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STATISTICAL OPTIMIZATION OF ERYTHROMYCIN PRODUCTION BY *Saccharopolyspora erythraea* UNDER SOLID STATE FERMENTATION OF AGRO-INDUSTRIAL MATERIALS USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

In this work, erythromycin production by *Saccharopolyspora erythraea* NCIMB 12462 was investigated under solid state fermentation (SSF) using beet sugar root (BSR) and agro-industrial materials. Among them oat meal (OM) has given maximum yield. The combination of OM with BSR (3:3 g/flask) proved to be an efficient mixture for erythromycin production as they gave the highest erythromycin level (6531 µg/flask) when compared to BSR and peptone. Supplementation of BSR and OM mixtures with sugarcane bagasse (SCB) as inert support with different inoculum size and additional water enhanced erythromycin production with 3.1-fold higher production than BSR- OM medium. However, increasing SCB content more than 3g/flask always showed an inhibitory effect on erythromycin production. Box-Behnken response surface methodology was applied to further optimize SCB content, inoculum size and moisture level for erythromycin production. Statistical analysis of the results showed that the linear of the selected terms, their interactions and quadric terms of these three variables had significant effects. The moisture level was the key factor influencing erythromycin production, due to its largest *F*-value (48.01) and the lowest *p*-value (0.0002) among the three variables. The interactions between SCB content and moisture level had also the most significant effect (*F*-value = 32.51) on erythromycin production. The optimized medium produced 29884 ug/flask of erythromycin under SSF, which is 1.1% higher than the unoptimized medium.

Keywords: Box-Behnken design, Erythromycin, Medium optimization, Solid state fermentation, *Saccharopolyspora erythraea* NCIMB 12462

INTRODUCTION

Erythromycin, a 14-member lactone ring belongs to macrolide family, is commercially produced by submerged fermentation (SmF) (**El-Enshasy** *et al.*, **2008**; **Zou** *et al.*, **2009** and **2010**). However, SmF process requires high energetic expenditures. It is widely used in pharmaceutical and veterinary applications in treatment of respiratory infectious diseases (**Hoyt and Robbins**, **200**1) caused by bacteria. The annual production of erythromycin is about 4000 tons. Most of the produced erythromycin is chemically converted into some semi synthetic derivatives such as azithromycin, clarithromycin and roxithromycin **(Minas, 2005**). Moreover, great and continuous efforts are being made to decrease its production cost by process optimization using agro-industrial by-products through different fermentation processes in submerged and solid state cultures **(Zou** *et al.*, **2010**; **Hamedi** *et al.*, **2015**; **Said** *et al.*, **2017**). In the search for more economical fermentation processes with high antibiotic activity, solid-state fermentation (SSF) has gained interest in recent years due to the advantages that presents over SmF such as higher product yields, less energy requirements, easier aeration, less waste water generation, reduced bacterial contamination and easier product recovery (**Pandey, 2003**; **Pérez-Guerra** *et al.*, **2003**; **Nigam and Pandey, 2009**).Agro-industrial wastes rich in sugars, cellulose, hemicellulose, proteins, minerals and microelements have been reused as solid support in SSF processes by microorganisms both from environmental and economical view points for the production of different value-added compounds (**Barrios-Gonzalez and Mejia, 1996; Dominguez** *et al.*, **2001; Mahalaxmi** *et al.*, **2010; Vastrad and Neelagund, 2011 and 2014**). Hence, most of the agricultural wastes and wastes from industrially processed foods have been exploited as solid substrates for the production of most important antibiotics (**Arumugam** *et al.***, 2014**).
An attempt was done to optimize erythromycin production by *S. erythraea* NCIMB 12462 grown under semi-solid-state fermentation conditions in a medium containing BSR as solid substrate (**Farid** *et al.*, **2015**; **Noor El Deen** *et al.*, 2015) Therefore, an extended study on the effect of addition agro-industrial by-products as carbon- nitrogen source and inert support on the production of erythromycin was investigated in SSF. Subsequently, the most factors affecting erythromycin production were optimized using Box–Behnken design and response-surface analyses. To the best of our knowledge, there is not enough information concerning optimization of erythromycin production by *S. erythraea* in SSF using statistical experimental designs.

MATERIAL AND METHODS

Microorganisms

Saccharopolyspora erythraea NCIMB 12462 used in this study was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK. This strain was maintained on starch nitrate agar medium of the following composition (g/L): starch, 20; $NaNO_3$, 2; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.5; $CaCO_3$, 3; $FeSO_4.7H_2O$, 0.01; agar, 20. The pH was adjusted to 7.0 before sterilization. The slants were inoculated and incubated at 30-32°C for 10 days in order to obtain a heavy sporulated growth. After that time, spores were suspended in 20% (w/v) glycerol and stored in vials at –86°C.
Bacillus subtilis NRRL B-543 was obtained from Northern Regional Research Laboratory (NRRL, Peoria, Illinois, USA). This bacterium was used as a test organism for the determination of the produced erythromycin by *S. erythraea* NCIMB 12462 using the agar diffusion method.

Solid state fermentation

The composition of the semi solid medium used for production of erythromycin was as described before (**Farid** *et al.*, **2015**) using ten grams of homogenized fresh BSR (equivalent to 3g dry BSR) moistened with 10 mL mineral salt solution composed of (g/L): $CaCO_3$ 5; NaCl 2.5; pH 7, and autoclaved at 121°C for 30 min in a 250 mL Erlenmeyer flask. Each flask was inoculated with the prepared inoculum and incubated at 30-32°C for 10 days. The moisture content of the medium after inoculation was 84.33% including the moisture content of BSR. Unless otherwise specified, these fermentation conditions were maintained throughout the experiment. All experiments were performed in duplicate.

Inoculum preparation

The spores from a fully sporulated 10-day-old slant of *S. erythraea* NCIMB 12462 grown on ISP-2 agar slants at 28°C were dispersed in 5 mL of sterile distilled water by dislodging them with a sterile loop under aseptic conditions. The spore suspension was used as inoculum for each 250 mL Erlenmeyer flask containing the semi solid medium. Unless otherwise stated, for pure culture, each flask containing 10 g fresh BSR substrate was inoculated with 5 mL spore suspension (10^6–10^7 spores/mL). Spore count was measured using the dilution plate count method (**Parkinson** *et al.*, **1971**).

Effect of inoculum size

Different inoculum sizes of *S. erythraea* NCIMB 12462 were used to inoculate sterile solid medium. The inoculum sizes used were 2.5 $X10^6$, 5x 10^6 and 5 x 10^{12} spore/mL. Unless otherwise specified, the moisture content of the medium after inoculation was 84.33% including the moisture content of BSR. Incubation was at 30-32°C for 10 days with stirring once daily. The samples were analysed for pH change and bioassay of erythromycin.

Effect of different addition of agricultural wastes on erythromycin production

Different agricultural wastes like black sun flower seeds, wheat bran, OM, corn bran (collected from local markets of Cairo, Egypt) and SCB (obtained from Egyptian Sugar and Integrated Industries Company, Giza, Egypt) were oven-dried at 65°C for one day to reduce the moisture content and then milled to achieve the size of less than 1 cm. Corn steep liquor (CSL) obtained from the Egyptian Starch-Glucose Factory, Mostorod, Cairo, Egypt was used in different volumes. The different wastes were added at two concentrations (0.5 and 1.0 g/flask) separately on the same BSR medium. Among the feeds tested addition of CSL with concentration of 0.5 and 1mL/flask besides the other component of BSR medium. All experiments were compared with control cultures without addition of agricultural wastes. Based on addition optimization results, the best agro waste were applied on the aforementioned SSF during fermentation time. Erythromycin titers have been measured throughout the whole process.

Effect of different levels of SCB and moisture level with BSR and OM on erythromycin production

In these experiments different weights (1, 2, 3 and 4 g/flask) of SCB (as inert support) with different excess water (2.5, 5 and 7.5 mL/g SCB) were used with BSR 10g/flask, and oatmeal 3g/flask as carbon and nitrogen sources. The inoculum size was used as 1mL/g SCB as follows;
Series A consists of 1g of SCB with different water (2.5, 5 and 7.5mL).
Series B consists of 2g SCB with different water (5, 10 and15 mL).
Series C consists of 3g SCB with different water (7.5, 15 and 22.5 mL).
Series D consists of 4g SCB with different water (10, 20 and 30 mL).

Optimization of erythromycin production by response surface methodology Box–Behnken design

From the previous study of the variables, the most significant factors for erythromycin production were further optimized by the Box–Behnken statistical design (**Box** and **Behnken, 1960**).They were further analyzed at three levels of concentration to find out the most optimal values for producing erythromycin. The three levels were coded as -1, 0 and +1 representing low, middle and high concentrations, respectively, as shown in Table 4. According to the design, 17 combinations were tested and their observations were fitted to the following second order equation as represented in Eq. 1 as follows,

$$Y=\beta 0+\beta iXi+\beta ijXiXj+\beta iiXi^2 \quad (1)$$

Where Y is the predicted response variable; β0, βi, βii, βij are constant regression coefficients of the model, and Xi, Xj (i=1, 3; j=1, 3, i j) represent the independent variables in the form of coded values. Statistical software package Design-Expert (Version 8.0.2, State-Ease, Minneapolis, MN, USA) was used to design and analyze the experiment. A 2^3 factorial design, with five replicates at the centre point with total number of 17 trials, was employed. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2.

Erythromycin extraction and bioassay

Extraction of erythromycin from fermented culture was carried out according to **Farid** *et al.* (**2015**) as follows: At the end of fermentation process, the fermented matter in the whole flask was mixed with 20 mL of distilled water and shaken in an orbital shaker 200 rpm at room temperature for 1 h. The whole content of each flask was centrifuged at 5000 rpm for 15 min at 4°C and the final clear supernatant was used as the antibiotic source**.**

Erythromycin assay

The agar diffusion bioassay method **(Perez** *et al.*, **1990**) that utilizes the antibacterial property of erythromycin to produce a zone of inhibition against *B. subtilis* was used. 100µl of clear extract filtrate fill the agar hole (0.9 mm diameter) punched in the nutrient agar plates (The antibiotic assay medium (Difco) composed of (g/L): glucose, 10.0; peptone, 10.0; meat extract, 2.5; yeast extract, 5.0; NaCl, 10.0 and agar, 20.0) freshly seeded with 0.1 mL of *Bacillus subtilis* NRRL B-543 strain of 24h old as the test organism. The inhibition zone diameter was measured in mm after incubation of plates at 30°C for 24 h and the concentration of erythromycin was calculated through standard erythromycin (Sigma Aldrich) calibration curve. All experiments were conducted in duplicate, and the mean of the two reading is represented as micrograms of produced erythromycin.

RESULTS AND DISCUSSION

Evaluation of different agro-industrial wastes for erythromycin production

This work was started by evaluating the production of erythromycin using different agro industrial wastes with BSR as the main carbon source. The results in Table 1 showed that all the used substrates had effect on erythromycin production in spite of variation in the degree of production. Maximum erythromycin production (3239.98 µg/flask) was achieved with added OM followed by sunflower seed and barley (2594.96 and 2405.19 µg/flask, respectively) while the minimum production was noticed with CSL, wheat bran and corn bran. Furthermore, combination between BSR with different concentrations of OM to enhance the erythromycin production was tested. This combination enhanced the antibiotic production up to 6531ug/flask; hence mixed substrate was used for further studies (Table 2).

Table 1 Effect of different agriculture byproducts as carbon and nitrogen source on erythromycin production

Treatment	Agr.by-product g/flask	Final pH	Total water* mL	Erythromycin µg/flask
Control 1		7.8	22	1067.78
Control 2		7.8	22	2078.35
Sunflower seed	1.0	6.6	22	2594.96
	0.5	7.5	22	1294.96
Corn steep liquor**	1.0	8.3	22	1235.70
	0.5	8.0	22	1024
Wheat bran	1.0	7.6	22	1664.58
	0.5	7.5	22	855.20
Oat meal	1.0	7.6	22	3239.98
	0.5	7.5	22	2078.347
Corn bran	1.0	7.3	22	1333.19
	0.5	7.0	22	634.85
Barley	1.0	7.3	22	2405.19
	0.5	7.3	22	989.69

Control 1: 10 grams of homogenized fresh BSR (equivalent to 3g dry BSR) moistened with 10 mL mineral salt solution and 0.04g/flask peptone, pH 7.
Control 2: 10 grams of homogenized fresh BSR (equivalent to 3g dry BSR) moistened with 10 mL mineral salt solution and 0.08 g/flask peptone, pH 7.
Total water* 7 mL +5 mL Inoc. size +10 mL water
Extraction was carried out using 20 mL water/flask.
** Corn steep liquor was added in mL

By comparing the process parameters between the medium with peptone, OM and the medium with CSL, the differences in erythromycin yield were observed. These phenomena could result from the difference in terms of the ingredients between peptone; OM and CSL. It was well known that the soluble phosphate played an important role in fermentation process optimization and erythromycin biosynthesis. The previous data showed that the phosphorus content in CSL was greater than in the other ingredients. In addition, the organic acids in CSL should possibly play a role in the cell growth (**Zhang** *et al.*, **2014**). Compared with conventional nitrogen products such as soybean flour, peptone, and yeast extract, agro-industrial waste materials possesses more stable quality, considerable nitrogen, carbon and minerals content and their price is cheaper than conventional nitrogen products.

Table 2 Effect of different concentrations of OM on erythromycin production

Oat conc. g/flask	Final pH	Total water* mL	Erythromycin µg/flask
0.25	7.4	22	
0.5	7.4	22	407
1	6.9	22	1543
2	7.5	22	3260
3	7.0	22	6531
4	6.0	22	4682
5	7.4	22	32.6

Total water* 7 mL +5 mL Inoc. size +10 mL dist. water
Extraction was carried out using 20 mL/flask.

Effect of different levels of SCB and moisture level with BSR and OM on erythromycin production

Natural substrates specially those contain starch, however, have a major disadvantage. During the growth of the microorganism, the solid medium is degraded, and as a result, the geometric and physical characteristics of the medium change (**Ooijkaas** *et al.*, **2000**). Oats, for example, shrink owing to the degradation of starch and the evaporation of water, and consequently channel formation can occur resulting in reduced mass and heat transfer (**Weber** *et al.*, **1999**). This disadvantage can be overcome by the use of an inert support with a more or less constant physical structure throughout the process, enabling improved control of heat and mass transfer. So, different weights of SCB (as inert support) with different excess water were used with fresh BSR 10g/flask, and OM 3g/flask as carbon and nitrogen sources. The inoculum size was used as indicated in Table 3.

The results in Table 3 indicate that increasing inoculum/g dry substrate (3 g dry BSR and 3 g OM) ratio strongly stimulated erythromycin production in cultures of Series A, B and C, but this operation showed a strong negative effect on the cultures of Series D. On the other hand, regarding to inoculum/g inert support (SCB) maximum production of erythromycin was recorded for series B and C. The decrease in nutrients % had a negative effect on erythromycin production in all cultures series. However,

when SCB content was increased erythromycin production always increased in cultures of Series A, B and C, but decreased in Series D. Maximum production of erythromycin in cultures of Series A, B, and C (9907, 19814 and 20502 ug/flask, respectively) was obtained at moisture % of 65-68, 70-75 and 73.5, and nutrient % 30-26.6, 22.2- 18.75 and 17.6, respectively. The higher erythromycin production obtained using SCB as natural inert support was likely due to the porosity of the SCB which allowed a better diffusion of oxygen and soluble nutrients from BSR and OM into the cultures during fermentation process, thus favoring the production of the antibiotic. Distribution of substrates particles (BSR and OM) in the presence of SCB in the fermentation process would provide a larger surface area per volume and allow full contact of microorganism with the nutrients in cultures of Series A, B and C but the diffusion of oxygen may be affected in series D. However, the most important finding was that water content and nutrients concentration are not individually responsible for the control of erythromycin production in SSF. It was demonstrated that high values of water content can stimulate or inhibit erythromycin production, depending on the strategy used. Conversely, increasing SCB content showed an inhibitory effect on erythromycin production. This means that SCB content strongly controls erythromycin production in this SSF system. Similarly, the thickness of the inert support layer, SCB in this fermentation, under any natural fermentation conditions plays a key role in the desired end product formation due to varied temperature gradients and O_2 transfer implications (**Raghavarao** *et al*., **2003**). It was reported that at larger bed thicknesses, the oxygen concentration at the bottom of the bed falls to zero within first 24 hrs of fermentation depending on the height of the bed which leads not only to inefficient use of the substrates but also to undesirable situation like anaerobiosis and cell lysis (**Raghava Rao** *et al.*, **1993**). On the other hand, the action of extracellular enzymes produced by microorganisms in degrading the solid state substrate into soluble fragments is a key factor in SSF (**Knapp and Howell**, **1980**). The outer surface of SCB may act not only as inert support but also as immobilized support for both microorganism and exoenzymes which facilitate erythromycin production.

Table 3 Variation of bagasse and moisture content on erythromycin production

Treatment	SCB g/flask	Inoc. mL	Added water mL/flask	Water %	Nutrients %	Nutrients /SCB (w/w)	Eryth. µg/flask
Series A							
1	1.0	1.0	2.5	60.00	34.28	6	7102.62
2			5.0	65.00	30.00	6	9907.24
3			7.5	68.88	26.66	6	9907.24
Series B							
1	2.0	2.0	5	63.63	27.27	3	9907.24
2			10	70.37	22.22	3	19814.00
3			15	75.00	18.75	3	19814.00
Series C							
1	3.0	3.0	7.5	66.03	22.64	2	18243.00
2			15	73.53	17.60	2	20502.09
3			22.5	78.31	14.45	2	16420.78
Series D							
1	4.0	4.0	10	67.74	19.35	1.5	2402.63
2			20	75.61	14.63	1.5	3707.08
3			30	80.31	11.76	1.5	2969.07

BSR 10g/flask and OM 3g/flask
Nutrients %= Total dry substrates (BSR+OM)/ (BSR+ OM+ Bagasse + Total water + Inoc.).

Optimization of erythromycin production by response surface methodology

In order to examine for the optimum combination of major process factors, using Box Behnken experimental design, a total of 17 experiments with different combinations of sugarcane SCB (inert support), inoculum size and added water were performed according to the Box Behnken experimental plan. The actual values and coded values of the sovereign process parameters are given in Table 4.

Table 4 Coded values of variables used in Box-Behnken design

Independent variables	Level		
	-1	0	1
X_1: SCB g/flask	2	3	4
X_2: Inoculum mL/flask	2.5 x 10^6	5x 10^6	5x10^{12}
X_3: Added water mL/flask	17.5	20	22.5

The experimental results of studying the effects of the three parameters on erythromycin production are presented in Table 5 along with the observed and predicted response. The results were analyzed using the analysis of variance (ANOVA) as competent to the experimental design used. The calculated regression equation for the optimization of process parameters showed erythromycin production units were functions of the levels of SCB content (X_1) inoculum volume (X_2) and the moisture content (X_3).

$$Y=+27056.94 - 1171.32X_1 + 837.65X_2 - 2190.13X_3 - 1867.35X_1X_2 - 2548.70X_1X_3+1190.10X_2X_3 +1092.507X_1^2 2197.8017X_2^2-2553.7917X_3^2 \quad (2)$$

Erythromycin yield varied from 17135.1 to 29884 µg/flask according to different levels of the selected factors. The lowest yield was observed for run No. 5 while the highest yield was obtained with run No. 12. The positive coefficient of X_2, quadratic term X_1 and the interaction X_2X_3 indicate linear effect to increase erythromycin production, while negative coefficients for X_1 and X_3 show a linear effect to decrease enzyme production. However, quadratic terms (X_2 and X_3) have also negative effects along with interaction terms (X_1X_3 and X_2X_3) that

decrease erythromycin production. These results suggest that the moisture content strongly affect erythromycin yield.

Table 5 Box-Behnken design matrix with experimental and predicted values of erythromycin production by *S. erythraea* NCIMB 12462 under SSF

Std. run	Run	X_1 SCB g/flask	X_2 Inoculum mL/flask	X_3 Moisture mL/flask	Erythromycin yield µg/flask	
					Experimented	Predicted
1	1	-1	-1	0	24795.9	24417.98
4	2	1	1	0	23372.7	23750.63
12	3	0	1	1	21512.5	22142.98
5	4	-1	0	-1	25400	26408.40
11	5	0	-1	1	17135.1	18087.47
9	6	0	-1	-1	25478.4	24847.92
14	7	0	0	0	27056.9	27056.90
15	8	0	0	0	27056.9	27056.90
8	9	1	0	1	20693.9	19685.50
10	10	0	1	-1	25095.4	24143.03
16	11	0	0	0	27056.9	27056.90
3	12	-1	1	0	29884	29827.89
2	13	1	-1	0	25754	25810.03
17	14	0	0	0	27056.9	27056.94
6	15	1	0	-1	28588.7	29163.15
13	16	0	0	0	27056.9	27056.94
7	17	-1	0	1	27700	27125.55

The statistical significance of Eq. (2) was checked by *F*-test, and the analysis of variance (ANOVA) for the response surface quadratic model is shown in Table 6. The Model F-value of 21.39 implies the model is significant. There is only a .03% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case X_1, X_2, X_3, X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2 and X_3^2 are significant model terms. The Student *t*-distribution and the corresponding *P* value, along with the parameter estimate, are given in Table 6. The *P*-values are used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The parameter estimates and the corresponding *P*-values showed that among the independent variables, X_1 (SCB content), X_2 (inoculum volume) and X_3 (moisture content) their quadratic and interaction terms had a significant effect on erythromycin production. It was also included that X_3 (moisture content) was the key factor influencing erythromycin production, due to its largest F-value (48.01) among the three variables. The quadric term of these three variables also had a significant effect. As could be seen, evident interactions existed in X_1, X_2 and X_3, were found to contribute to the response at a significant level, and also could be seen from the P values in Table 4.

Table 6 Regression analysis for erythromycin production for quadratic response surface model fitting (ANOVA)

Source	Sum of squares	Degrees of freedom	Mean squares	F Value	p-value Prop>F
Model	1.539E+008	9	1.710E+007	21.39	0.0003*
X_1	1.098E+007	1	1.098E+007	13.73	0.0076*
X_2	5.613E+006	1	5.613E+006	7.02	0.0329*
X_3	3.837E+007	1	3.837E+007	48.01	0.0002*
X_1 X_2	1.395E+007	1	1.395E+007	17.45	0.0042*
X_1 X_3	2.598E+007	1	2.598E+007	32.51	0.0007*
X_2 X_3	5.665E+006	1	5.665E+006	7.09	0.0324*
X_1^2	5.026E+006	1	5.026E+006	6.29	0.0405*
X_2^2	2.034E+007	1	2.034E+007	25.45	0.0015*
X_3^2	2.746E+007	1	2.746E+007	34.36	0.0006*
Residual	5.595E+006	7	7.992E+005		
Lack of fit	5.595E+006	3	1.865E+006		
Pure error	0.000	4	0.000		
Cor. Total	1.595E+008	16			

Std. Dev.	894.00	R-Squared	0.9649
Mean	25335.02	Adj R-Squared	0.9198
C.V. %	3.53	Pred R-Squared	0.4387
PRESS	8.952E+007	Adeq Precision	17.123

Note: X_1: SCB; X_2: Inoculum; X_3: Moisture. Cor.: Correlation, * Values of "probability >F" less than 0.05 indicate model terms are significant.

So, compared with the traditional 'one-variable at- a-time' approach which is unable to detect the frequent interactions occurring between two or more factors although they often do occur, RSM has immeasurable effects and tremendous advantages.

The closer the value of R-Squared to 1, the better is the correlation between the experimental and predicted values. Value of R^2 0.9649 demonstrating reasonable correlation erythromycin predicted and experiential outcome. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 17.123 indicates an adequate signal. Compared with that under the original culture condition, erythromycin production increased 54.64%.

The strong agreement between the yield predicted by the final quadratic model and the experimental results (Table 5), and the variance analysis of the second-order polynomial model and the value for lack of fit (Table 6) indicate that the accuracy and general ability of the polynomial model are very good. The analysis of response trends using the model is considered to be reasonable. Design Expert predicted the maximum erythromycin yield to be 29827.89 µg/g of substrate in optimized medium which is very close to the actual level of erythromycin produced in the optimized medium, which was 29884µg/flask. Erythromycin yield in the initial medium before optimization was only 27056.9 µg/flask, which is 1.1 fold less compared to the optimized level.

The three-dimensional response surface plots described by the regression model were drawn to illustrate the effects of the independent variables and the interactive effects of each independent variable on the response variables. These plots showed the effects of two factors on the response at a time when the third variable was fixed (0 level). The shape of the corresponding contour plots indicates whether the mutual interactions between the independent variables are significant or not. An elliptical nature of the contour plots indicates that the interactions between the independent variables are significant. Since interactions between the three independents are observed from Table 6, elliptical contour plots are found from Figs. 1a, 1b and 1c. From the three-dimensional response surface plots and the corresponding contour plots, the optimal values of the independent variables could be observed, and the interaction between each independent variable's pair can be easily understood. The interactions between the three parameters (SCB content, inoculum size and moisture content) and erythromycin production were revealed by response surface plots and contour plots, as shown in Figure 1. Figure 1a represents the effects of SCB content and inoculum size levels individually and their mutual interaction on the erythromycin production. Varying SCB content and inoculum size concentration mutual interactions had a significant effect on the erythromycin production value. The increase in SCB content and inoculum size concentrations enhanced the production of erythromycin initially, but then, with increasing their concentrations further, the erythromycin production could decrease. The highest response value was observed at 2g SCB/flask and $5x10^{12}$ mL spore/flask (Figure 1a). However, the increase in SCB content and decreasing moisture content has similar phenomenon as observed in Figure 1b.From the three-dimensional response surface plots and the corresponding contour plots, the optimal values of the independent variables could be observed, and the interaction between each independent variable's pair can be easily understood. The interactions between the three parameters (SCB content, inoculum size and moisture content) and erythromycin production were revealed by response surface plots and contour plots, as shown in Figure 1. Figure 1a represents the effects of SCB content and inoculum size levels individually and their mutual interaction on the erythromycin production. Varying SCB content and inoculum size concentration mutual interactions had a significant effect on the erythromycin production value.

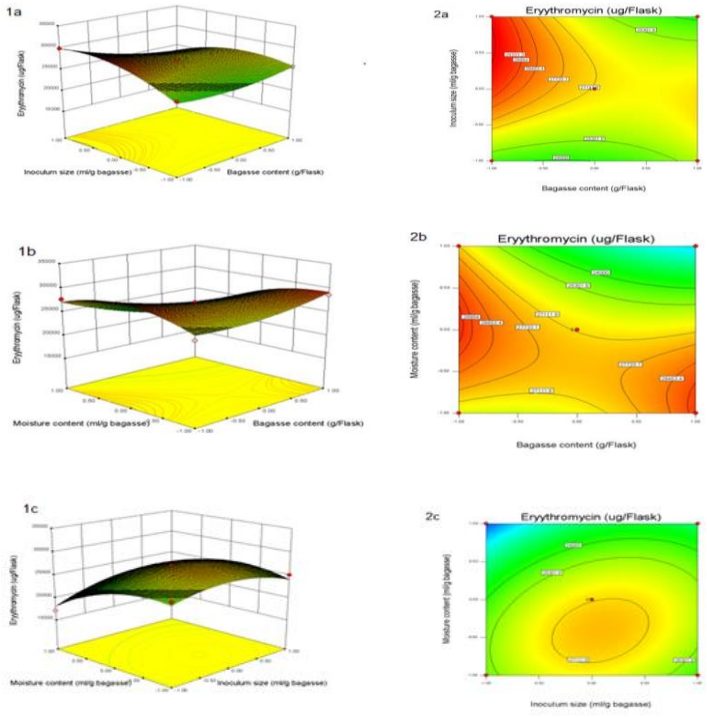

Figure 1 Response surface plot and contour plot of the combined effects of:
a) Bagasse content and inoculum size with constant moisture content,
b) Bagasse content and moisture content with constant inoculum size,
c) Inoculum size and moisture content with constant bagasse content, on erythromycin production by *S. erythraea* NCIMB 12462 under SSF using BSR, OM and SCB.

The increase in SCB content and inoculum size concentrations enhanced the production of erythromycin initially, but then, with increasing their concentrations further, the erythromycin production could decrease. The highest response value was observed at 2g SCB/flask and $5x10^{12}$ mL spore/flask (Figure 1a). However, the increase in SCB content and decreasing moisture content has similar phenomenon as observed in Figure 1b.
Fig. 1c represents the interaction between inoculum size and moisture level. Lower and higher levels of both variables did not result in higher erythromycin yields. The shape of the response surface curves showed a moderate interaction between these tested variables.

CONCLUSION

The results showed the use of cheap agro-residues as substrate for fermentation production of erythromycin by *S. erythraea* NCIMB 12462 under SSF, thus contributing to the reduction in the cost of production medium. Introducing SCB as inert support enhanced erythromycin production. The statistical approach showed significant results for optimizing the process parameters for maximal erythromycin production under SSF. The present study identified the effect of various process parameters on the erythromycin yield and the production was found to be significantly influenced by SCB content, inoculum size and moisture level.

Conflict of interest

The authors declare that the research was conducted in absence of any conflict of interest.

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