Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.47750/jptcp.2023.30.06.034

# Optimization Of the Culture Medium of Althiomycin-Producing *Myxococcus stipitatus* GL41 Using Plackett-Burman Design and Response Surface Methodology

Yen Thi Ngoc Nguyen[1], Chung Dinh Duong[1]*, Thai Minh Nguyen[2], Nga Đinh Nguyen[2], Anh Tu Nguyen[2]

[1] Faculty of Pharmacy, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam.

[2] Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh City, Ho Chi Minh City, Vietnam

***Corresponding author:** Chung Dinh Duong, Faculty of Pharmacy, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam, Email: ddchung@ntt.edu.vn

**Submitted: 14 February 2023; Accepted: 25 March 2023; Published: 06 April 2023**

## ABSTRACT

Althiomycin is a thiazole antibiotic with broad-spectrum activity against Gram-positive and Gram-negative bacteria through protein synthesis inhibition at the site of the 50S ribosomal subunit. This study was conducted to improve althiomycin production in a wild myxobacterial strain (*Myxococcus stipitatus* GL41) by optimization of the fermentation medium. The Plackett-Burman design screened eleven critical factors that affect althiomycin content in extracts. The factors with significant effect ($p < 0.05$) were employed in the Box-Behnken model to clarify the interaction between the independent variables and identify the optimal value for each variable. Three factors (soluble starch, baker's yeast and hepes buffer) determined from the Plackett-Burman design regression analysis results showed significant effects on althiomycin production. The RSM model predicted the optimal fermentation medium's parameters, including 6.09 g/L of soluble starch, 4.05 g/L of baker's yeast, and 20.65 g/L of hepes buffer. Model validation was performed to estimate the althiomycin content showed that the value reached 3.3 mg/L, which is 4.7 times higher than the initial P medium. The mathematical model is well compatible with the experimental results showing that optimization of the fermentation medium by statistical algorithms is an effective solution to promote the production yield of natural compounds.

**Keywords:** *Myxococcus stipitatus, althiomycin, optimization, Plackett-Burman, Response surface methodology*

## INTRODUCTION

For decades, natural products derived from microorganisms have played an essential role in revealing biological compounds that can both prevent and treat diseases. Accordingly, many bioactive metabolites were found in Actinobacteria and Myxobacteria.

Even so, studies of Myxobacteria were considerably less extensive than those of Actinobacteria (1). Nevertheless, these microorganisms have contributed to the discovery of hundreds of compound families that demonstrate the potential for producing active secondary metabolites (2).

Around 100 structural frameworks and 600 derivatives from about 7500 isolates, mainly polyketide and non-ribosomal peptide molecules, were described (3).

Althiomycin is naturally produced by *Streptomyces althioticus* (1957) (4), subsequently by members of the Myxococcus genus, *Cystobacter fuscus* (1982), *Myxococcus virescens*, *Myxococcus fulvus* (1983) (5). This is a potential agent that has been shown to be broad-spectrum antimicrobial and can be used against gram-positive (*Staphylococcus aureus*, *Streptococcus pyogenes*) and gram-negative (*E. coli*, Shigella, Proteus) bacteria (6). Althiomycin is a polyketide containing pentapeptides (cysteine, glycine, and serine) that is synthesized by the combination of non-ribosomal peptide and polyketide synthesis systems and inhibits the biosynthesis of bacterial proteins by hampering the puromycin reaction at the site of peptide bond formation (7, 8).

The efficiency of althiomycin production in wild-type strains is low (5). Previous studies have attempted to unravel the cluster of genes that encode the althiomycin biosynthesis pathway in the *Myxococcus xanthus* strain (9). Nevertheless, the impact of culture media components on althiomycin yield in wild Myxococcus producers has yet to be observed, probably due to challenges in manipulating and cultivating the Myxobacteria species (10, 11). Indeed, myxobacteria are an order of unusual microorganisms with complicated social behaviours, sliding movement, and hunting in groups, forming multicellular fruiting bodies depending on the population (12, 13).

Following the advancement of contemporarily statistical and mathematical methods, many response-associated input factors are initially screened in the factorial models as Plackett-Burman (1946) (14) or Taguchi (1957) and optimized utilizing response surface methodology (Box-Behnken, BB; Central composite design, CCD) (15, 16). Furthermore, optimization has advantages because it reduces the number of experimental tests, adapts to multiple input variables, investigates the correlation between factors, identifies the most suitable conditions, and predicts the values of target responses contents/ concentrations. Therefore, the experimental design has been widely used to optimize the substrate composition in producing natural substances from microorganisms and scale up to industrial fermentation (17).

Usually, culture medium optimization will increase the desired secondary metabolites. Therefore, the sufficient addition of nutrients and growth factors to the fermentation medium is one of the most efficient ways to promote the accumulation of natural products (18). In this study, we examined the relationship between the production of althiomycin in *Myxococcus stipitatus* GL41 (Accession number ON076907) and the composition of the culture medium and then further optimized the concentrations of these input substrates. Since the effects of nutrients on althiomycin production have yet to be investigated in detail, it is believed that the synthesis of althiomycin by *M. stipitatus* GL41 has significantly improved when determining the nutrient compositions that have the greatest impact on productivity.

## MATERIALS AND METHODS

### *Materials*

The chemicals (acetonitrile, methanol) were of chromatographic analytical grade. Water was deionized and purified using a Cascada III-I water purification system (Pall, USA).

Althiomycin as a standard was isolated from *Myxococcus stipitatus* GL41 and characterized the molecular structure by UV, MS and NMR spectra data. The stock standard solution was prepared by dissolving standard althiomycin in methanol to obtain the final concentration of 1 mg/mL. The working solutions were prepared by dilution of stock solution with mobile phase to obtain the decreasing concentrations of 10-250 µg/mL of althiomycin, which were used to establish the linear regression relationship. Woking solutions were filtered through a 0.45 µm millipore membrane filter and stored in the refrigerator at 4°C for one week without affecting the stability.

***Microorganism and cultivation***

The wild *Myxococcus stipitatus* GL41 is an althiomycin-producing strain that was supplied by Microbiology department at Nguyen Tat Thanh University. Cells were reactivated from cryo-vial and subcultured on VY/2 medium (g/L, baker's yeast 5.0, $CaCl_2.H_2O$ 1.0, cyanocobalamin 0.0005, agar 15.0, and the final pH 7.2) for 3-7 days at ambient temperature.

Strain GL41 colonies were scraped off and transferred into 200-mL seed medium (g/L, baker's yeast 2.0, $CaCl_2.H_2O$ 1.0, ferric EDTA 0.008, Hepes 23.8, $MgSO_4.7H_2O$ 1.0, peptone 2.0, soluble starch 4.0, yeast extract 2.0, pH 7.5), then shaked at 160 rpm for three days. The suspension was provided as the seed of cultivation. Following the experimental design, each 10-mL aliquot was inoculated into 90-mL Erlenmeyer flasks containing various medium compositions. The media was agitated at ambient temperature and then supplemented with 1-2% autoclaved Amberlite XAD-16N resin at the $4^{th}$-day of cultivation. The adsorbent resin was collected from the culture after $10^{th}$-day fermentation, washed with distilled water, dried naturally in the air, and extracted with 30 mL of acetone-methanol mixture (50:50, v/v, repeat three times). The extracts were concentrated under a vacuum at 40°C, then dissolved in methanol and titrated to 2 mL, filtered through a 0.45 μm membrane filter, and analyzed using a high-performance liquid chromatography (HPLC) system.

***Liquid chromatography conditions for determination of althiomycin***

Althiomycin was quantitated by the HPLC system (Agilent 1260 Infinity, USA) consisting of a Gemini C18 column (250×4.6 mm, 5 μm, Phenomenex, USA).

The injection volume was 20 μL. Chromatographic elution with a mobile phase contained acetonitrile (solvent A) and water (solvent B) at the various ratio of the gradient programme. Initial isocratic elution was 2% A in 5 min, then the gradient mode increased to 100% A within 40 min and maintained with 100% A in 5 min. The flow rate was 1.0 mL/min, and the column oven temperature was set at 45°C. Detection was at the wavelength of 280 nm. Data were analyzed by Chemstation software.

***Optimization experiment***

The published medium compositions were referred to establish the variables that might impact the althiomycin concentration and the range of these parameters. Initially, the Plackett-Burman design was used to identify the crucial factors of althiomycin production. The Box-Behnken response surface technique was subsequently employed to approach the optimal level of the chosen variables. Eventually, empirical tests were conducted to validate the reliability of the experimental model.

**TABLE 1:** Levels of the factors employed in Plackett-Burman design

| Codes | Factors | Low (-1) | High (+1) |
|---|---|---|---|
| X1 | Soluble starch (g/L) | 3 | 8 |
| X2 | Baker's yeast (g/L) | 0 | 5 |
| X3 | Glucose (g/L) | 0 | 2 |
| X4 | Yeast extract (g/L) | 1 | 3 |
| X5 | Casiton (mg/L) | 0 | 3 |
| X6 | $CaCl_2.2H_2O$ (g/L) | 0.5 | 1 |
| X7 | $MgSO_4.7H_2O$ (g/L) | 1 | 2 |
| X8 | Hepes (g/L) | 10 | 23.8 |
| X9 | Trace solution (mL) | 0 | 0.5 |
| X10 | Cyanocobalamin (mg/L) | 0 | 1 |
| X11 | XAD 16N (%) | 1 | 2 |

***Plackett-Burman (PB) design***

Eleven ingredients were selected to investigate the significant factors affecting the althiomycin content in the extract with a higher confidence ($p < 0.05$): soluble starch, glucose, yeast extract, baker's yeast, casiton, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes buffer), cyanocobalamin (vitamin B12), trace solution, and Amberlite XAD 16N resin. Table 1 sets the low (-1) and high (+1) values for each factor.

The experiment matrix created using the Plackett-Burman design with eleven factors and twelve runs are shown in Table 2. The response was althiomycin peak area (APA, mAU.s) that was determined from chromatographic spectra. The parameters with confidence levels greater than 95% ($p = 0.05$) were considered to affect APA significantly and were included in subsequent optimization using Response surface methodology (RSM). The mathematical correlation was determined using the following first-degree regression equation:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i \quad (1)$$

Where Y was the response (APA, mAU.s); $X_i$ was input variables; $b_0$ was the intercept value; $b_i$ was the first-degree coefficient of each variable.

***Box-Behnken design***

A factorial analysis gathered information based on the significant effects and interaction coefficients between selected factors that positively affect althiomycin production. Three distinct variables were employed in Box-Behnken 15 runs to determine the optimal value of each variable will enhance APA to the maximum extent. The three variables chosen from PB were soluble starch, baker's yeast, and hepes buffer encoded as A, B, and C, respectively. Each factor was optimized at three levels (-1, 0, and +1) in RSM (Table 4).

The response function was chosen as the althiomycin peak region in the extract (mAU.s). A quadratic equation represents modelling:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum \sum_{i<j} b_{ij} X_i X_j + \sum_{i}^{k} b_{ii} X^2 \quad (2)$$

Where Y was the response (APA, mAU.s); $X_i$, $X_j$ were independent factors; $b_0$ was the intercept value; $b_i$ was the first-degree coefficient; $b_{ii}$ was the quadratic coefficient; $b_{ij}$ was the interaction coefficient of each pair of factors.

The data were analyzed using Stat-Ease Inc's Design Expert 11.0.0® software. The appropriate level of components for maximal althiomycin production was determined according to the analysis outcomes.

***Verification experiments***

Three parallel experiments were conducted using the optimal formulation for the fermentation medium to confirm the reliability of the optimization results. The results are the average of three replicates compared to the model's predicted response value.

## RESULTS AND DISCUSSION

***Screening of main factors using PB***

According to the Plackett-Burman analysis, the peak area of althiomycin in the extract ranged from 332 to 1103 mAU.s (Table 2). The Design Expert software was used to statistically calculate the impact of each factor on althiomycin content (Table 3). The factors with positive and considerable influence values, including soluble starch (X1), baker's yeast (X2), and hepes buffer (X8), were selected since they significantly affected the APA at a 95% confidence level. Based on the coefficient of influence, it was found that hepes buffer had the most potent effect on althiomycin production (0.0001), followed by soluble starch (0.0062) and baker's yeast (0.0480). The factors such as glucose, yeast extract, casiton, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, cyanocobalamin, trace solution, and XAD 16N resin were found to have no significant effect on APA at $p < 0.05$. Therefore, we chose soluble starch, baker's yeast and hepes buffer as the

factors that strongly influence the althiomycin peak area on the chromatogram to optimize by RSM experimental design. The model equation for APA (mAU.s) could be written as:

APA (mAU.s) = 190.43 + 35.70X1 +53.58X2 – 52.25X4 + 27.57X8 – 168.33X10

The results of a statistical analysis of experimental data using the ANOVA are presented in Table 3. The coefficient $R^2$ of 0.9483 indicated that the model is significant. This value was comparable to the adjusted $R^2$ value of 0.9052.

**TABLE 2:** The experimental matrix according to Plackett-Burman design

| Run | Factors | | | | | | | | | | | Peak area (mAU.s) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | X1 | X2 | X3 | X4 | X5 | X6 | X7 | X8 | X9 | X10 | X11 | Actual | Predicted |
| 1 | 3 | 0 | 0 | 1 | 0 | 0.5 | 1 | 10 | 0 | 0 | 1 | 550 | 521 |
| 2 | 3 | 0 | 2 | 1 | 3 | 1 | 1 | 23.8 | 0.5 | 1 | 1 | 842 | 817 |
| 3 | 8 | 5 | 0 | 1 | 0 | 1 | 1 | 23.8 | 0.5 | 0 | 2 | 1091 | 1103 |
| 4 | 8 | 5 | 0 | 3 | 3 | 1 | 1 | 10 | 0 | 1 | 1 | 749 | 702 |
| 5 | 3 | 5 | 0 | 3 | 3 | 0.5 | 2 | 23.8 | 0.5 | 0 | 1 | 817 | 820 |
| 6 | 8 | 0 | 0 | 1 | 3 | 0.5 | 2 | 23.8 | 0 | 1 | 2 | 1129 | 1080 |
| 7 | 3 | 0 | 0 | 3 | 0 | 1 | 2 | 10 | 0.5 | 1 | 2 | 289 | 332 |
| 8 | 3 | 5 | 2 | 3 | 0 | 0.5 | 1 | 23.8 | 0 | 1 | 2 | 963 | 904 |
| 9 | 8 | 5 | 2 | 1 | 0 | 0.5 | 2 | 10 | 0.5 | 1 | 1 | 698 | 723 |
| 10 | 8 | 0 | 2 | 3 | 0 | 1 | 2 | 23.8 | 0 | 0 | 1 | 858 | 976 |
| 11 | 3 | 5 | 2 | 1 | 3 | 1 | 2 | 10 | 0 | 0 | 2 | 562 | 628 |
| 12 | 8 | 0 | 2 | 3 | 3 | 0.5 | 1 | 10 | 0.5 | 0 | 2 | 569 | 511 |

**TABLE 3:** Statistical analysis of factors for the Plackett-Burman model

| Factors | Df | Sum of squares | Mean square | Effect | Coefficient estimate | Standard error | f-value | p-value |
|---|---|---|---|---|---|---|---|---|
| Model | 5 | 618394.42 | 123678.88 | | | | 22.02 | 0.0009[a] |
| X1 | 1 | 95586.75 | 95586.75 | 178.5 | 89.25 | 21.64 | 17.02 | 0.0062[a] |
| X2 | 1 | 34454.08 | 34454.08 | 107.17 | 53.58 | 21.64 | 6.13 | 0.0480[a] |
| X3 | 1 | 1474.08 | 1474.08 | -22.17 | -11.08 | 21.64 | - | 0.3754[b] |
| X4 | 1 | 32760.75 | 32760.75 | -104.5 | -52.25 | 21.64 | 5.83 | 0.0522[b] |
| X5 | 1 | 3996.75 | 3996.75 | 36.5 | 18.25 | 21.64 | - | 0.1926[b] |
| X6 | 1 | 9352.08 | 9352.08 | -55.83 | -27.92 | 21.64 | - | 0.1290[b] |
| X7 | 1 | 14076.75 | 14076.75 | -68.5 | -34.25 | 21.64 | - | 0.1168[b] |
| X8 | 1 | 434340.75 | 434340.75 | 380.5 | 190.25 | 21.64 | 77.32 | 0.0001[a] |
| X9 | 1 | 21252.08 | 21252.08 | -84.17 | -42.08 | 21.64 | - | 0.2496[b] |
| X10 | 1 | 4144.08 | 4144.08 | 37.17 | 18.58 | 21.64 | 3.78 | 0.0997[b] |
| X11 | 1 | 660.08 | 660.08 | 14.83 | 7.42 | 21.64 | - | 0.1000[b] |
| Residue | 6 | 33703.83 | 5617.31 | | | | | |
| Total | 11 | 652098.25 | | | | | | |
| $R^2$ - 0.9483; [a] significant at $\alpha = 0.05$; [b] not significant at $\alpha = 0.05$ | | | | | | | | |

The model p-value of 0.0009 indicates that the model was significant. The model f-value of 22.02 indicates that the model is statistically significant, and the probability of the model f-value occurring due to noise is only a 0.09% chance. Each parameter significance was evaluated using p-value (Probability > F). Thus, the "Prob > F" value less than 0.05 indicated that the model terms affecting althiomycin production were significant (APA). X1, X2, and X8 are

important model terms in this circumstance (Table 4).

### *Optimization of factors for maximum APA*

**TABLE 4:** Observed levels of 03 factors used in RSM

| **Factors** | | **Level** | | |
|---|---|---|---|---|
| | | **-1** | **0** | **+1** |
| A | Soluble starch (g/L) | 3 | 5.5 | 8 |
| B | Baker's yeast (g/L) | 0 | 2.5 | 5 |
| C | Hepes (g/L) | 10 | 16.9 | 23.8 |

After identifying the primary factors influencing APA, experimental planning was conducted using RSM, analyzed by Design Expert® 11.0.0. Table 5 presents the experimental and predicted response values according to the model.

**TABLE 5:** Experimental results according to Box-Behnken

| **Run** | **Factors** | | | **Peak area (mAU.s)** | |
|---|---|---|---|---|---|
| | **A** | **B** | **C** | **Actual** | **Predicted** |
| 1 | 8 | 2.5 | 23.8 | 1103 | 1108.1 |
| 2 | 5.5 | 5 | 10 | 782 | 790.0 |
| 3 | 3 | 2.5 | 10 | 501 | 554.4 |
| 4 | 5.5 | 0 | 23.8 | 855 | 831.0 |
| 5 | 5.5 | 0 | 10 | 454 | 433.8 |
| 6 | 8 | 2.5 | 10 | 752 | 710.9 |
| 7 | 3 | 0 | 16.9 | 592 | 596.1 |
| 8 | 5.5 | 5 | 23.8 | 1151 | 1187.3 |
| 9 | 8 | 5 | 16.9 | 1113 | 1108.9 |
| 10* | 5.5 | 2.5 | 16.9 | 1220 | 1218.0 |
| 11 | 3 | 2.5 | 23.8 | 969 | 951.6 |
| 12* | 5.5 | 2.5 | 16.9 | 1243 | 1218.0 |
| 13* | 5.5 | 2.5 | 16.9 | 1191 | 1218.0 |
| 14 | 8 | 0 | 16.9 | 562 | 602.1 |
| 15 | 3 | 5 | 16.9 | 842 | 801.9 |
| (*): Center point | | | | | |

**TABLE 6:** Regression results of the Box-Behnken design

| **Source** | **Sum of squares** | **Df** | **Mean square** | **f-value** | **p-value** |
|---|---|---|---|---|---|
| Model | 1053740.83 | 7 | 150534.40 | 88.96 | < 0.0001[a] |
| A | 48984.50 | 1 | 48984.50 | 28.95 | 0.0010[a] |
| B | 253828.13 | 1 | 253828.13 | 150.01 | < 0.0001[a] |
| C | 315615.13 | 1 | 315615.13 | 186.53 | < 0.0001[a] |
| A * B | 22650.25 | 1 | 22650.25 | 13.39 | 0.0081[a] |
| A * A | 162830.77 | 1 | 162830.77 | 96.23 | < 0.0001[a] |
| B * B | 196599.00 | 1 | 196599.00 | 116.19 | < 0.0001[a] |
| C * C | 115349.77 | 1 | 115349.77 | 68.17 | < 0.0001[a] |

| | | | | | |
|---|---|---|---|---|---|
| Residual | 11844.50 | 7 | 1692.07 | - | - |
| Lack of fit | 10486.50 | 5 | 2097.30 | 3.09 | 0.2625[b] |
| Pure error | 1358.00 | 2 | 679.00 | | |
| Total | 1065585.33 | 14 | | | |
| $R^2$ - 0.9889; C.V. % - 4.63; [a] significant at α = 0.05; [b] not significant at α = 0.05 | | | | | |

Regression analysis was obtained from experimental data, and the following second-order polynomial equation was implied to be used as a model to predict the APA:

$$APA\ (mAU.s) = -1769.68 + 370.80A + 189.63B + 154.27C + 12.04AB - 33.60A^2 - 36.92B^2 - 3.71C^2$$

Whereas APA is althiomycin peak area (mAU.s), A, B, and C are the concentration of soluble starch, baker's yeast, and hepes (g/L), respectively.

Analysis of variance (ANOVA) exhibited p-value ($p < 0.0001$) indicates that the regression model is statistically significant and consistent with the experimental observations (Table 6). The model calculated the corresponding responses with an accuracy of 98.89%, as suggested by the correlation coefficient $R^2$ = 0.9889. The predicted $R^2$ value ($R^2$ = 0.9211) reasonably correlated with the adjusted $R^2$ ($R^2$ = 0.9778). The f-value for Lack of Fit was 0.2625, noticed that the Lack of Fit was not significant.

The model terms such as A, B, C, AB, $A^2$, $B^2$, and $C^2$ were found to have "Prob > F" values < 0.05, suggesting that soluble starch, baker's yeast, and hepes, and the interactions of soluble starch and baker's yeast were significant. 2D contour plots and 3D response surface graphs showing the interaction of these factors were presented in Figure 1c, d. The non-significant regression coefficients ($p > 0.05$) were excluded (AC and BC).

With the objective function of maximum biomass APA, the Design Experts software predicted the optimal parameters: soluble starch concentration of 6.09 g/L, baker's yeast concentration of 4.05 g/L, and hepes buffer concentration of 20.65 g/L. Consequently, the regression equation calculated that the yield of althiomycin obtained from the fermentation medium is 1313 (mAU.s).

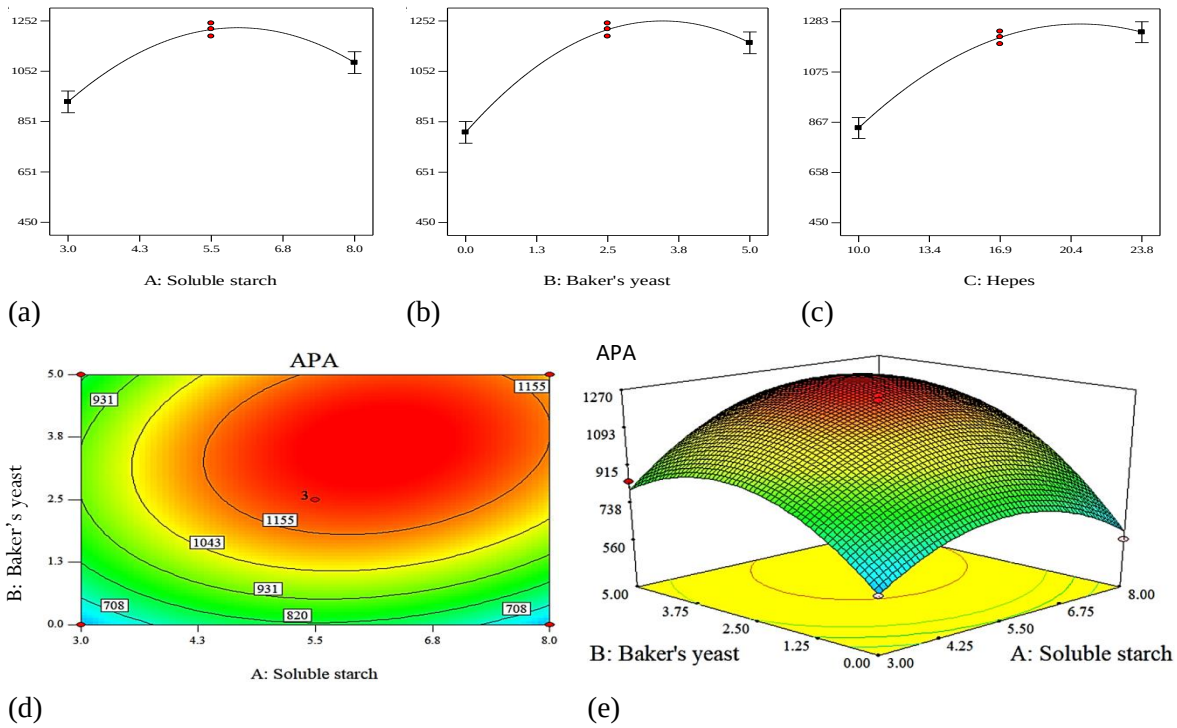


**FIGURE 1:** Factors affecting the althiomycin peak area

J Popul Ther Clin Pharmacol Vol 30(6):e287–e296; 06 April 2023.

a-c: Effect of the concentration of soluble starch (A), baker's yeast (B), and hepes buffer (C) on APA, respectively; d-e: Contour plot and response surface show the interactions between soluble starch (A) and baker's yeast (B) on APA, respectively.

***Verification experiment***

The statistical model was re-evaluated with the optimal values of three key ingredients in actual experiments to determine APA (mAU.s). A confirmation test was performed in triplicate. A linear calibration curve used to estimate the althiomycin concentration (µg/mL) from APA (mAU.s) was $y = 7.669x - 1.215$. The average yield of althiomycin was 1266 (mAU.s) after ten days of fermentation.

## DISCUSSION

This study identified soluble starch, baker's yeast, and hepes buffer as important factors for althiomycin production by Plackett-Burman design. Then these variables were further optimized by RSM. Figures 1a, b, and c show that the line representing the first-order effect of three factors on APA is a straight line at the beginning of the curve with a slope (the regression coefficient is 0.0026-0.0450), indicating that the factors have a proportional impact on APA. In contrast, the regression coefficients for all three factors are -33.60, -36.92, and -3.71 for soluble starch, baker's yeast and hepes, respectively (p-value = 0.05), deducing that there are adverse second-order effects. When the concentrations of these factors are high within the survey range, the APA/ althiomycin concentration tends to decrease. The highest regression coefficient of hepes (154.27) exhibits the greatest impact on APA.

The response surface between the two elements (A and B) reveals the remarkable influence of soluble starch and baker's yeast on althiomycin concentration as a convex pattern with a downward opening in graphical representations (Figure 1d, e).

Starch is significant organic carbon and energy source for some microorganisms and plays an important role in microbial metabolism. There are reasonable explanations for which the polysaccharide (soluble starch) played a more prominent role compared with glucose in the screening model. Firstly, in the mixture of many carbon sources, a simple carbohydrate (like glucose) is rapidly assimilated during the exponential phase to multiply cultured cells. After glucose was depleted, starch, known as a slowly metabolized carbon source, was used (19). Simultaneously, secondary synthesis frequently accumulates at the growth curve's steady phase (20). In detail, antibiotic formation usually occurs during the late growth phase, commonly caused by nutrient deficiency and/or growth retardation (19). Secondly, some members of the genus Myxococcus may not use effectively mono- and disaccharides as a source of carbon and energy, as some studies reported on members of *Myxococcus xanthus* (21, 22).

Baker's yeast is a source of microbial cells, which myxobacteria would normally lyse for feeding (23). Enzymatic systems are responsible for degrading biological macromolecules, necessitating multicellular communication as the cell density in the culture medium increases (24). The medium supplemented with microorganisms such as bacteria or yeasts has been fully determined with the necessary nutritional composition for the growth of some myxobacterial strains. All bacteriolytic myxobacteria require other microbial cells for peptides and amino acids for nitrogen, carbon, and energy (25). As a result, a relatively large amount of ammonia is released and severely limits growth and yield (23). Therefore, the stabilization of pH can account for the increase in althiomycin production that was induced by the hepes buffer. Indeed, hepes, a crucial organic buffer with a pH range of 6.8-8.0, is frequently used to maintain the ideal condition for Myxobacterial growth and the biosynthesis of secondary metabolites during fermentation (23).

Althiomycin accumulation in the post-optimized medium was higher during cultivation and came to 1266 (mAU.s), compared with 1313 (mAU.s) of prediction. After validation of the optimal model, the difference in APA values between the predicted and the test model was found to be

96.4%, proving that the model was well compatible with the experiments. A linear calibration curve was used to convert the experimental APA (mAU.s) to althiomycin concentration (mg/L) in culture media, which resulted in the equivalent value of 3.3 mg/L. This value is 4.7 times greater compared with the althiomycin content cultured on original peptone media (0.7 mg/L) and 1.2 times greater than the production capacity of *Cystobacter fuscus* (2.7 mg/L) (5).

## CONCLUSION

Based on optimizing the fermentation medium based on an evolutional algorithm, the production of althiomycin by *M. stipitatus* GL41 was greatly increased in this work. Significant factors in improving althiomycin content include soluble starch, baker's yeast, and hepes buffer. The optimal fermentation medium contained 6.09 g/L of soluble starch, 4.05 g/L of baker's yeast, and 20.65 g/L of hepes buffer, with the maximum peak area of althiomycin reaching 1266 (mAU.s), equivalent to 3.3 mg/L, which is approximately 4.7 times higher than the initial medium. The finding suggests that medium optimization is feasible to enhance althiomycin production.

## ACKNOWLEDGEMENTS

None.

## CONFLICT OF INTEREST

None.

***Financial support***

Yen Thi Ngoc Nguyen was funded by the Master, PhD Scholarship Programme of Vingroup Innovation Foundation (VINIF), code VINIF.2022.TS150.

***Ethics statement***

None.

## REFERENCES

1. Landwehr W, Wolf C, Wink J. How to overcome the antibiotic crisis. United States: Springer, 2016.
2. Herrmann J, Abou Fayad A, Müller R. Natural products from myxobacteria: novel metabolites and bioactivities. Nat Prod Rep. 2017;34(2):135-60. https://doi.org/10.1039/c6np00106h
3. Saadatpour F, Mohammadipanah F. Bioprospecting of indigenous myxobacteria from Iran and potential of Cystobacter as a source of anti-MDR compounds. Folia Microbiol (Praha). 2020;65(4):639-48. https://doi.org/10.1007/s12223-019-00768-2
4. Yamaguchi H, Nakayama Y, Takeda K, et al. A new antibiotic, althiomycin. J Antibiot (Tokyo). 1957;10(5):195-200. https://doi.org/10.11554/antibioticsa.10.5_195
5. Kunze B, Reichenbach H, Augustiniak H, et al. Isolation and identification of althiomycin from Cystobacter fuscus (Myxobacterales). J Antibiot (Tokyo). 1982;35(5):635-6. https://doi.org/10.7164/antibiotics.35.635
6. Inami K, Shiba T. Total synthesis of antibiotic althiomycin. Bull Chem Soc Jpn. 1985;58(1):352-60. https://doi.org/10.1016/S0040-4039(01)90099-1
7. Fujimoto H, Kinoshita T, Suzuki H, et al. Studies on the mode of action of althiomycin. J Antibiot (Tokyo). 1970;23(6):271-5. https://doi.org/10.7164/antibiotics.23.271
8. Kirst HA, Szymanski EF, Dorman DE, et al. Structure of althiomycin. J Antibiot (Tokyo). 1975;28(4):286-91. https://doi.org/10.7164/antibiotics.28.286
9. Cortina NS, Revermann O, Krug D, et al. Identification and characterization of the althiomycin biosynthetic gene cluster in Myxococcus xanthus DK897. Chembiochem. 2011;12(9):1411-6. https://doi.org/10.1002/cbic.201100154
10. Bode HB, Müller R. Analysis of myxobacterial secondary metabolism goes molecular. J Ind Microbiol Biotechnol. 2006;33(7):577-88. https://doi.org/10.1007/s10295-006-0082-7
11. Rouhizohrab N, Mohammadipanah F. Suppression of predominant interfering bacteria in the purification process of myxobacteria. Iran J Microbiol. 2022;14(5):721-9. https://doi.org/10.18502/ijm.v14i5.10968

12. Muñoz-Dorado J, Marcos-Torres FJ, García-Bravo E, et al. Myxobacteria: moving, killing, feeding, and surviving together. Front Microbiol. 2016;7:781. https://doi.org/10.3389/fmicb.2016.00781
13. Findlay BL. The chemical ecology of predatory soil bacteria. ACS Chem Biol. 2016;11(6):1502-10. https://doi.org/10.1021/acschembio.6b00176
14. Vanaja K, Shobha Rani R. Design of experiments: concept and applications of Plackett Burman design. Clin Res Regul Aff. 2007;24(1):1-23. https://doi.org/10.1080/10601330701220520
15. Dean A, Voss D, Draguljić D, et al. Design and analysis of experiments. 10th ed. United States: Springer, 2017.
16. Keskin Gündoğdu T, Deniz İ, Çalışkan G, et al. Experimental design methods for bioengineering applications. Crit Rev Biotechnol. 2016;36(2):368-88. https://doi.org/10.3109/07388551.2014.973014
17. Mason RL, Gunst RF, Hess JL. Statistical design and analysis of experiments: with applications to engineering and science. United States: John Wiley & Sons, 2003.
18. Murthy HN, Lee E-J, Paek K-Y. Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. Plant Cell, Tissue and Organ Culture. 2014;118:1-16. https://doi.org/10.1007/s11240-014-0467-7
19. Sánchez S, Chávez A, Forero A, et al. Carbon source regulation of antibiotic production. J Antibiot (Tokyo). 2010;63(8):442-59. https://doi.org/10.1038/ja.2010.78
20. Ali S, Syeda T, Syeda F, et al. Strategies and kinetics of industrial fermentation for the mass production of various primary and secondary metabolites from microbes. Eur J Pharm Med Res. 2018;6:595-606.
21. Bretscher AP, Kaiser D. Nutrition of Myxococcus xanthus, a fruiting myxobacterium. J Bacteriol. 1978;133(2):763-8. https://doi.org/10.1128/jb.133.2.763-768.1978
22. Watson BF, Dworkin M. Comparative intermediary metabolism of vegetative cells and microcysts of Myxococcus xanthus. J Bacteriol. 1968;96(5):1465-73. https://doi.org/10.1128/jb.96.5.1465-1473.1968
23. Shimkets LJ, Dworkin M, Reichenbach H. The myxobacteria. United States: Springer, 2006.
24. DeLong EF, Lory S, Stackebrandt E, et al. The Prokaryotes: Deltaproteobacteria and Epsilonproteobacteria. Germany: Springer Berlin Heidelberg, 2014.
25. Rosenberg E. Myxobacteria: development and cell interactions. United States: Springer, 2012.