Optimization of *Streptomyces Clavuligerus* Medium and Conditions for Clavulanic Acid Production

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**Abstract:** The purpose of the present work was to use 3-level factorial design for optimizing the medium constituents and condition for production of clavulanic acid from *Streptomyces clavuligerus* (Strep. clavuligerus) NRRL 27064. A two-factor, three-level factorial design was used for the optimization procedure with the amount of olive oil (X1) and the initial pH of the fermentation medium (X2) as the independent variables. The regression equation generated for Y (clavulanic acid yield after 120 h) was Y = – 507.6 – 0.26X1 + 146.9X2 – X1^2 – 10X2^2 + 0.07X1X2. Optimization was done by maximizing clavulanic acid yield at 120 h.

**Key words:** *Streptomyces clavuligerus*, Optimization, Clavulanic acid, Factorial design

**INTRODUCTION**

Traditionally, experiment involves maximum effort and time especially when complex processes are to be developed. It is desirable to develop acceptable process parameters in shortest possible time with minimum effort and raw materials. A number of literatures are available describing techniques of experimental design combined with optimization[1-3]. The technique of factorial design is an efficient method of having relative significance of a number of variables and their interactions. In the factorial design, each variable is evaluated at several levels of all other variables. The levels of the factors involved can be set at will. Under such conditions, a complete factorial study is possible, but would involve a large number of experiments. A fractional factorial design can give a solution to this problem[4]. Varieties of pathogenic Gram-positive and Gram-negative bacteria have the ability to produce β-lactamases and lead to remove the antibiotic activity through the hydrolysis of the active β-lactam ring. Clavulanic acid and has been used in combination with conventional β-lactam antibiotics to inhibit β-lactamases [5]. The combination of clavulanic acid with amoxicillin is the most common and efficient example with high levels of antibacterial activity[6]. Clavulanic acid can be produced industrially by fermentation of *Strep. clavuligerus* NRRL 27064 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[7].

The most common carbon source that is generally used for the production of clavulanic acid in *Strep. clavuligerus* fermentation is glycerol with arginine or soybean used as a nitrogen source[8,9]. It has been reported that glycerol has been widely utilized as carbon source in the process with clavulanic acid titres up to 3.25 g/L[10,11]. Using of carbohydrates may lead to decrease in the rate of biosynthesis as a result of rapid catabolism[7]. The best alternative to maintaining the level of glycerol for long periods is substituting lipids for glycerol[5]. Substituting lipids for glycerol may avoid the fast carbon catabolite. The addition of oil is preferred on an energy basis, because typical oil contains around 2.4 times the energy of glycerol. It can also act as antifoam, enhance secondary metabolism for their stimulation of bacterial growth and product synthesis[12,13]. In addition, they are the cheapest available alternative carbon sources. According to Large *et al*[12] independently of the microorganism involved or the localization of the enzyme, carbon source which is essential to lipase activity, may act as stimulant or inhibitor to this activity. In the process of clavulanic acid production by *Strep. clavuligerus*, utilizing medium containing lipid and glycerol, the lipid consumption starts only after glycerol exhaustion, indicating that glycerol is a repressor of the lipase synthesis. Utilization of oil in growth medium has some disadvantages that occur due to the presence of higher residual oil level which may lead to increase medium viscosity and warrant additional downstream processing. Reduction in residual oil levels may further
increase antibiotic titres and significantly reduce downstream processing costs [12]. Also the initial pH influences the apparent decomposition of clavulanic acid and pH changes may alter culture conditions for most fermentation processes [14], it is important to determine the optimal pH for cell growth and metabolite production [15].

The objective of this study was to prepare a production medium containing a suitable amount of olive oil and initial pH using a combination of statistical strategies involving the use of factorial design in an attempt to optimize the production of clavulanic acid by *Streptomyces clavuligerus* in a fermentation system.

**MATERIALS AND METHODS**

**Microbial strains:** Vegetative cells of *Strep. clavuligerus* NRRL 27064 (5 g/L dry weight), stored in cryotubes with 10% (v/v) glycerol at −70 °C, were used throughout the present work.

**Preparation of seed culture:** The seed medium was consist of: glycerol, 10 g/L, bacto peptone, 10 g/L, malt extract, 10 g/L, yeast extract, 1 g/L, K2HPO4, 2.5 g/L, MgSO4 7H2O, 0.75 g/L, MnCl2 4H2O, 0.001 g/L, FeSO4 H2O, 0.001 g/L, ZnSO4 7H2O, 0.001 g/L, 100 mM MOPS buffer - 3-[N-Morpholino]-propanesulfonic acid - 21 g/L. The pH of the medium was adjusted at 7 with NaOH solution prior to being autoclaved at 121 °C for 20 min. The medium used in the inoculum cultivation was equivalent in composition to the corresponding production culture medium as described below.

The production culture medium was based on that described by Maranesi et al [16]. Two different medium have been prepared the first one consist of : starch 10 g/L, soybean flour 20 g/L, olive oil 19, 23 or 27 g/L, glycerol 15 g/L, and phosphate 1.2 g/L in addition to 0.001 g/L ZnSO4, H2O, 0.001 g/L FeSO4 7H2O, 0.001 g/L of MnCl2 4H2O of trace elements, and 1 mL/L silicon as antifoam. The medium was adjusted at pH 6.8, 7 or 7.2 and sterilized at 121 °C for 20 min. The medium used in the inoculum cultivation was equivalent in composition to the corresponding production culture medium as described below.

The production culture medium was transferred at predetermined time intervals using high performance liquid chromatography (HPLC) as described by Foulstone and Reading [17] which has been modified by Tabuk et al [18]. Clavulanic acid in the form of lithium salt (SmithKline Beecham Pharmaceuticals) has been used as standard.

**Experimental Design and Data Treatment:** To investigate the relationship between olive oil content and initial pH of the medium in the production of clavulanic acid by the *Strep. clavuligerus*, a 3-level factorial design was used. The media compositions were selected according to the experimental design. The factors levels were allocated into three categories (low, medium and high). For the response surface methodology and the polynomial regression, the coded values were changed as follows: -1=low, 0=medium and 1=high and the analysis performed with the experimental design module of Statgraphics plus Version 5.1 (Statpoint Technologies, Inc., Warrenton, VA, USA).

Statistical optimization method for fermentation process could overcome the limitations of classic empirical methods and was proved to be a powerful
tool for the optimization of the *Strep. clavuligerus*. In this study, response surface methodology (RSM) model (3-level factorial design) was proposed to study the combined effects of olive oil content and initial pH of culture media on clavulanic acid production. Validation experiments were carried out to verify the validity and the accuracy of the models.

**RESULTS AND DISCUSSION**

The use of olive oil as a sole source of carbon in the production of clavulanic acid as an alternative of glycerol have a positive effect and proven to be a promising choice. In this work clavulanic acid was produced from medium containing olive oil and glycerol at initial pH value of 7 (the clavulanic acid amounts from both media were calculated after 120 hours). Production of clavulanic acid in medium containing olive oil gave nearly about twofold higher (1.07 g/L) in clavulanic acid production than glycerol medium (0.511 g/L). According to Efthimiou et al. [13], the triglycerides in olive oil are hydrolyzed by lipases produced by the bacteria to release glycerol and un-esterified fatty acids into the culture medium that can then be taken up by the bacteria.

For optimization process nine experiments are required for the RSM based on the 3-level factorial design when there are two factors at three levels each. The independent factors are shown in table 1, while the dependent variable Y (response) was clavulanic acid yield after 120 hours of fermentation. The experimental media with observed response are shown in table 2. Based on the experimental design, the factor combinations yielded different response.

The mathematical relationships in the form of polynomial equation relating the response Y and the independent variables were: $Y = -507.6 - 0.26X_1 + 146.9X_2 - X_1^2 - 10X_2^2 + 0.07X_1X_2$. The above equation represents the quantitative effect of process variable ($X_1$ and $X_2$) and their interaction on the response Y. The values of the coefficient $X_1$ and $X_2$ are related to the effect of these variables on the response Y. Coefficients with more than one factor term and those with higher order terms represent interaction terms and quadratic relationship respectively. A positive sign represents a synergistic effect, while a negative sign indicates an antagonistic effect. Figure 2 shows the main effects of $X_1$ and $X_2$ on the clavulanic acid yield.

From the data obtained in these experiments it could be concluded that media containing 27 g/L olive oil with either initial pH value of 6.8 or 7.2 produced the lowest clavulanic acid yield (0.178 and 0.236 g/L respectively) compared to other tested medium which could be due to either increase in the pH of the medium or the higher oil content which increase the viscosity value of the medium (high residual oil level) and as a result decrease the production of clavulanic acid. According to this observation the optimum initial pH value of the medium could be used with 27 g/L olive oil concentration id pH of 7 which produce 0.431 g/L.

In the other hand, media containing 23 g/L olive oil (regardless of the initial pH value) produces high amount of clavulanic acid yield compared to media containing 27 g/L olive oil. The highest amount being produce from medium has initial pH value of 7 (1.07 g/L).

In the present work the highest clavulanic acid production was obtained from medium containing 19 g/L olive oil and having initial pH value of 7 that probably could be due to that olive oil content does not affect on the viscosity of the medium which allow more production of clavulanic acid and over more the initial pH of 7 is the optimum for the microorganism and allow add farther production of clavulanic acid.

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<th>Table 1: Independent variables in factorial design</th>
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<td>pH value ($X_2$)</td>
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<th>Table 2: Olive oil content and initial pH of the prepared media.</th>
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<tr>
<td>9</td>
<td>27</td>
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Fig. 1: Standardized pareto chart for clavulanic acid yield

Fig. 2: Main effects plot shows the main effect of $X_1$ and $X_2$ on the clavulanic acid yield.

Fig. 3: Interaction plot shows the main effect of $X_1$ and $X_2$ on the clavulanic acid yield.

Fig. 4: Normal probability plot for clavulanic acid yield.

Fig. 5: Response surface plot showing the effect of $X_1$ and $X_2$ on clavulanic acid yield.
After generating the polynomial equations relating the dependent and independent variables (table 1), the process was optimized for the response Y. Optimization was performed to obtain the levels of X₁ and X₂, which maximized Y. From the optimization process it was found that the highest clavulanic acid yield was obtained from the medium.

**Conclusions:** In addition to establishing optimal fermentation medium composition for scale up, the present work makes it possible to predict both amount and productivity under different conditions by means of the response and contour surfaces and the polynomial model. This is useful not only for the additional knowledge supplied about the process, but also for the potential in medium engineering and evaluation under economic constrains of medium composition, yield and productivity. The results strongly support the use of RSM for fermentation condition optimization. The chosen method of optimization of fermentation condition was efficient, relatively simple and time and material saving. This work should help to build more rational control strategy, possibly involving scale-up of production of clavulanic acid by *Strep. clavuligerus*. From this work one could concluded that the highest amount of clavulanic acid production could be obtained from a medium containing 19 g/L olive oil at initial pH value of 7.

**REFERENCES**


