# OPTIMIZATION OF KEY PROCESS VARIABLES FOR ENHANCED REFAMYCIN B PRODUCTION IN SOLID STATE FERMENTATION BY AMYCOLATOPSIS MEDITERRANE MTCC 14 USING STATISTICAL METHODS

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## Abstract

*In the present study of solid media conditions for the refamycin B yield by solid state fermentation was studied and optimized using  both classical method  and statistical design of experiments). Statistical analysis of the results of Plackett–Burman showed that the lower level of initial moisture , initial pH, barbital, glucose and to solid media, or increase in the concentration of xylose in the range tested, results in significant effect in refamycin B yield of Amycolatopsis rifamycinica MTCC 14 by solid state fermentation. The effect of change in the levels of initial moisture, initial pH, barbital, glucose and xylose on the rfefamycin B yield was studied using central composite design methodology. Statistical analysis of the data showed that all the independent process had significant effect on refamycin B yield. The interaction between initial moisture  and initial pH,  between initial moisture  and barbital, between initial moisture  and glucose, between initial moisture  and xylose, between initial pH and xylose,  between barbital and glucose, between barbital  and xylose, and between glucose and xylose were significant when the response was refamycin B.*

**Key words:** *Refamycin B; olive oil cake; solid state fermentation;Amycolatopsis Mediterrane MTCC 14; response surface methodology*

## 1.Introduction

Refamycin B is a less toxic systemic antibacterial antibiotic. This antibiotic is widely employed for treating a variety of mycobacterial infections. It is used for the treatment of tuberculosis, leprosy and AIDS-related mycobacterial infections [1]. Refamycin antibiotic was first isolated from *Streptomyces mediterranei* [2]. An extensive literature is available on refamycin B production by *Nocardia* species [3,4,5,6], but *Amycolatopsis mediterranei* was found to be the best strain for refamycin B production.

Antibiotics production is an increasing field of biotechnology. Most antibiotic manufacturers produce antibiotics by submerged fermentation (SmF) techniques. However, in the last decades there has been an increasing trend towards the utilization of the solid-state fermentation (SSF) technique to produce several antibiotics [4,8]. Solid-state fermentation can be defined as a fermentation that takes place on a solid or semisolid substrate support [9]. The most fundamental advantages related with SSF as compared with submerged fermentation (SmF) are improved product recovery, reduced energy requirements and simplicity of the equipment used [10].Sunflower oil cake, sesame oil cake, soy bean cake, coconut oil cake, mustard oil cake, palm kernel cake, groundnut oil cake, cottonseed cake, canola oil cake, olive oil cake, rapeseed cake are agricultural wastes which are abundant in several countries and can be used as solid substrates for developing biotechnological process of industrial interest [11]. Olive oil cake is useful source of protein and energy for livestock. They are normally used in animal feed, especially for ruminants. Olive oil cake has high nutritional value, as they possess high protein content (6.3%), crude fiber (40%) and ash (4.2%) [12]. They are economically cheap, stable and dependable sources available in large quantities throughout the year. For that we selected olive oil cake as a solid substrate.

Classical methods of optimization of processes involve changing one independent variable while fixing the others at given levels. This single-dimensional search technique is clear, but often requires a consequential amount of work and time, and frequently fails to yield optimized conditions because it does not consider possible interactions among process parameters. Recently, statistical experimental strategies including Plackett-Burman Design (PBD) and response surface methodology (RSM) have been well engaged for optimization of SSF [13, 14,15, 16]. These strategies are particularly appropriate for optimization of SSF because they are generally subjective to less incidental errors which are often introduced by the complex nature of the solid components in SSF. In this study, we used both classical method and statistical strategies to optimize the SSF medium conditions for maximum production of refamycin B by *Amycolatopsis Mediterrane* MTCC 14.

## 2.Materials and method

### 2.1. Microorganisms and maintenance medium

*Amycolatopsis rifamycinica* MTCC 14was obtained from the Institute of Microbial Technology, Chandigarh, India. *Amycolatopsis rifamycinica* MTCC 14 was maintained on agar slants containing (g/L) dextrose 20, glycerol 20, yeast extract 5, beef extract 3, casein acid hydrolysate 3, peptone 2.5,malt extract 1, agar 20. The organisms were sub cultured every month and preserved at $4 \pm 1^{o}$C.

### 2.1. Preparation of inoculum

The spores from a fully sporulated slant were spread in 10 ml of 0.1% Tween 80 by dislodging them with a sterile loop under aseptic conditions. The spore suspension was used as inoculum. Viable spores present in the suspension were determined by serial dilution followed by plate count (which showed $8 \times 10^{8}$ spores/ml)

### 2.2 . Preparation of substrate

Olive oil cake was procured as gift from Malaysia. The collected olive oil cake was dried and mechanically milled with a lab mill (Ultra Centrifugal Mill) and sieved through standard mesh sieves (200–500 µm) using an electronic sieve shaker to obtain the powder of 200–500 µm

particle sizes.

## 2.3. Solid-state fermentation

For SSF under the basal medium condition, 5 g of olive oil cake was placed in a 100-ml conical flask and autoclaved twice at 120°C for 20 min. The flask was cooled to room temperature, the flasks were inoculated with $2.0x10^6$ spores/mL under sterile conditions and the following sterilized solutions were added to supplement the carbon and nitrogen sources, barbital and adjusted the pH and moisture content. The contents of the flasks were, fully mixed and incubated at $28\pm0.5^oC$ for predetermined time period and used for extraction and estimation of rifamycin B[17].

## 2.4. Scanning electron microscope

Samples were fixed by 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), dried by a critical point dryer and placed on brass stub. The samples were then coated with gold in a ion sputter and investigated on a scanning electron microscope (Model JEOL JSM T-330, Japan).

## 2.5. Antibiotic extraction

Ten volumes of distilled water were added to each flask. The extraction of the refamycin B was carried out on a rotary shaker (250 rpm) at $28 \pm 2^oC$ for 1 h. The slurry was squeezed through cheese cloth. The extract was clarified by centrifugation at 12,000xg ($4^oC$) for 15 min. The clear supernatant was used for refamycin B determination.

## *2.6. Determination of refamycin B*

Refamycin B was estimated according to the method of Pasqualucci *et al*., 1970 [18]. Reaction mixture contained 0.1 ml of suitably diluted antibiotics + 5 ml of 0.2 M acetate buffer (pH 4.63) and read the absorbance at 425 nm using UV–Visible spectrophotometer (SHIMADZU UV-1800), against 0.1 ml of antibiotic solution diluted to 5 ml with acetate buffer, pH 4.63 containing 0.1% $NaNO_2$. Refamycin B production under SSF was expressed as g /kgds dry fermented mass. Each sample was tested in duplicate.

## 2.7. Screening of carbon and nitrogen sources, initial pH, initial moisture content using classical methodology

Before arranging an experimental design with a response surface methodology (RSM), the effects of process variable tested by classical methodology. The influence of various carbon and nitrogen sources, barbitol, pH, moisture content was done using classical methodology. Carbon sources (glucose, maltose, galactose, ribose and xylose) at the concentration of 15% (w/w) were evaluated. The nitrogen sources (pea nutmeal, beef extract, soyabean meal, ammonium sulphate and ammonium choloride ) at a concentration of 20% (w/w) were evaluated. The concentration of the barbital (1%, 2%, 3%, 4% and 5%) were evaluated. The pH (7.0, 7.5, 8.0, 8.5 and 9.0) were evaluated. The moisture content ( 40%, 45%, 50%, 55% and 60%) were evaluated.

## *2.8. Statistical Optimization procedure*

The statistical optimization of process parameters for refamycin B production by *Amycolatopsis rifamycinica* MTCC 14 was carried out in two stages as described below.

## 2.9. Identification and selection of important process parameters using Plackett–Burman design

To identify the independent process variables that were most important for antibiotics production Plackett–Burman design (PBD) was used [19]. These critical independent process variable of the solid medium were selected on the basis of the optimized medium conditions, which were concluded from the classical variable method. In the present experiment, eleven independent process variables were selected for the screening in 20 trials. Each independent process variable was represented at two levels, i.e., high (+1) and low (-1) (Table 1 ). The effect of each independent process variable was determined by the following equation:

| | Independent process variables | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | X1 | X2 | X3 | X4 | X5 | X6 | X7 | X8 | X9 | X10 | X11 |
| Low Level (-1) | 15 | 15 | 15 | 15 | 20 | 20 | 20 | 20 | 2.0 | 7.5 | 50 |
| High Level (+) | 20 | 20 | 20 | 20 | 25 | 25 | 25 | 25 | 3.0 | 8.5 | 55 |

$$E(X_i) = \frac{2(\sum Mi_{+1} - \sum Mi_{-1})}{N} \Rightarrow (1)$$

Where  $E(X_i)$ the coefficient of effect of the tested ($i^{th}$) independent process variable.

$M_{+1}$  is the refamycin B yield from the trials where the independent process variable was present at the high level, $M_{-1}$ is the refamycin B yield from the trials where the independent process variable present at the low level, and N is the total number of trials (experiments,
i.e., 20).

Five independent process variables, which were found to be most effective independent process variables in the refamycin B production on the basis of PBD, were selected to identify the optimized conditions for the maximum production of refamycin B using the response surface methodology (RSM) [20].

**Table 1.** Independent process  variable screened in PBD and their real values
X1,Glucose ( % w/w) ; X2, Maltose( % w/w); X3, Ribose( % w/w); X4, Xylose( % w/w) ; X5, Soyabean Meal( % w/w) ; X6, Peanut Meal( % w/w) ; X7, Ammonium Chloride( % w/w) ; X8, Ammonium Sulphate( % w/w) ; X9,Babitol( % w/w) ; X10, Initial pH ;   X11 Initial moisture (%)

**Table 2.** PBD of independent process variable with refamycin B yield as response

| Std Order | X1 | X2 | X3 | X4 | X5 | X6 | X7 | X8 | X9 | X10 | X11 | Yield |
|-----------|----|----|----|----|----|----|----|----|----|-----|-----|-------|
| 17 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1.97 |
| 16 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 2.94 |
| 5 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | 9.46 |
| 10 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1.96 |
| 4 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 8.35 |
| 6 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 7.37 |
| 18 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 2.78 |
| 12 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 3.26 |
| 19 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 7.32 |
| 20 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 9.37 |
| 15 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | 1.43 |
| 13 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 8.97 |
| 9 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 7.85 |
| 3 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | 3.67 |
| 11 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | 8.32 |
| 7 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | 1.85 |
| 2 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 6.35 |
| 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | 2.76 |
| 8 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | 5.24 |
| 14 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | 4.32 |

## 2.10. Optimization of selected process parameters using response surface methodology

To find out the optimum level of the preferred independent process variables (initial moisture, initial pH , barbital , glucose and xylose).The levels of five independent process variables, viz. initial moisture ($X11$), initial pH ($X10$), barbital ($X9$), glucose ($X1$) and xylose ($X4$) were optimized by CCD experimental plan using the statistical software package The unscramblar Trial version 9.7 ( CAMO, Woodbridge, NJ, USA). Each independent process variable in the design was studied at five different levels (Table 4 ). A $2^5$ factorial design, with ten axial points, 32 cube points and ten replicates at the centre point with a total number of 31 experiments were employed (Table 5). The action of the system was explained by the following quadratic equation:

$$Y = \beta_o + \beta_1 X10 + \beta_2 X9 + \beta_3 X8 + \beta_4 X4 + \beta_{11} X10^2 + \beta_{22} X9^2 + \beta_{33} X8^2 + \beta_{44} X4^2 +$$

$$\beta_1\beta_2 X10 X9 + \beta_1\beta_3 X10 X8 + \beta_1\beta_4 X10 X4 + \beta_2\beta_3 X9 X8 + \beta_2\beta_4 X9 X4 + \beta_3\beta_4 X8 X4 \Rightarrow (2)$$

$$Y = \beta_o + \beta_1 X10 + \beta_2 X9 + \beta_3 X8 + \beta_4 X4 + \beta_{11} X10^2 + \beta_{22} X9^2 + \beta_{33} X8^2 + \beta_{44} X4^2 +$$

$$\beta_1\beta_2 X10 X9 + \beta_1\beta_3 X10 X8 + \beta_1\beta_4 X10 X4 + \beta_2\beta_3 X9 X8 + \beta_2\beta_4 X9 X4 + \beta_3\beta_4 X8 X4 \Rightarrow (2)$$

where, Y is predicted response, $\beta_o$ is intercept,$\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$ are linear coefficients,$\beta_{11}$, $\beta_{22}$, $\beta_{33}$, $\beta_{44}$ are squared coefficient,$\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_1\beta_4$, $\beta_1\beta_5$,$\beta_2\beta_3$, $\beta_2\beta_4$, $\beta_2\beta_5$, $\beta_3\beta_4$ , $\beta_3\beta_5$ ,$\beta_4\beta_5$,are interaction

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coefficients and X10, X9, X8, X4,X10$^2$, X9$^2$, X8$^2$, X4$^2$, X5$^2$,X11X10, X11X10, X11X9, X11X1, X11X4, X10X9, X10X1, X10X4, X9X1, X0X4 are interaction independent  process variables.

**Table 4.** Design of independent process variables and there levels  ls

| Independent process variable (Coded) | Independent process variable Name | Units | Range | and | Level | | |
|---|---|---|---|---|---|---|---|
| | | | Lower Axial (0.31) | Low (1.00) | Central (1.50) | High (2.00) | Higher Axial (2.68) |
| X11 | Initial moisture | (%) | 36.55 | 40 | 42.50 | 45 | 48.44 |
| X10 | Initial pH | | 6.15 | 3.00 | 6.75 | 7.0 | 7.34 |
| X9 | Barbital | % (w/w) | 0.15 | 0.5 | 0.75 | 1.0 | 1.34 |
| X1 | Glucose | % (w/w) | 1.55 | 5 | 7.50 | 10 | 13.44 |
| X4 | Xylose | % (w/w) | 21.55 | 25 | 27.50 | 30 | 33.44 |

**Table  5.** Central composite design with experimental and predicted values of refamycin  B yield

| Design type | Experimental Run | X11 | X10 | X9 | X1 | X14 | Rifamycin B (g/kgds) ( actual yield) | Rifamycin B (g/kgds (predicted Yield) | Residual |
|---|---|---|---|---|---|---|---|---|---|
| L:X11-a | 1 | 0.31 | 1.50 | 1.50 | 1.50 | 1.50 | 8.52 | 8.41 | 0.11 |
| H:X11-a | 2 | 2.68 | 1.50 | 1.50 | 1.50 | 1.50 | 9.06 | 9.06 | 0.00 |
| L:X10-a | 3 | 1.50 | 0.31 | 1.50 | 1.50 | 1.50 | 3.07 | 2.32 | 0.75 |
| H:X10-a | 4 | 1.50 | 2.68 | 1.50 | 1.50 | 1.50 | 3.76 | 4.40 | 0.64 |
| L:X9-a | 5 | 1.50 | 1.50 | 0.31 | 1.50 | 1.50 | 6.09 | 6.25 | 0.16 |
| H:X9-a | 6 | 1.50 | 1.50 | 2.68 | 1.50 | 1.50 | 5.21 | 4.94 | 0.27 |
| L:X1-a | 7 | 1.50 | 1.50 | 1.50 | 0.31 | 1.50 | 3.97 | 3.84 | 0.13 |
| H:X1-a | 8 | 1.50 | 1.50 | 1.50 | 2.68 | 1.50 | 1.98 | 2.01 | -0.03 |
| L:X4-a | 9 | 1.50 | 1.50 | 1.50 | 1.50 | 0.31 | 7.85 | 6.14 | 1.71 |
| H:X4-a | 10 | 1.50 | 1.50 | 1.50 | 1.50 | 2.68 | 3.07 | 4.67 | -1.6 |
| Cube001a | 11 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 4.07 | 5.43 | -1.36 |
| Cube002a | 12 | 2.00 | 1.00 | 1.00 | 1.00 | 1.00 | 9.10 | 9.53 | -0.43 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Cube003a | 13 | 1.00 | 2.00 | 1.00 | 1.00 | 1.00 | 5.90 | 5.49 | 0.41 |
| Cube004a | 14 | 2.00 | 2.00 | 1.00 | 1.00 | 1.00 | 7.54 | 7.29 | 0.25 |
| Cube005a | 15 | 1.00 | 1.00 | 2.00 | 1.00 | 1.00 | 3.54 | 3.81 | -0.27 |
| Cube006a | 16 | 2.00 | 1.00 | 2.00 | 1.00 | 1.00 | 9.43 | 9.13 | 0.3 |
| Cube007a | 17 | 1.00 | 2.00 | 2.00 | 1.00 | 1.00 | 6.03 | 6.04 | -0.01 |
| Cube008a | 18 | 2.00 | 2.00 | 2.00 | 1.00 | 1.00 | 8.02 | 9.06 | -1.04 |
| Cube009a | 19 | 1.00 | 1.00 | 1.00 | 2.00 | 1.00 | 4.43 | 4.33 | 0.1 |
| Cube010a | 20 | 2.00 | 1.00 | 1.00 | 2.00 | 1.00 | 2.86 | 3.02 | -0.16 |
| Cube011a | 21 | 1.00 | 2.00 | 1.00 | 2.00 | 1.00 | 5.46 | 6.56 | -1.1 |
| Cube012a | 22 | 2.00 | 2.00 | 1.00 | 2.00 | 1.00 | 3.43 | 2.95 | 0.48 |
| Cube013a | 23 | 1.00 | 1.00 | 2.00 | 2.00 | 1.00 | 4.21 | 4.51 | -0.3 |
| Cube014a | 24 | 2.00 | 1.00 | 2.00 | 2.00 | 1.00 | 3.65 | 4.41 | -0.76 |
| Cube015a | 25 | 1.00 | 2.00 | 2.00 | 2.00 | 1.00 | 9.32 | 8.91 | 0.41 |
| Cube016a | 26 | 2.00 | 2.00 | 2.00 | 2.00 | 1.00 | 5.88 | 6.52 | -0.64 |
| Cube017a | 27 | 1.00 | 1.00 | 1.00 | 1.00 | 2.00 | 7.23 | 6.08 | 1.15 |
| Cube018a | 28 | 2.00 | 1.00 | 1.00 | 1.00 | 2.00 | 8.98 | 9.01 | -0.03 |
| Cube019a | 29 | 1.00 | 2.00 | 1.00 | 1.00 | 2.00 | 6.06 | 5.72 | 0.34 |
| Cube020a | 30 | 2.00 | 2.00 | 1.00 | 1.00 | 2.00 | 6.09 | 6.36 | -0.27 |
| Cube021a | 31 | 1.00 | 1.00 | 2.00 | 1.00 | 2.00 | 1.08 | 1.41 | -0.33 |
| Cube022a | 32 | 2.00 | 1.00 | 2.00 | 1.00 | 2.00 | 5.53 | 5.57 | -0.04 |
| Cube023a | 33 | 1.00 | 2.00 | 2.00 | 1.00 | 2.00 | 3.03 | 3.22 | -0.19 |
| Cube024a | 34 | 2.00 | 2.00 | 2.00 | 1.00 | 2.00 | 6.22 | 5.08 | 1.14 |
| Cube025a | 35 | 1.00 | 1.00 | 1.00 | 2.00 | 2.00 | 6.97 | 7.08 | -0.11 |
| Cube026a | 36 | 2.00 | 1.00 | 1.00 | 2.00 | 2.00 | 4.78 | 4.60 | 0.18 |
| Cube027a | 37 | 1.00 | 2.00 | 1.00 | 2.00 | 2.00 | 9.56 | 8.89 | 0.67 |
| Cube028a | 38 | 2.00 | 2.00 | 1.00 | 2.00 | 2.00 | 4.32 | 4.12 | 0.2 |
| Cube029a | 39 | 1.00 | 1.00 | 2.00 | 2.00 | 2.00 | 4.34 | 4.20 | 0.14 |
| Cube030a | 40 | 2.00 | 1.00 | 2.00 | 2.00 | 2.00 | 3.03 | 2.95 | 0.08 |
| Cube031a | 41 | 1.00 | 2.00 | 2.00 | 2.00 | 2.00 | 8.32 | 8.19 | 0.13 |
| Cube032a | 42 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 5.32 | 4.64 | 0.68 |
| Cent-a | 43 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 9.94 | 9.93 | 0.01 |
| Cent-b | 44 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 9.93 | 9.93 | 0.00 |
| Cent-c | 45 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 9.93 | 9.93 | 0.00 |
| Cent-d | 46 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 9.96 | 9.93 | 0.03 |
| Cent-e | 47 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 9.93 | 9.93 | 0.00 |
| Cent-f | 48 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 9.94 | 9.93 | 0.01 |
| Cent-g | 49 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 9.91 | 9.93 | -0.02 |
| Cent-h | 50 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 9.94 | 9.93 | 0.01 |
| Cent-i | 51 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 9.92 | 9.93 | -0.01 |
| Cent-j | 52 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 9.98 | 9.93 | 0.05 |

## 2.11. ANOVA and model fitting

Statistical significance of the model and the regression coefficients were estimated by analysis of variance (ANOVA). Probability p-value of $< 0.05$ was reasoned as statistically significant. It was reasoned as significant if the F-value becomes greater and the p-value becomes smaller. Lack of fit was also determined to check the quality of the model. The accuracy of the model was checked by the coefficient of determination $R^2$ with 95 % confidence limits ($\alpha=0.05$), which measures

`goodness of fit` of the model. When $R^2$ approaches unity, the empirical model fits the actual experimental data. The relationships between the response and the variables were also envisioned by two-dimensional contour or three-dimensional (3D) response surface plots to see the relative influence of the independent process variables and predict experimental results for other combinations as well. The optimal values of the independent process variables were obtained by solving the regression equation together with analyzing the response 3D surfaces and 2D contour plots. Subsequently, the experimentally optimized medium was prepared to verify the responses predicted by the equation, therefore evaluating the validity of the model [21].

## 2.12 Verification of model

Optimal conditions for the production of refamycin B from solid state fermentation depended on initial moisture, initial pH , barbital , glucose and xylose were obtained using the predictive equations of RSM. The experimental and predicted values were compared in order to determine the validity of the model.

# 3.0 Results and discussion

## 3.1. Optimization by classical methods

Although classical methods are tedious, and overlook the interaction between different process variables, this method was beneficial for the choice of ranges in PBD, making the result more economical and convincing.

Considering the presence of only 5–7% of carbohydrate in olive oil cake, it was considered appropriate to supplement ready sugar for better growth and antibiotic production.

Simple and complex carbon sources, viz. glucose maltose, galactose, ribose and xylose, were used as carbon sources to investigate their effect on refamycin B production (Figure. 1). Refamycin B production was found to be good with all the five carbon sources tested with glucose to be the best carbon source increasing the refamycin B production to 6.78 g/kgds at day eight of incubation period. These results were in agreement with Mahalaxmi *et al*., 2010 [17], who affirmed that glucose is optimum as carbon supplementation for maximum refamycin B production by *Amycolatopsis* sp. RSP 3 in solid-state fermentation.
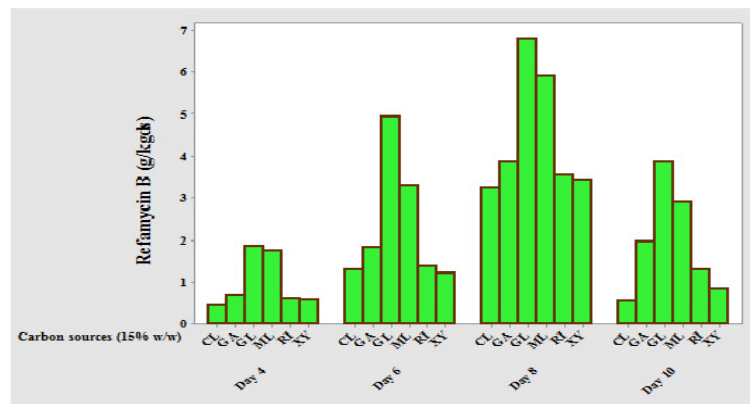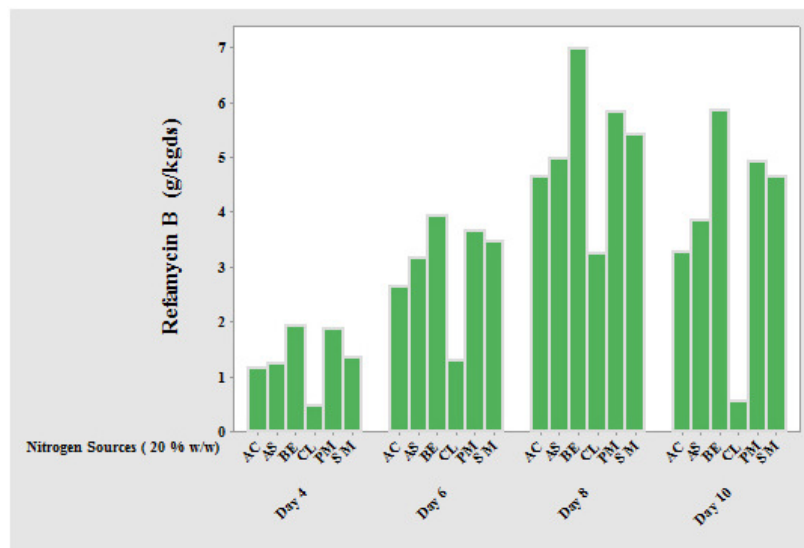


**Figure 1** Effect of carbon sources on refamycin B production by *Amycolatopsis rifamycinica* MTCC 14 in SSF. CL, control; GL, glucose; ML, maltose; GA, galactose; RI, ribose; XY, xylose

Generally, the high concentration of nitrogen sources in media is effective in decorative the

production of refamycin B by *Amycolatopsis mediterranei* [22].Effect of supplementation using different nitrogen sources on the production of refamycin B is illustrated in Figure 2. Ammonium sulphate and ammonium chloride as inorganic and peanut meal, beef extract, soyabean meal were used as complex nitrogen source, for supplementation of additional nitrogen sources. Based on the results it was found that beef extract, peanut meal, soyabean meal and ammonium sulphate were the best nitrogen sources and its supplementation led to further increase in refamycin B production to 6.98, 5.84, 5.43 and 4.97 g/kgds at day eight of incubation period.

Although the oil seed cake contained good amount of crude protein as per composition, the supplemented nitrogen may be better accessed and utilized leading to better refamycin B production.



**Figure 2.** Effect of different nitrogen sources on refamycin B production by *Amycolatopsis Mediterrane* MTCC 14 in SSF. CL control ; PM, peanut meal; BE, beef extract; SM, soyabean meal; AS, ammonium sulphate; AC, ammonium chloride

Generally, the concentration of barbital in media was effective in enhancing the production of refamycin B by *Nocardia* sp RSP-3 and *Amycolatopsis mediterranei* M18 [23,24]. A range of concentrations of barbital were tested to evaluate their capacity to support *Nocardia Mediterrane* MTCC 14 growth and refamycin B production. The supplementation of 3% (w/w) of barbital gave the highest refamycin B production of 8.45 g/kgds, which contributed to 1.5-fold enhancement in refamycin B yield compared to the control medium ( 0 % w/w) (Figure.3 ).

The optimum  initial pH of the medium was found to be 8.5, up to which there was an increase in refamycin B production followed by a decrease if further increase in pH (Figure.4 ). Mahalaxmi *et al*., 2010 [17] reported an optimum pH of 8.0 for maximum refamycin B production by *Amycolatopsis* sp. RSP 3 under SSF.
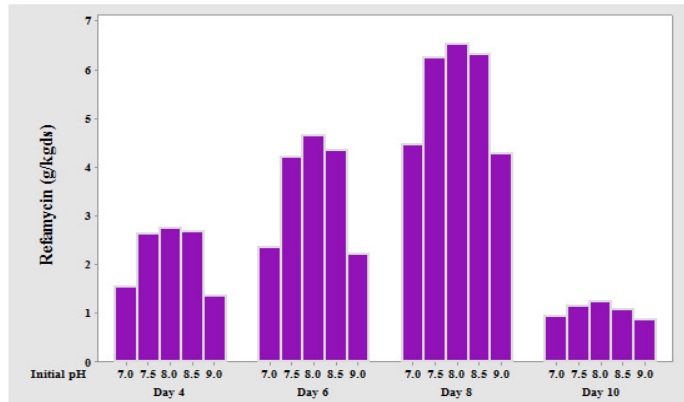
**Figure 4.** Effect of initial pH of the fermentation medium on refamycin B production in SSF.

As shown in Figure 5 , initial moisture content of the either substrate (olive oil cake) has a great influence on refamycin B production by *Amycolatopsis rifamycinica* MTCC 14 strain. With an increase in the initial moisture content of the substrate from 40% to 55% refamycin B production was concomitantly enhanced, and further increase in the intial moisture content of substrate resulted in a steady decline in refamycin B yield. Regular embellishment in refamycin B production was reported by the augmentation in moisture content of fermentation system [17]. The moisture level in the solid-state fermentation greatly affects the process due to its hindrance in the physical properties of the solid particle.Increased moisture is believed to abbreviate the porosity of substrate[25], thus limiting the oxygen transfer [26,27].
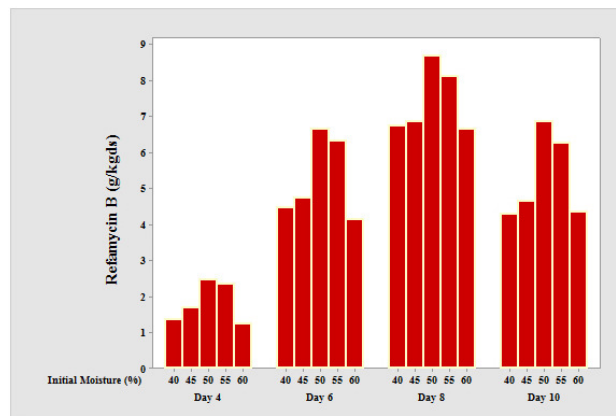


**Figure 5.** Influence of initial moisture (%) content of the substrates on refamycin B production.

The scanning electron microscope observation  of *Amycolatopsis rifamycinica* MTCC 14  growth on  olive oil cake in solid state fermentation (Figure 6).
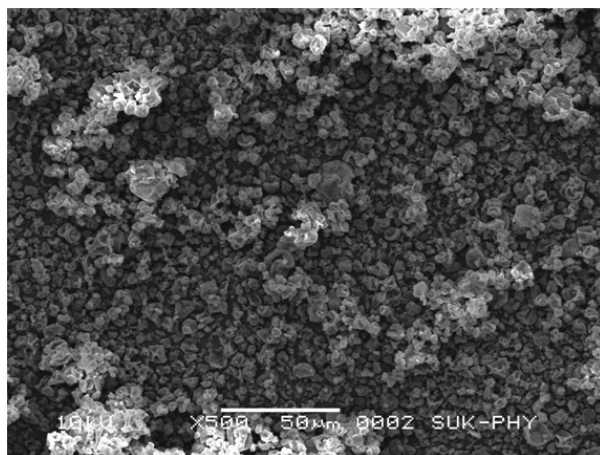
**Figure 6.** Scanning electron micrograph of *Amycolatopsis rifamycinica* MTCC 14 after 8 days of culture on olive oil cake

## 3.2. Statistical optimization

In order to find the best functioning independent process variables, two experimental designs were conducted: the first was a Plackett-Burmrn design for determining the most influencing independent process variables (Experiments 1 to 20 in Table 2), and the second was a CCD (Experiments 1 to 52 in Table 5) for determining the response surfaces model for the more influencing independent process variables and then obtaining the optimal functioning conditions.

## 3.3. Screening of medium conditions using Plackett–Burman design

Plackett–Burman design is a well-accepted and extensively used statistical design for the screening of the medium conditions [28]. Initial experiments were carried out according to the Plackett-Burmrn design matrix as shown in Table 2. The *Amycolatopsis rifamycinica* MTCC 14 was cultured under 20 different culture conditions. Refamycin B yield (g/kgds) were taken as the responses for each run (Table 2). Table 3 shows the resulting effects of the independent process variables on the responses, and the associated t-values, p valules and significant levels. Confidence levels were approved only when above 95%. As shown in Table, initial moisture, initial pH, barbital, glucose and xylose in the tested range had significant effects on refamycin B yield. The Pareto chart allows one to detect the independent process variables and which are most important to the process or design optimization study one has to deal with [29]. In this chart ( Figure 7), the lengths of each bars are proportional to the actual value of the estimated effects [30]. The effects of all studied independent process as can be discussed from the Pareto chart illustrated by inspection of these results revealed that not all independent process variables effects are significant for the refamycin B yield. .Based on these results, the independent process variables having the greatest impacts on the production of refamycin B by *Amycolatopsis rifamycinica* MTCC 14 were identified as initial moisture, initial pH, barbital, glucose and xylose.

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**Table 3.** Coefficients of independent process variables in PBD refamycin B production and associated statistical tests

| Term | Effect | Coef | SE Coef | T | P |
|---|---|---|---|---|---|
| Constant | | 5.277 | 0.3223 | 16.37 | 0.000 |
| X1 | -1.954 | -0.977 | 0.3223 | -3.03 | 0.016 |
| X2 | 1.390 | 0.695 | 0.3223 | 2.16 | 0.063 |
| X3 | -1.238 | -0.619 | 0.3223 | -1.92 | 0.091 |
| X4 | 1.584 | 0.792 | 0.3223 | 2.46 | 0.039 |
| X5 | 0.546 | 0.273 | 0.3223 | 0.85 | 0.422 |
| X6 | -1.124 | -0.562 | 0.3223 | -1.74 | 0.119 |
| X7 | 0.544 | 0.272 | 0.3223 | 0.84 | 0.423 |
| X8 | 0.884 | 0.442 | 0.3223 | 1.37 | 0.207 |
| X9 | -2.108 | -1.054 | 0.3223 | -3.27 | 0.011 |
| X10 | -2.214 | -1.107 | 0.3223 | -3.43 | 0.009 |
| X11 | -2.510 | -1.255 | 0.3223 | -3.89 | 0.005 |

$$S = 1.44128 \quad PRESS = 103.864$$
$$R\text{-}Sq = 89.42\% \quad R\text{-}Sq(pred) = 33.84\% \quad R\text{-}Sq(adj) = 74.86\%$$



**Pareto Chart For standardized effects**
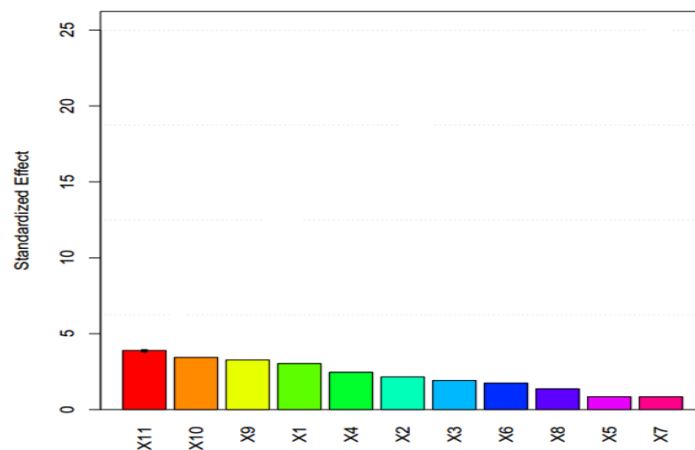
Figure 7. Pareto chart of the Plackett-Burman design models used for the production of refamycin B in solid state fermentation by *Amycolatopsis rifamycinica* MTCC

## 3.4 Optimization of medium conditions using central composite design

Based on the results of the Plackett-Burmrn design where the five independent process variables had negative and positive effects within the ranges tested, the central point and range of level for each independent process variables were chosen (Table 4). The levels of non significant medium conditions were set at their corresponding low levels in the Plackett-Burmrn design.

The preferred independent process variable variables (initial moisture, initial pH , barbital , glucose and xylose) were further optimized by RSM using central composite design (CCD).The results obtained for refamycin B production were fed into the unscramblar trial version 9.7 software ( CAMO Woodbridge, NJ, USA )and analyzed. The results were analyzed using the analysis of variance (ANOVA) as applicable to the experimental design used. The range of coded

as well as actual values of preferred independent process variables are conferred in Table 4. The calculated regression equation for the optimization of medium conditions showed the refamycin B production (Y) as a function of dependent variable. By applying multiple regression analysis on the experimental data, the following equation was found to explain refamycin B production:

$Y =9.9326+ 0.1367X11 + 0.4359X10 - 0.2755 X9 - 0.3859 X1- 0.3092X4 - 0.2097X11*X11 - 1.1607X10*X10 - 0.7656 X9*X9 - 1.2385X1*X1 -0.7984X4*X4 - 0.5744X11*X10 + 0.3056X11*X9 - 1.3537X11*X1- 0.2894 X11*X4 + 0.5437 X10*X9 + 0.5444X10*X1- 0.1037X10*X4 +0.4481X9*X1 - 0.7625X9*X4 + 0.5256X1*X4$          (3)

Where Y, represents the response, i.e., refamycin B yield  and X11, X10, X9, X1, X4,  X11X11, X10X10, X9X9, X1X1, X4X4, X11X10, X11X9, X11X1, X11X4, X10X9, X10X1, X10X4, X9X1, X9X4,  and X1X4 are the independent process variables.

Table 5 outlines the design matrix of the central composite design (CCD) and the results obtained for refamycin B production. The central point was repeated ten times (run 43–52) to check the experimental errors. The experimental levels of refamycin B production are given in Table 5, along with the predicted data. The goodness of fit of the regression model was evaluated by a test for lack of fit at 95% level of significance. The goodness of the model can be checked using different criteria. Table 6  lists the estimates of coefficients and the associated t-values and significant levels. In this work, only the estimates with significant levels higher than 90% (P< 0.10) were included in the final model [Eq. (3)]. Thus, the reduced models which describe the responses as functions of the more significant independent process variables were generated. The coefficient of determination ($R^2$) was 0.9535 for refamycin B production, which explained 96.60% variability in the model. The $R^2$ value should be between 0 and 1. Relatively high values of $R^2$ were obtained for these models. The nearer the $R^2$ values is to 1.0, the stronger the model and better it predicts the response [31]. The  adjusted determination coefficient (Adj $R^2$) corrects the $R^2$ value for the sample size and the number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted $R^2$ may be noticeably smaller than the $R^2$. The adjusted $R^2$ in this study was 0. 9234 , which was close to $R^2$ value . The mean squares are obtained by dividing the sum of squares of each of the two sources of variation, the model and the error variance, by the respective degrees of freedom. F-test shows that the models (Table 7)  were authentic because their significant levels were greater than 99% [32].  The model F-value of 31.76 and values of p >F (<0.0001) indicated that the model is significant. The model was found to be adequate to the data at the probability level of 95%.

The corresponding analysis of variance values are presented in the Table 6. Among model terms, X10, X9,  X1, X9X9, X1X1, X4X4,  X11X10, X11X9, X11X1, X11X4, X10X4, X9X1, X9X4 and X1X4 were significant with a probability of more than 99% confidence level ; X4, was significant with a probability of more than 95% confidence level. The model terms, X1, X1X1 and X10X9, X10X1, however, had no significant influence on refamycin B yield.

Table  6. Regression coefficients for refamycin B yield

| Term | Coef | SE Coef | T | P |
|---|---|---|---|---|
| Constant | 9.9326 | 0.23179 | 42.852 | 0.000 |
| X11 | 0.1367 | 0.11206 | 1.220 | 0.232 |
| X10 | 0.4359 | 0.11206 | 3.890 | 0.000 |
| X9 | -0.2755 | 0.11206 | -2.459 | 0.020 |
| X1 | -0.3859 | 0.11206 | -3.443 | 0.002 |
| X4 | -0.3092 | 0.11206 | -2.759 | 0.010 |
| X11*X11 | -0.2097 | 0.09640 | -2.175 | 0.037 |

| | | | | |
|---|---|---|---|---|
| X10*X10 | -1.1607 | 0.09640 | -12.041 | 0.000 |
| X9*X9 | -0.7656 | 0.09640 | -7.942 | 0.000 |
| X1*X1 | -1.2385 | 0.09640 | -12.848 | 0.000 |
| X4*X4 | -0.7984 | 0.09640 | -8.282 | 0.000 |
| X11*X10 | -0.5744 | 0.13037 | -4.406 | 0.000 |
| X11*X9 | 0.3056 | 0.13037 | 2.344 | 0.026 |
| X11*X1 | -1.3537 | 0.13037 | -10.384 | 0.000 |
| X11*X4 | -0.2894 | 0.13037 | -2.220 | 0.034 |
| X10*X9 | 0.5437 | 0.13037 | 4.171 | 0.000 |
| X10*X1 | 0.5444 | 0.13037 | 4.176 | 0.000 |
| X10*X4 | -0.1037 | 0.13037 | -0.796 | 0.432 |
| X9*X1 | 0.4481 | 0.13037 | 3.437 | 0.002 |
| X9*X4 | -0.7625 | 0.13037 | -5.849 | 0.000 |
| X1*X4 | 0.5256 | 0.13037 | 4.032 | 0.000 |

$$S = 0.737482 \quad PRESS = 68.0545$$
$$R\text{-}Sq = 95.35\% \quad R\text{-}Sq(pred) = 81.22\% \quad R\text{-}Sq(adj) = 92.34\%$$

Table 7. Analysis of variance (ANOVA) for the selected quadratic model

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Regression | 20 | 345.454 | 345.454 | 17.2727 | 31.76 | 0.000 |
| Linear | 5 | 22.917 | 22.917 | 4.5835 | 8.43 | 0.000 |
| X11 | 1 | 0.809 | 0.809 | 0.8093 | 1.49 | 0.232 |
| X10 | 1 | 8.231 | 8.231 | 8.2306 | 15.13 | 0.000 |
| X9 | 1 | 3.288 | 3.288 | 3.2876 | 6.04 | 0.020 |
| X1 | 1 | 6.449 | 6.449 | 6.4489 | 11.86 | 0.002 |
| X4 | 1 | 4.141 | 4.141 | 4.1410 | 7.61 | 0.010 |
| Square | 5 | 194.505 | 194.505 | 38.9011 | 71.53 | 0.000 |
| X11*X11 | 1 | 0.216 | 2.573 | 2.5733 | 4.73 | 0.037 |
| X10*X10 | 1 | 54.518 | 78.854 | 78.8543 | 144.98 | 0.000 |
| X9*X9 | 1 | 21.727 | 34.309 | 34.3092 | 63.08 | 0.000 |
| X1*X1 | 1 | 80.741 | 89.776 | 89.7764 | 165.07 | 0.000 |
| X4*X4 | 1 | 37.303 | 37.303 | 37.3028 | 68.59 | 0.000 |
| Interaction | 10 | 128.031 | 128.031 | 12.8031 | 23.54 | 0.000 |
| X11*X10 | 1 | 10.557 | 10.557 | 10.5570 | 19.41 | 0.000 |
| X11*X9 | 1 | 2.989 | 2.989 | 2.9890 | 5.50 | 0.026 |
| X11*X1 | 1 | 58.644 | 58.644 | 58.6444 | 107.83 | 0.000 |
| X11*X4 | 1 | 2.680 | 2.680 | 2.6796 | 4.93 | 0.034 |
| X10*X9 | 1 | 9.461 | 9.461 | 9.4613 | 17.40 | 0.000 |
| X10*X1 | 1 | 9.483 | 9.483 | 9.4830 | 17.44 | 0.000 |
| X10*X4 | 1 | 0.344 | 0.344 | 0.3445 | 0.63 | 0.432 |
| X9*X1 | 1 | 6.426 | 6.426 | 6.4261 | 11.82 | 0.002 |

| | | | | | | |
|---|---|---|---|---|---|---|
| X9*X4 | 1 | 18.605 | 18.605 | 18.6050 | 34.21 | 0.000 |
| X1*X4 | 1 | 8.841 | 8.841 | 8.8410 | 16.26 | 0.000 |
| Residual Error | 31 | 16.860 | 16.860 | 0.5439 | | |
| Lack-of-Fit | 22 | 16.857 | 16.857 | 0.7662 | 1868.80 | 0.000 |
| Pure Error | 9 | 0.004 | 0.004 | 0.0004 | | |
| Total | 51 | 362.314 | | | | |

SS- sum of squares; DF- degrees of freedom; MS- mean square

## 3.5. Model diagnostics

Before accepting any model, the appropriateness of the adopted model should be checkered by the advantageous statistical method. The major diagnostic method is residual (observed minus predicted) examination as shown in Figres 8-9, supplying diagnostics for residual actions . There are several residuals graphs to test the model assumptions. The basic analysis is to examine a normal probability plot of the residuals, that is, the number of standard deviations of the measured values from their respective predicted values (Figure 8A.). The normal probability plot is engaged to calculate whether the residuals follow a normal distribution, which is the most considerable impression for statistical modeling and model appropriateness checking.

The next examination is to appearance at the residuals plotted versus the predicted responses (Figure 8B.). There should be no systemic pattern in the plot, and the points should fall   within a horizontal band centred at zero. Departure from this may indicate a contravention of the constant variance impression. The magnitude of the residual should be independent of its predictive value, which means that the disperse should be about the same across all levels of the predicted values. Actual versus predicted displays the real response data plotted against the predicted responses (Figure 9A). Points above or below the diagonal line mean areas of over or under prediction. Residuals versus sample (run order ) graphs communicate any time-based effects or sequential component (Figure 9B.) Residuals versus independent process variable graphs also demand to be examined to see if there might be variance changes (dispersion effect) with any independent process variable or level of independent process variable.

There were no expressive violations of the model assumptions found in this residual examination except one outlier as shown in Fig.. This design point seems to be due to extent  error rather than random experimental error. So the modelling could be used for further studies such as medium formulation, process optimization, independent process variable analysis, or simulation without any distort.
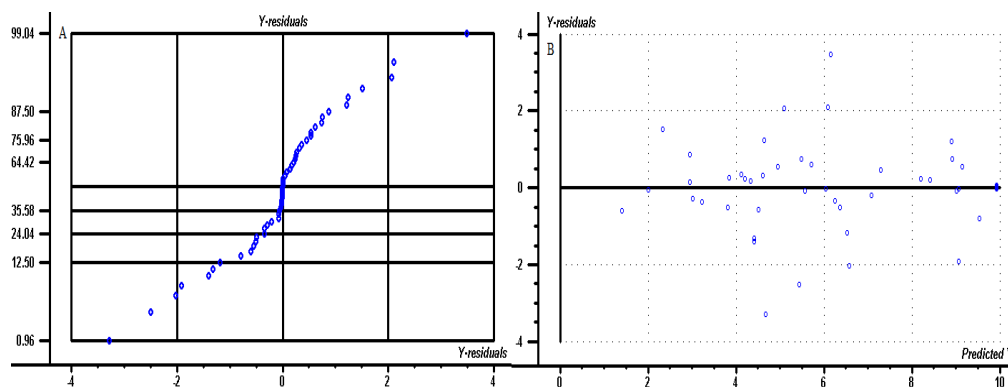
**Figure 8.** Residual diagnostics of crossed model for refamycin B yield: (A) normal probability; (B) residuals vs. predicted
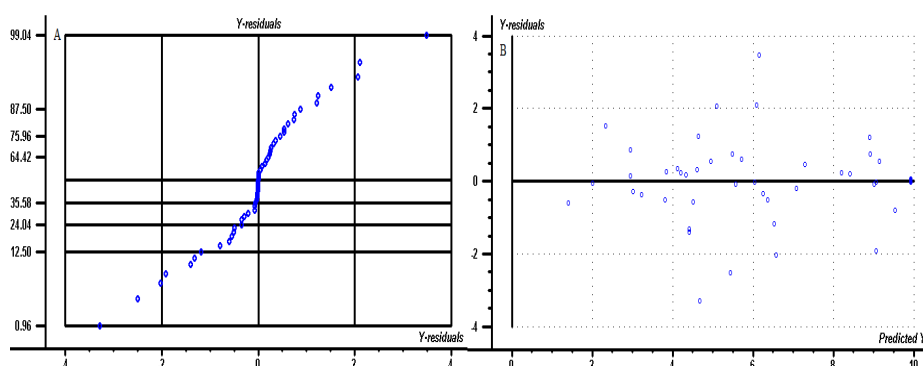


**Figure 8.** Residual diagnostics of crossed model for refamycin B yield: (A) normal probability; (B) residuals vs. predicted

## 3.6. Three-dimensional (3D) response surface and  (2D) contour plots

The three-dimensional response surface  and  two-plots are a function of two factors, maintaining all other independent process variables at fixed levels, conferred as a solid surface framed in a three-dimensional space (Figures 10-19). This graphic presentation helps to understand the main and the interaction effects of those two independent process variable [33, 34]. At the same time, 3D response surfaces and their corresponding contour plots can assist the consistent examination of the effects of the experimental independent process variables on the responses[35]. In this work, since the regression model had five independent process variables, three independent process variables were held constant at the centre level.

## 3.6.1 Effects of effect of initial moisture and initial pH

An elliptical response surface in the entire region was found from the second order quadratic equation for the refamycin B production with the interaction of initial moisture level and initial pH level (Figure 10). The results showed the refamycin B production, which was considerably affected by varying the level  of initial moisture and initial pH. The maximum refamycin B yield was obtained at the point of intersection of the major and minor axes of the ellipse. The maximum yield of refamycin B was predicted at given ranges of both initial moisture and initial pH level. The refamycin B yield decreased at the maximum and minimum values of ranges considered in both independent process variables. About 9.353 g/kgds was obtained from the response surface as the maximum refamycin B yield  at the initial moisture, initial pH, barbital, glucose and xylose concentration was about 43% 6.5, 0.75% (w/w), 7.50% (w/w) and 27.50% (w/w), respectively.
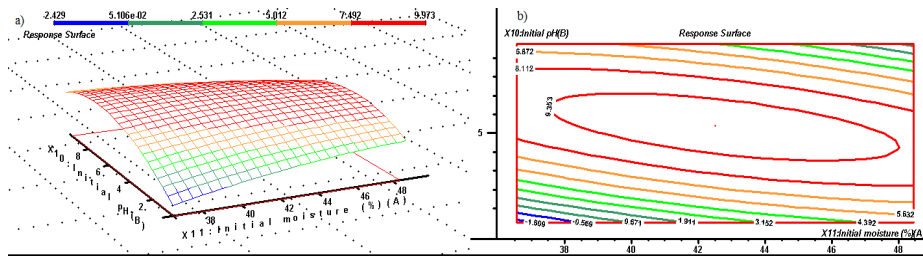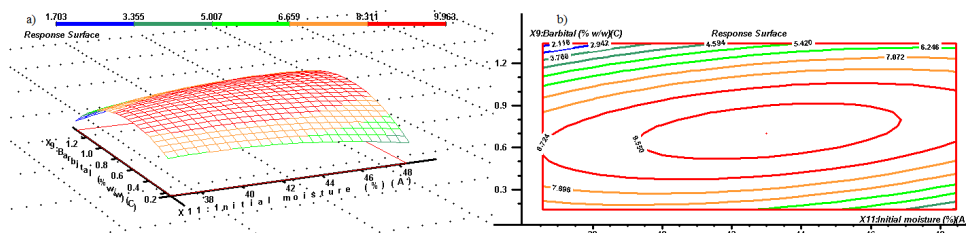
28

**Figure 10.** Three-dimensional surface plot (a) and two- dimensional counter plot (b) of refamycin B as a function of X11:initial moisture and X10:initial pH (X9:barbital, X1:glucose and X4:xylose were kept constant)

## 3.6.2 Effects of effect of initial moisture and barbital

Figure 11 shows the elliptical response surface plot of refamycin B production as a function of initial moisture  level and barbital concentration. The predicted refamycin B production decreased at the higher and lower values of ranges for both initial moisture  level and barbital concentration. Maximum refamycin B production was obtained near the center points of the response surface. The maximum production of refamycin B of about 9.550g/kgds was predicted at the initial moisture  level and barbital concentration level of about 43% and 0.6% (w/w) while initial pH, glucose and xylose  were 5.0 ,7.50% (w/w) and 27.50% (w/w).



**Figure 11.** Three-dimensional surface plot (a) and two- dimensional contour plot (b) of refamycin B as a function of X11:initial moisture and X9:barbital (X10:initial pH, X1:glucose and X4:xylose were kept constant)

## 3.6.3 Effects of effect of initial moisture and glucose

Figure 12 is the response surface plot for variation in the refamycin B production, as a function of initial moisture level and glucose concentration by keeping the level of initial pH, barbital and xylose  at 5.0, 0.75% (w/w) and 27.50% (w/w). The production of refamycin B was affected by the concentration of initial moisture level and glucose. The production of refamycin B increased with increasing the level of initial moisture from 46 to 48 %.  Similarly, refamycin B yield increased upon increasing the concentration of glucose from 9 to 12% (w/w).
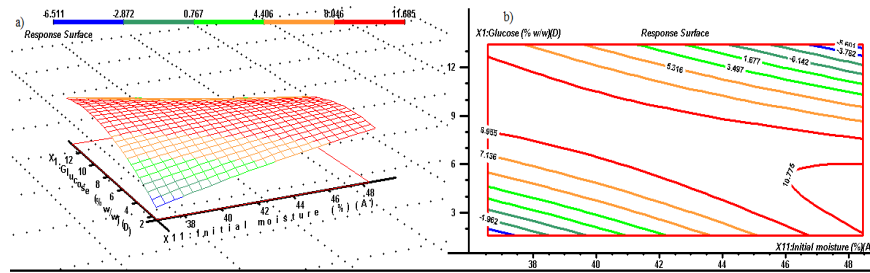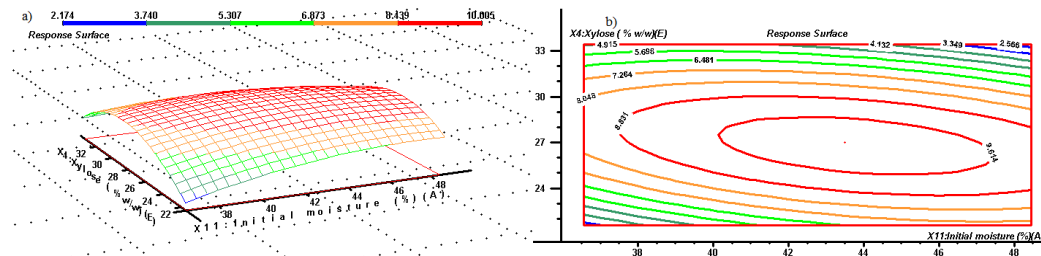
**Figure 12.** Three-dimensional surface plot (a) and two- dimensional contour plot (b) of refamycin B as a function of X11:initial moisture and X1:glucose(X10:initial pH, X9:barbital and X4:xylose were kept constant)

### 3.6.4 Effects of effect of initial moisture and xylose

Figure 13 denotes the three and two dimensional surfaces plots of effect of initial moisture and xylose on response. As can be seen, enhancing the initial moisture from 40 to 46% could increase the refamycin B yield, but a further increase in initial moisture would lead to a decline of the refamycin B yield. Also, the effect of xylose on the refamycin yield was significant. The % (w/w)increased as the xylose concentration went from 26 to 30 % (w/w), however, the % (w/w) slightly decreased by further increasing xylose concentration.



**Figure 13.** Three-dimensional surface plot (a) and two- dimensional contour plot (b) of refamycin B as a function of X11:initial moisture and X4:xylose (X10:initial pH, X9:barbital and X1:glucose were kept constant)

### 3.6.5 Effects of effect of initial pH and barbital

Figure 14 depicts the effect of initial pH and barbital and their interactive effects on the refamycin B yield. From the analysis of response surface plots, the interaction between initial pH and barbital was significant. It was observed that at low initial pH (<2), refamycin B yield decreased with the increase in xylose concentration. Also, refamycin B yield decline was observed at higher initial pH (>8).
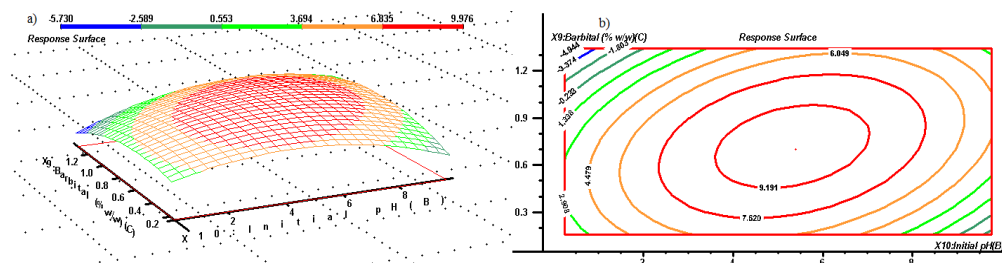


**Figure 14.** Three-dimensional surface plot (a) and two- dimensional contour plot (b) of refamycin B as a function of X10:initial pH and X9:barbital (X10:initial moisture, X1:glucose and X4:xylose were kept constant)

30

### 3.6.6 Effects of effect of initial pH and glucose

The effect of initial pH and glucose concentrations on refamycin B yield are shown in Figure 15. At the middle initial pH range (4 to 6) and middle barbital concentration (6 to 10 % w/w) optimal refamycin B yield was attained (9.04 g/kgds).
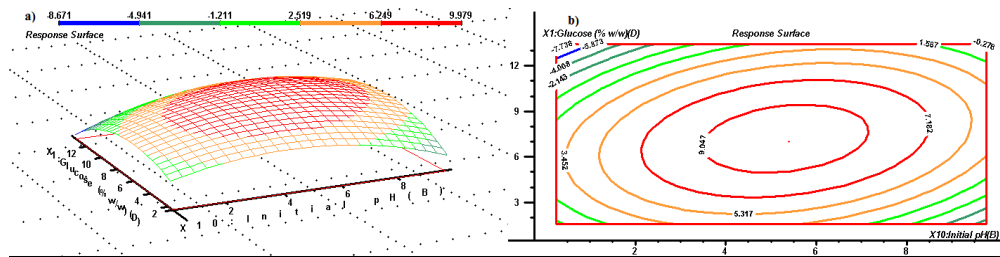


**Figure 15.** Three-dimensional surface plot (a) and two- dimensional contour plot (b) of refamycin B as a function of X10:initial pH and X1:glucose (X10:initial moisture, X9:barbital and X4:xylose were kept constant)

### 3.6.7 Effects of effect of initial pH and xylose

The initial pH and xylose effects on the response at fixed optimum initial moisture, barbital and glucose of 42.5%, 0.75% (w/w) and 7.5% (w/w) were illustrated in Figure 16. The refamycin B yield was remarkably low at low values of initial pH and xylose. Increase in both initial pH and xylose an increase in the response surface. The response value reached its highest level of initial pH at 6 while xylose concentration showed a maximum at 28% (w/w).
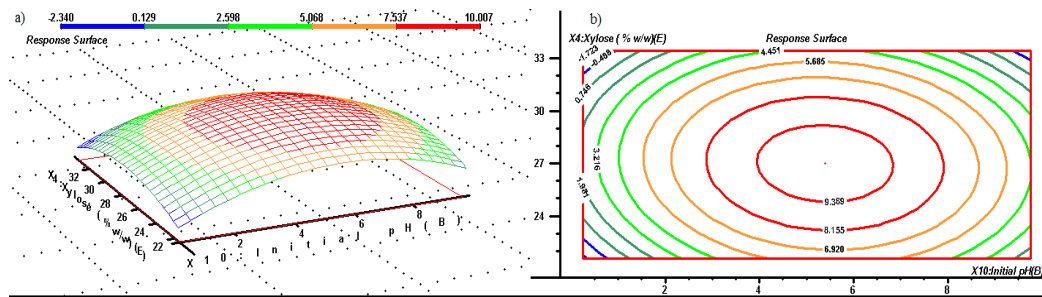


**Figure 16.** Three-dimensional surface plot (a) and two- dimensional contour plot (b) of refamycin B as a function of X10:initial pH and X4:xylose (X10:initial moisture, X9:barbital and X1:glucose were kept constant)

### 3.6.8 Effects of effect of barbital and glucose

The effect of the barbital and the glucose on the response at optimum initial moisture, initial pH and xylose  of 42.5% , 5.0 and 27.5% (w/w) were demonstrated in Figure 17. The response surface of refamycin B yield showed a net peak of 9.201 g/kgds at 8% (w/w) of glucose and 8 % (w/w) of barbital, but after 10% (w/w)  of glucose and1% (w/w)  of barbital  the response decreased.
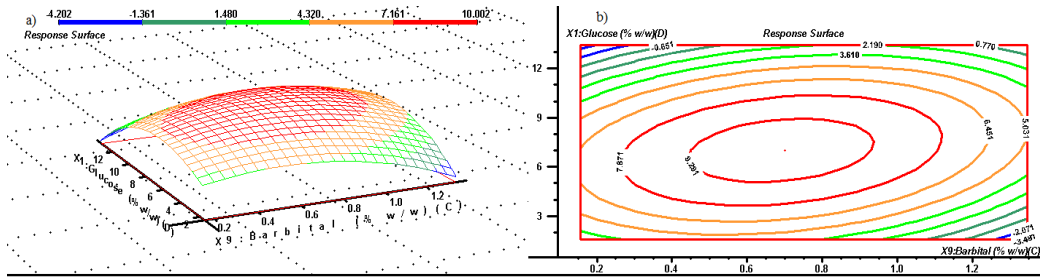
**Figure 17.** Three-dimensional surface plot (a) and two- dimensional contour plot (b) of refamycin B as a function of X9:barbital and X1:glucose (X10:initial moisture, X10:initial pH and X4:xylose were kept constant)

### 3.6.9 Effects of effect of barbital and xylose

The effects of barbital % (w/w) and xylose% (w/w)   on the response are presented at an optimum temperature of initial moisture, initial pH and glucose  of 42.5% , 5.0 and 7.5 % (w/w) and are shown in Figure 18. The response surface of initial refamycin B  yield showed a peak with 9.232 g/kgds value at barbital 0.8 % (w/w)  and 28% (w/w)  of xylose.
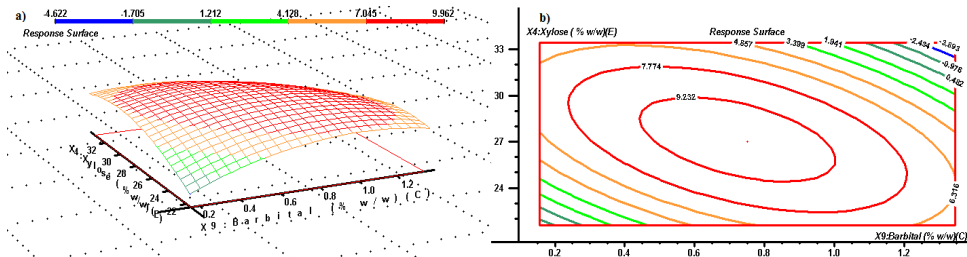


**Figure 18.** Three-dimensional surface plot (a) and two- dimensional contour plot (b) of refamycin B as a function of X9:barbital and X4:xylose (X10:initial moisture, X10:initial pH and X1:glucose were kept constant)

### 3.7. Effects of effect of glucose and xylose

Figure 19    illustrates  the  effects  of  glucose  and  xylose  concentration  on  the  refamycin B yield.  The  response  surface  plot  indicated  that  the  increase  of  xylose concentration  up  to 27 % (w/w)  enhanced   the  refamycin B yield. However,  the  using  high  concentration  of xylose  (>30% (w/w) )  had  a  negative  effect  on  the  refamycin B yield.  As shown  in  Figiure 19.  ,  the  refamycin B  yield  improved  with  increasing  the  concentration  of  glucose. However,  the  using  high  concentration  of  glucose  (>10% (w/w) )  had  a  negative  effect  on the  refamycin B yield.
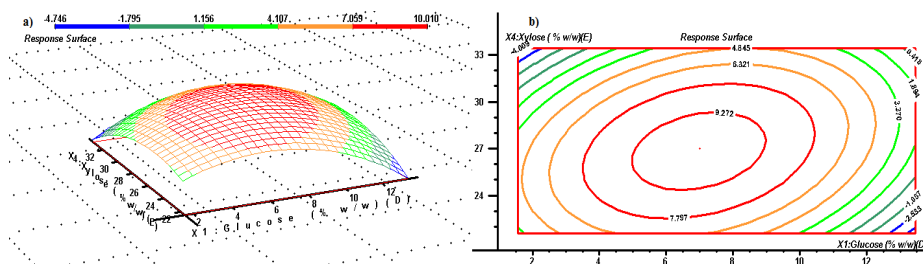


**Figure 19.** Three-dimensional surface plot (a) and two- dimensional contour plot (b) of refamycin B as a function of X1:glucose and X4:xylose (X10:initial moisture, X10:initial pH and X9:barbital were kept constant)

### 3.8. Optimization and validation

Based on the second-order polynomial Model presented in Eq.3, the predicted maximum refamycin B was 9.93 g/kgds at initial moisture 42.50 %, initial pH 6.75, barbital 1.34% (w/w) , glucose7.50% (w/w) and xylose 27.50% (w/w). The predicted condition was validated by duplicate experiments, and the actual refamycin B yield was 9.96 g/kgds. The prediction was close to validation of actual results, indicating that the model can be used to guide and optimize the solid culture medium of *Amycolatopsis rifamycinica* MTCC 14.

## 4. Conclusions

At first, the classical method was taken. Although it was laborious, inadequate and neglected in much research, this method was helpful in the choice of range in PBD. We then selected the main process variables and found the initial proximity of the optimums. Finally the optimal solid medium was obtained. In the optimal conditions, the refamycin increased one fold.

The statistical method used in this study was scientific, convincing and virtue applying in other research fields. The results obtained in this study may be useful for a highly effective production of refamycin B.

The results of Plackett–Burman DOE showed that the increment of initial moisture , initial pH, barbital and glucose to solid media, or decrease in the concentration of xylose in the range tested, results in significant effect in refamycin B yield of *Amycolatopsis rifamycinica* MTCC 14 by solid state fermentation whereas the supplement of maltose, ribose, soyabean meal, peanut meal, ammonium chloride and ammonium sulphate to the solid media had no significant effect.

The optimal production condition to produce refamycin B was successfully investigated using RSM-CCD. The second-order polynomial model gave a acceptable description of the actual data. An optimized condition for simultaneous maximum production of refamycin B was initial moisture 42.50 %, initial pH 6.75, barbital 0.75 %(w/w) , glucose7.50% (w/w) and xylose 27.50% (w/w). The obtained values of dependent process variable were close to the predicted yield values. This study can be useful in the development of industrial production process.

## 5. Acknowledgment

## 6. Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## REFERENCES

[1] A.L. Pozniak and R. Miller,"The treatment of tuberculosis in HIV-infected persons," AIDS,vol. 13, no. 4, pp. 435–445,1999.

[2] P. Margalith and G. Beretta,"Rifomycin. XI Taxonomic study on Streptomyces mediterranei nov. Sp.," Mycopathologia et mycologia applicata, vol. 8, no. 4 pp. 321–330,1960.

[3] H.C. Bong, N.C Ho,and H.K. In , "Rifamycin B production by Nocardia mediterranei immobilized in a dual hollow fibre bioreactor," Enzyme and Microbial Technology , vol.9, no. 6, pp. 345–349,1987.

[4] G.Venkateswarlu, P.S.Murali, S.Gita, and L.VenkateswarRao, "Improvement of rifamycin B production using mutant strains of Amycolatopsis mediterranei," Bioprocess Engengineering, vol.23, no.4, pp. 315-318, 2000.

[5] J.L. Kye and T.R.Yong, "Quantitative analysis of mycelium morphological characteristics and rifamycin B production using Nocardia mediterranei," Journal of Biotechnol, vol. 36, no.3, pp. 239–245,1994.

[6] M.B.Prashant, V.S.Sujata, A.M.Tessa, and P.W.Pramod "A cybernetic model to predict the effect of freely available nitrogen substrate on rifamycin B production in complex media," Applied Microbiology Biotechnology, vol.72, no.4, pp.662-670,2006.

[7] P. Ellaiah, B.Srinivasulu, and K. Adinarayana,"Optimization studies on neomycin production by a mutant strain of Streptomyces marinensis in solid state fermentation," Process Biochemistry, vol.39, no.5, pp. 529–534,2004.

[8] K.Shazia, R.Nosheen, A.Kalsoom, and A.G. Muhammad, "Production of tylosin in solid-state fermentation by Streptomyces fradiae NRRL-2702 and its gamma-irradiated mutant (c-1)," Letters in Applied Microbiology, vol.49, no.5, pp.635–640,2009.

[9] D.H.D Farah, V. Graciele, C.B.Marcelo, A.M.David, and K. Nadia, "A mathematical model describing the effect of temperature variations on the kinetics of microbial growth in solid-state culture," Process Biochemistry, vol.40, no. 5,pp. 801–807,2005.

[10] S.Virupakshi, B.K.Gireesh, R.G.Satish, and G.R.Naik, "Production of a xylanolytic enzyme by a thermoalkaliphilic Bacillus sp. JB-99 in solid state fermentation," Process Biochemistry, vol.40, no. 1,pp.431–435,2005.

[11] R. Sumitra, K.S. Sudheer, L. Christian , R.S. Carlos, and P. Ashok, "Oil cakes and their biotechnological applications –A review," Bioresource Technology, vol.98,no. 10, pp.2000–2009,2007.

[12] B. Maymone, A. Battaglini, and M. Tiberio, "Ricerche sul valore nutri-tivo della sansa d"olive,"Alimentazione Animale ,vol. 5,pp. 219–250,1961.

[13] M.Shinji and S.S. Makoto, "Medium optimization of antifungal lipopeptide, iturin A, production by Bacillus subtilis in solid-state fermentation by response surface methodology," Applied Microbiology Biotechnology, vol.76, no.1, pp.101–108,2007.

[14] B.J. Naveena, A. Md, K. Bhadriah, and G. Reddy, "Selection of medium components by Plackett–Burman design for production of L(+) lactic acid by Lactobacillus amylophilus GV6 in SSF using wheat bran," Bioresource Technology, 96,no. 4,pp.485–490,2005.

[15] B.J. Naveena, A. Md, K. Bhadrayya, S.S. Madhavendra, R. Gopal, "Direct fermentation of starch to L(+) lactic acid in SSF by Lactobacillus amylophilus GV6 using wheat bran as support and substrate:medium optimization using RSM," Process Biochemistry, vol.40, no.2, pp. 681–690, 2005.

[16] I.U. Fatma, T. Canan, and G. Nihan, "Solid-state production of polygalacturonase by Aspergillus sojae ATCC 20235," Journal of Biotechnology, vol.127 no. 2, pp.322–334,2007.

[17] Y. Mahalaxmi, T. Sathish, S.R. Ch, and R.S. Prakasham, "Corn husk as a novel substrate for the production of rifamycin B by isolated Amycolatopsis sp. RSP 3 under SSF," Process Biochemistry,vol.45, no. 1, pp.47–53,2010.

[18] C.R. Pasqualucci, A. Vigevani, P. Radelli, and G.G. Gallo, "Imp roved differential spectrophotometric determination of rifamycins," Journal of Pharmaceutical Sciences, vol.59, no. 5, pp.685–687,1970.

[19] B. Jia, Z.H. Jin, and L.H. Mei, "Medium Optimization Based on Statistical Methodologies for Pristinamycins Production by Streptomyces pristinaespiralis," Applied Biochemistry Biotechnology, vol.144, no. 2, pp.133–143, 2008.

[20] B. Jaspreet, P. Vandana, S. Vineeta, C.K.M. Tripathi, and B. Vinod, "Studies on medium optimization for the production of antifungal and antibacterial antibiotics from a bioactive soil actinomycete," Medicinal Chemistry Research, 17 no.2, pp.425–436,2008.

[21] T. Pramila, and B. Subhash "Catalytic cracking of palm oil for the production of biofuels: Optimization studies" Bioresource Technology, vol.98, no.18, pp.3593–3601, 2007.

[22] J.Z. Hua, L.G. Ping, and C.P. Lin, "Scale-up of rifamycin B fermentation with Amycolatopsis mediterranei," Journal of Zhejiang Univesity Science, vol.5,no.12, pp.1590–1596,2004.

[23] Y. Mahalaxmi, R.C. Subba, G.S. Laxmi, T. Satish, P. Sudhakar, and R.S. Prakasham, "Rifamycin B production pattern in Nocardia RSP-3 strain and influence of barbital on antibiotic production," Current Trends in Biotechnology and Pharmcy, Vol.2,pp. 208–216,2008.

[24] A. Mejia, V.G. Gonzalez, and B.J. Gonzalez, "Biochemical mechanism of the effect of barbital on rifamycin-B biosynthesis by Amycolatopsis mediterranei (M18 strain)," Journal of Bioscience and Bioengineering, vol.95, no. 3, pp.288–292,2003.

[25] S. Amit, V. Vivekanand, and P.S. Rajesh,"Solid-state fermentation for gluconic acid production from sugarcane molasses by Aspergillus niger ARNU-4 employing tea waste as the novel solid support," Bioresource Technology, vol. 99, no. 9,pp. 3444–3450,2008.

[26] V. Carrizales, H. Rodriguez, and Sardina, "Determination of specific growth rate of molds as semi solid culture," Biotechnology and Bioengineering, vol.23, no.2, pp.321–323,1981.

[27] A, Pandey, C.R. Soccol, and D. Mitchell, "New developments in solid-state fermentation: I-bioprocess and products," Process Biochemistry, vol.35, no.10, pp.1153–1169,2000.

[28] R.I. Plackett and J.P. Burman, "The design of optimum multifactorial experiments," Biometrika, vol.33,no. 4,pp.305–325,1946.

[29] Antony J. Design of Experiments for Engineers and Scientists. Butterworth-Heinemann, ISBN: 0750647094, 2003

[30] Abhishek Kumar Singh• Mausumi Mukhopadhyay Optimization of Lipase-Catalyzed Glycerolysis for Mono and Diglyceride Production Using Response Surface Methodology Arab J Sci Eng (2014) 39:2463–2474

[31] S. Bijender and T. Satyanarayana, "Phytase production by a thermophilic mould Sporotrichum thermophile in solid state fermentation and its potential applications," Bioresource Technology, vol.99, no.8, pp.2824–2830,2008.

[32] Zhi-You Wen, Feng Chen Application of Statistically-Based Experimental Designs for the Optimization of Eicosapentaenoic Acid Production by the Diatom Nitzschia laevis BIOTECHNOLOGY AND BIOENGINEERING, VOL. 75, NO. 2, OCTOBER 20, 2001

[33] Z. Quan, S. Jinjiang, J. Haixiang, H. Xianqing, and X. Yuquan,"Optimization of phenazine-1-carboxylic acid production by agacA/qscR-inactivated Pseudomonas sp. M18GQ harboring pME6032Phz using response surface methodology," Applied Microbiology Biotechnology, 86 no. 6, pp.1761–1773,2010.

[34] K. Yetilmezsoy, S. Demirel, R.J. Vanderbei, Response surface modeling of Pb(II) removal from aqueous solution byPistacia veraL.: Box–Behnken experimental design, J. Hazard. Mater. 171 (2009) 551–562.

[35] J. Jiménez, Y. Guardia-Puebla, M.E. Cisneros-Ortiz, J.M. Morgan-Sagastume, G. Guerra, A. Noyola Optimization of the specific methanogenic activity during the anaerobic co-digestion of pig manure and rice straw, using industrial clay residues as inorganic additive Chemical Engineering Journal 259 (2015) 703–714

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