessible material. Microorganisms in particular situation are able to produce the secondary metabolites; therefore, the key point in producing the secondary metabolites, is the planning/designing of adequate culture medium. Limitation of nutritious sources in culture medium causes the microorganism growth limitation and product's production. There is a high relation between medium forming material and clavulanic acid production that among these materials, the carbon and nitrogen source prominently exert an effect on the amount and type of metabolites produced by Streptomyces clavuligerus.
Hamdi et al. (2011) had studied improvement of clavulanic acid production by Streptomyces clavuligerus with peanut combinations and reported the highest clavulanic acid concentration was obtained in medium containing soybean meal supplemented with peanut protein.
In a research conducted by Saudagar et al. (2006) clavulanic acid production was increased by 18% with the span of feeding glycerol and reached a maximum at 1.30 mg/ml glycerol feeding as compared to 1.10 mg/ml in the control.
In addition to establishing optimal fermentation medium composition for scale up, the present work made it possible to predict the best amount of wheat bran. Furthermore, the results of the experiment carried out with a modified form of this medium, in which the corn oil was replaced with wheat bran, showing that the production remained high. However, it was delayed, presumably due to the consequent lower availability of carbon source for growth.

CONCLUSIONS

Wheat bran used in the clavulanic acid production process as its intrinsic characteristics allow an adequate supply of carbon source and this finding is the potential of major economic significance for the pharmaceutical industry. Using wheat bran as a sole source of carbon can support bacterial growth and enhance the clavulanic acid production. Considering the price and abundance of wheat bran (compared to corn oil) accessibility and production of more clavulanic acid in culture medium containing wheat bran as the carbon source. Finally, we conclude that the wheat bran can be an appropriate substitute for corn oil. Since the wheat is produced in abundance in Iran and the wheat bran is considered a surplus and excessive product in wheat cultivation process, as a result, its use instead of corn oil is economic and a hopeful choice, having a positive impact. An appropriate feeding strategy for enhanced production of clavulanic acid by S. clavuligerus needs to be developed in the future.

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