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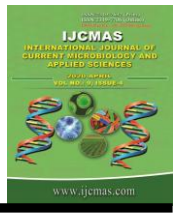

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Optimization of Nutritional Variables Using Response Surface Methodology for Enhanced Antifungal Metabolite Production by *Janibacter* sp. RC18 from Turmeric Rhizosphere

Ruth Chiamaka Osaro-Matthew*, Francis Sopuruchukwu Ire and Nnenna Frank-peterside

Department of Microbiology University of Port Harcourt Choba, Nigeria

**Corresponding author*

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A B S T R A C T

Antifungal metabolites from rare actinomycetes have been found attractive for application due to its novelty, potency and environmental friendliness. Optimization of process variables for enhanced production of antifungal metabolite in *Janibacter* sp. RC18 was carried out in this study. One factor at a time (OFAT) was used for preliminary optimization of fermentation variables (time, temperature, initial pH, carbon and nitrogen sources). A three factor central composite design (CCD) and response surface methodology (RSM) were employed for optimization of the selected significant nutritional variable (starch, soybean meal) and $CaCO_3$. The optimum antifungal metabolite production was obtained at day 7 incubation period inhibition, temperature 30 ^{o}C, pH 8, starch as carbon source and soybean meal as nitrogen source. Response surface analysis revealed that the optimum value of the variables were 10.76 g/l of starch, 11.95 g/l of soybean meal and 1.57 g/l of $CaCO_3$. Under this optimal condition antifungal metabolite production was 23.7 ± 0.09 mm which increased by 22.33 % compared to OFAT optimized medium (18.4 ± 0.06 mm). The present study has proved CCD and RSM is a reliable statistical tool for optimization of antifungal metabolite production in actinomycetes.

Introduction

The recent call for green or sustainable agriculture is a path to profitable and environmentally benign agriculture (FAO, 2017). The persistent use of synthetic agrochemicals leads to environmental and health problem. Most of these synthetic agrochemicals are recalcitrant xenobiotics persisting in the environment thereby causing environmental degradation. It also accumulates in the agricultural produce and can be passed to humans through food chain hence leading to health problems (EEA, 2005).

Microorganisms have gained global attention as agroactive agents which are alternative to synthetic agrochemicals. Actinomycetes which are Gram positive bacteria have proved to be a promising microorganisms for production of various bioactive agent of agricultural importance (Ahmad *et al*., 2008). The genus *Janibacter* which *Janibacter limosus* was the first specie reported by Martin *et al.,* (1997) belongs to the family intrasporangiaceae in the actinomycetales order. Some other species of this genus that have been reported are: *J. terrae* (Yoon et al., 2000), *J. brevis* (Imamura *et al*., 2000), *J. melonis* (Yoon *et al.,* 2004), *J. corallicola* (Kageyama *et al.,* 2007), *J. hoylei* (Shivaji *et al.,* 2009), *J. alkaliphilus* (Li *et al.,* 2012), *J. cremeus* (Hamada *et al*., 2013), and *J. indicus* Zhang *et al.,* 2014). Some investigations have shown *Janibacter* as potential biocontrol agents, *Janibacter melonis* was reported to be antagonistic against *Ralstonia solanacearum* by Achari and Ramesh, (2014), and Nimaichand *et al.,* (2015) reported *Janibacter* antagonistic against *Fusarium oxysporum* and *Rhizoctonia oryzae-sativea*.

In bioprospecting, one thing is to source for novel and potent strain another thing is sustainable production of bioactive metabolites by the strain. Antifungal metabolite production is affected by some physical (temperature, pH and incubation period) and nutritional factors (carbon, nitrogen and mineral) (Bundale *et al*., 2015). These factors are responsible to make fermentation conditions suitable for bacterial growth and metabolites production. One-factor-at-a-time (OFAT) technique which is a traditional method of optimization that fails to describe interactions between variables and response is suitable for preliminary study in order to select significant factors before proceeding to optimization.

Although, identifying significant variables for optimal production of bioactive compound in a strain by OFAT is important. But for sustainability, variables especially nutritional factors should be incorporated at the correct levels, also relationship between the dependent and independent variables must be established hence the need for RSM optimization. Response surface methodology (RSM) which is a collection of mathematical and statistical tools is useful for analysing and optimizing response of multivariate system (Kocheki, 2009).

Hence the aim of this study is to use OFAT for preliminary optimization investigations, RSM and central composite design (CCD) to model and statistically optimize process variables for maximum antifungal production in *Janibacter* sp strain RC18.

Materials and Methods

Actinomycetes and inoculum preparation

Janibacter sp strain RC18 (Genbank Accession number MK473882) isolated from turmeric rhizosphere was described in precious studies (Osaro-Matthew *et al*., 2020). Strain RC18 was inoculated into 50ml Starch Casein broth in a 100 ml Erlenmeyer flask. The flask was then incubated in a rotary incubator for 150 rpm at temperature 30^{o}C until absorbance of 0.2 was recorded at OD_{600} and 20 µl of this culture was used as seed inoculum.

Test phytopathogens

Alternaria pimpriana DSM 62023 and *Colletotrichum coccodes* DSM 2492 were obtained from DSMZ (German collection of microorganism and cell cultures). *Collectotricum coccodes* DMS 2924 was selected as the test organism for RSM experiment since it is a commonly found phytopathogen in Nigeria.

Experimental set up

The experiment was carried out in 100 ml Erlenmeryer flask containing 50 ml Glycerol beef extract broth of (Glycerol 10 g/l, Beef extract 10 g/l, NaCl 5 g/l, $CaCO_3$ 2 g/l and K_2HPO_4 2.5 g/l,). Fermentation was carried out in a rotary incubator at 150 rpm, all experiment was done in triplicates.

Bioassay

At the end of fermentation, antifungal activity was determined using Agar well diffusion technique (Barry and Thornsberry, 1985), on a PDA (Potato Dextrose Agar). A 5 mm diameter well was made by punching the agar (inoculated with test organisms) with a sterile steel borer and 50 µl of the culture supernatant obtained after centrifuging at 5000 rpm for 10 minutes was poured in the well. Incubation was carried out at 25 ^{o}C ± 2 ^{o}C for 5 days and the diameter zone of inhibition was measured and recorded in millimetres (mm).

Selection of fermentation conditions

Incubation period

Fermentation media inoculated with strain RC18 were incubated at 28 ^{o}C on a rotary shaker for 10 days for maximum antifungal metabolite production and antifungal assay was carried out every 24 hours till the day 10.

Incubation temperature

Effect of incubation temperatures on antifungal production of strain RC18 were studied at different temperature (25 ^{o}C, 30 ^{o}C, 35 ^{o}C, 40 ^{o}C and 45 ^{o}C). Fermentation was carried out in a Glycerol Beef extract (GBE) broth at the optimized fermentation period (day 7) and at the end bioassay was carried out as previously described.

Initial pH

Optimum pH was studied by adjusting initial fermentation media pH to different pH levels (4, 5, 6, 7, 8, 9, 10) using 1 M HCl and 1 M NaOH. The fermentation broth was inoculated with strain RC18 and incubated at optimized temperature (30^{o}C) and optimized incubation period (7 days).

Carbon sources

To select the best carbon source for maximum antifungal metabolite production five different carbon sources (glucose, lactose, fructose, sucrose and starch) were used in substituting glycerol which was the carbon source in the primary basal medium one at a time. Afterward, fermentation was carried out at optimum temperature (30 ^{o}C), incubation time (7 days) and pH 8 and bioassay was carried as previously described.

Nitrogen sources

In order to select the best nitrogen sources for enhancing the antifungal activity of the strain RC18. Various sources of nitrogen (malt extract, yeast extract, peptone, ammonium sulphate, and soybean meal) were used to substitute one by one for beef extract (nitrogen source) in the primary basal medium and fermentation was carried out using the best carbon source (starch), at the optimum temperature (30 ^{o}C), incubation time (7 days) and pH 8, thereafter, bioassay was carried as previously described

Experimental design for optimization by RSM

Central composite design

The optimization of antifungal production was carried out according RSM. The nutritional variables were optimized for

enhanced antifungal activity using the central composite design, the three selected variables were carbon, nitrogen and $CaCO_3$. For carbon and nitrogen sources, starch and soybean meal were selected based on the one factor at a time result, for $CaCO_3$ it was selected based on its role on slow growing actinomycetes.

The experimental runs comprise of 20 trials (8 factorial points, 6 axial points and 6 central point) based on the five-levels three variables. All fermentations were performed in a randomized order. RSM and second-order CCD for three-variables (Starch = X_1, Soybean meal = X_2, and $CaCO_3$ = X_3), five level combinations coded -1.68, -1, 0, +1, and +1.68 as modelled by Snedecor and Cochran (1980) was adopted to determine the effects of the independent variables on response variables (antifungal activity). The natural levels were calculated using the coded levels as outlined in Table 1, comprising of 20 experimental runs and different formulation concentrations.

Statistical and Mathematical Analysis

Data obtained from one factor at a time optimization was subjected to ANOVA and mean were separated using Duncan multiple range test using SPSS. To determine if there is a relationship between the independent variables and the dependent variables for the RSM, regression method was used to analyse the obtained data using MINITAB 14.13. The response factor (Yi) as a mathematical function of a few continuous factors was modelled using regression analysis. A second-order polynomial equation according to equation 1 was used to express the response.

$$Y=\beta_0 +\sum \beta_i X_i+\sum \beta_{ii} X_i^2+\sum \beta_{ij} X_i X_j$$

Equation (1)

Y represents the predicted response,$_0$is a constant β_i is the coefficient of linear effect, β_{ii} is the coefficient squared effect, is the coefficient of interaction effect, and is independent variables under study. This design was used to study the effects of the variables (main, interaction, and quadratic). It was also used to optimize the levels of variables for enhancing antifungal activity. The developed model was then studied for significance and lack-of-fit, while response surface plot was designed after removal of the non-significant terms with the same software (Ahsan *et al.*, 2017; Chirayu *et al*., 2018).

Validation of the experimental model

The prediction of the optimum concentration of starch, soybean and $CaCO_3$ was achieved by applying the regression analysis of equation 1, the obtained optimal value was evaluated and compared to that of OFAT optimized medium of Starch soybean broth of (Soybean meal, 10 g/l, starch 10 g/l, NaCl 5 g/l and $CaCO_3$ 2 g/l).

Results and Discussion

Time played an important role in the antimetabolite production in *Janibacter* sp RC18 (Fig. 1), it was observed that the incubation time for optimum production of antifungal metabolite in strain RC18 was day 7 with an inhibition zone of 15.0 ± 0.58 mm against *C. coccodes* and 12.7 ± 0.33 mm against *A. pimpriana*.

Janibacter sp RC18 was able to produce antifungal metabolite in the temperature range 20^oC – 40^oC, with an optimum at 30^oC and no activity at 45^oC (Fig. 2).

The effect of pH levels variation on antifungal metabolite production of strain RC18 as represented in (Fig. 3) showed that pH 8 was optimal as 14.8 ± 0.17 mm inhibition zone was obtained against *C. coccodes* and 15.8 ± 0.33 mm inhibition zone was obtained against

A. pimpriana. The minimum antifungal metabolite production was obtained at pH 6 while there was no antifungal production at pH 4, 5 and 10.

Optimum antifungal production was obtained when starch was used as sole carbon source as presented in (Fig. 4). Soybean meal was the best nitrogen source for antifungal production in strain RC18 (Fig. 5).

RSM optimization of process variable by CCD

The study adopted RSM using CCD in which 20 experimental runs to enable adequate measurement of the response variables (antifungal metabolite production) were performed as presented in Table 2.

The coded coefficient for antifungal production in *Janibacter* sp RC18 was presented in Table 3. The linear, interactive and quadratic effects of 3 independent variables starch (X_1) soybean (X_2) and $CaCO_3$ (X_3) at three levels on the response variable (antifungal production) of *Janibacter* sp RC18 were reported. The linear effects were all significant at (P<0.001), the quadratic effect of X_1X_2 (starch and soybean meal) was not significant. The T-value of X_3 and quadratic effect of the three variables were negative which indicates negative correlation.

From the analysis of variance (ANOVA) (Table 4) the model showed a high F- value (349.27), a small p-value (0.000), a non significant Lack of fit (0.509), Coefficient of Variation (0.324070), coefficient of determination (R^2) value 99.68 % and adjusted R^2 (R^2adj) value 99.40 %.

Analysis of residual to check regression assumptions

The graphical validation of the regression model using residual plots residual plot were presented in Fig. 6a-d. The normal probability plot showed linearity (Fig. 6a), the plots of residual versus fitted value (Fig. 6b) randomly scattered around 0 point. The plot of frequency versus residual (Fig. 6c) histogram showed a dumbbell shaped. The plot of residual versus observation order in (Fig. 6d) showed a symmetric pattern.

Response surface and contour plots

The 3D response surface and 2D contour plots of *Janibacter* sp. RC18 antifungal activity against *Collectotricum coccodes* are shown in Figure 7. The 2D contour plots of (Fig. 7a., Fig. 7c.) representing the interactive effect of starch and soybean meal, and starch and $CaCO_3$ were elliptical in shape. The interactive effect of soybean meal and $CaCO_3$ was circular in shape (Fig. 7e).

The three dimensional (3D) RSM plots (Fig. 7b, 7d, 7f.) indicated that maximum antifungal activity occurred at medium level of soybean meal (10 - 14 g/l), $CaCO_3$ (1 - 2.5 g/l) and starch (10 - 12 g/l).

Prediction of optimal level

The optimal plot (Fig. 8) displays the prediction of the optimal level based on the result of the regression model above. The optimal value of the response variable 24.48 mm was obtained with Carbon, Nitrogen and $CaCO_3$ source as 10.76 g/l, 11.95 g/l and 1.57 g/l respectively.

Correlation between the observed antifungal activity and the predicted values

The correlation between the observed antifungal activity and the RSM predicted values as represented in the parity plot. Figure 9 shows that from the experimental tests that the observed values were close to the predicted value.

Table.1 Coded and uncoded value for each variable

Variables		Coded levels of experimental variables				
Coded	Uncoded	_α	_1	0	+1	+α
X_1	Starch	1.59	5	10	15	18.41
X_2	Soybean meal	1.59	5	10	15	18.41
X_3	$CaCO_3$	0.32	1	2	3	3.68

Table.2 Central composite design plan in coded value, the observed and predicted response

Run	X_1: Starch (g/L)	X_2: Soy bean meal (g/L)	X3: $CaCO_3$ (g/L)	Inhibition Zone diameter (mm)	
				Observed	Predicted
1	-1	1	-1	22.30	22.15
2	0	-1.68	0	13.70	13.26
3	0	0	0	22.40	23.60
4	1	1	1	18.60	18.14
5	1	-1	1	15.00	15.21
6	0	0	0	23.60	23.60
7	-1	-1	1	12.00	11.98
8	-1	-1	-1	17.30	17.82
9	0	0	0	22.70	23.60
10	0	0	0	23.40	23.60
11	-1	1	1	14.50	14.46
12	1	1	-1	21.50	21.58
13	1	-1	-1	16.70	16.79
14	0	0	1.68	11.50	11.71
15	0	0	-1.68	19.80	19.51
16	1.68	0	0	22.00	22.08
17	0	0	0	23.00	23.60
18	-1.68	0	0	20.00	19.84
19	0	1.68	0	19.00	19.37
20	0	0	0	23.90	23.60

$$Y = 0.78 + 0.4098\ X_1 + 2.5657\ X_2 + 7.783\ X_3 + 0.00450\ X_1X_2 + 0.2125\ X_1X_3 - 0.0925\ X_1X_3 \quad \text{Equation (2)}$$

Table.3 Coded Coefficient for Antifungal Production

Term	Effect	Coef	SE Coef	T-Value	P-Value	VIF
Constant		21.512	0.131	164.16	0.000	
X_1	1.5413	0.7706	0.0676	11.41	0.000	2.37
X_2	4.3386	2.1693	0.0676	32.11	0.000	2.37
X_3	-5.236	-2.618	0.119	-21.93	0.000	1.85
X_1^2	-0.4669	-0.2335	0.0213	-10.94	0.000	1.97
X_2^2	-1.2889	-0.6445	0.0213	-30.20	0.000	1.97
X_3^2	-5.6507	-2.8254	0.0854	-33.10	0.000	1.02
X_1X_2	0.0563	0.0281	0.0286	0.98	0.349	1.85
X_1X_3	1.0625	0.5312	0.0573	9.27	0.000	1.43
X_2X_3	0.4625	-0.2313	0.0573	-4.04	0.002	1.43

Coef. = coefficient.

Table.4 Analysis of Variance (ANOVA) for Quadratic Model

SOURCES	DF	SS	MS	*F*-value	*P*-value
Model	9	330.107	36.679	349.25	0.000
Linear	3	160.869	53.623	510.59	0.000
X_1	1	13.663	13.663	130.10	0.000
X_2	1	108.263	108.263	1030.87	0.000
X_3	1	50.506	50.506	480.91	0.000
Square	3	194.773	64.924	618.20	0.000
X_1^2	1	12.568	12.568	119.67	0.000
X_2^2	1	95.769	95.769	911.90	0.000
X_3^2	1	115.041	115.041	1095.40	0.000
2-Way Interaction	3	10.844	3.615	34.42	0.000
X_1X_2	1	0.101	0.101	0.96	0.349^{ns}
X_1X_3	1	9.031	9.031	85.99	0.000
X_2X_3	1	1.711	1.711	16.29	0.002
Error	10	1.050	0.105		
Lack-of-Fit	5	1.050	0.210	5.00	0.0509^{ns}
Pure Error	5	0.030	0.001		
Total	19	331.157			

R^2 = 0.9968; significant at *P* value less than 0.05. ns= non-significant.
Model Summary = C.V. (coefficient of variation) = 0.324070
R^2 (99.68 %) R^2 (adjusted) (99.40 %) R^2 (predicted) (97.49 %).

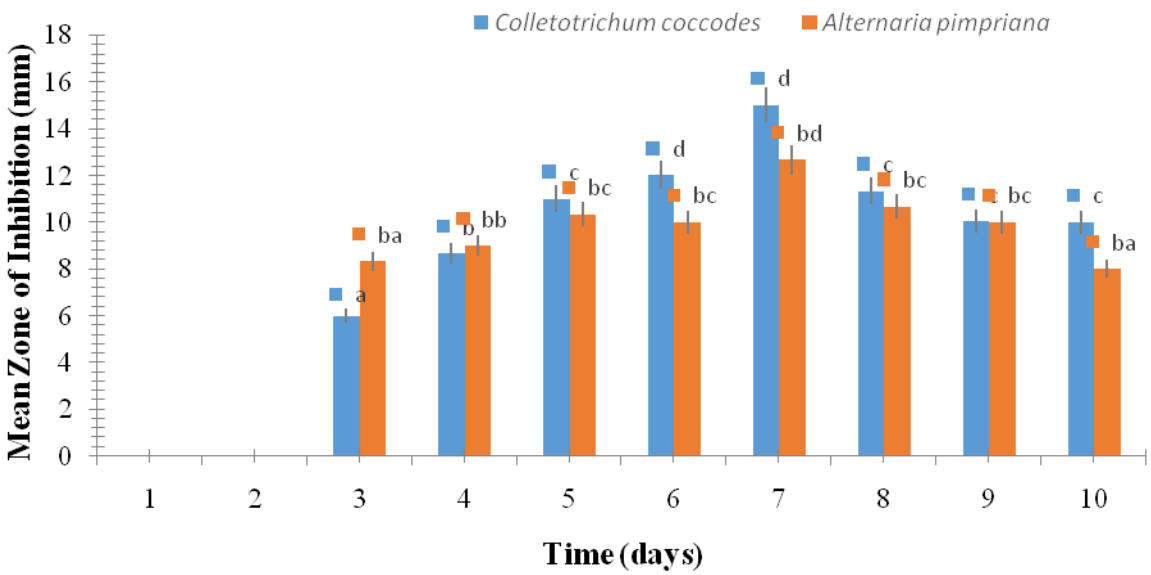


Fig. 1: Effect of incubation period on antifungal metabolite production by *Janibacter* sp. RC18

Bars represent mean ± SE of three replicates. Different letters on bars indicates significant difference between treatments, Using Duncan's multiple range test ($P \leq 0.05$).

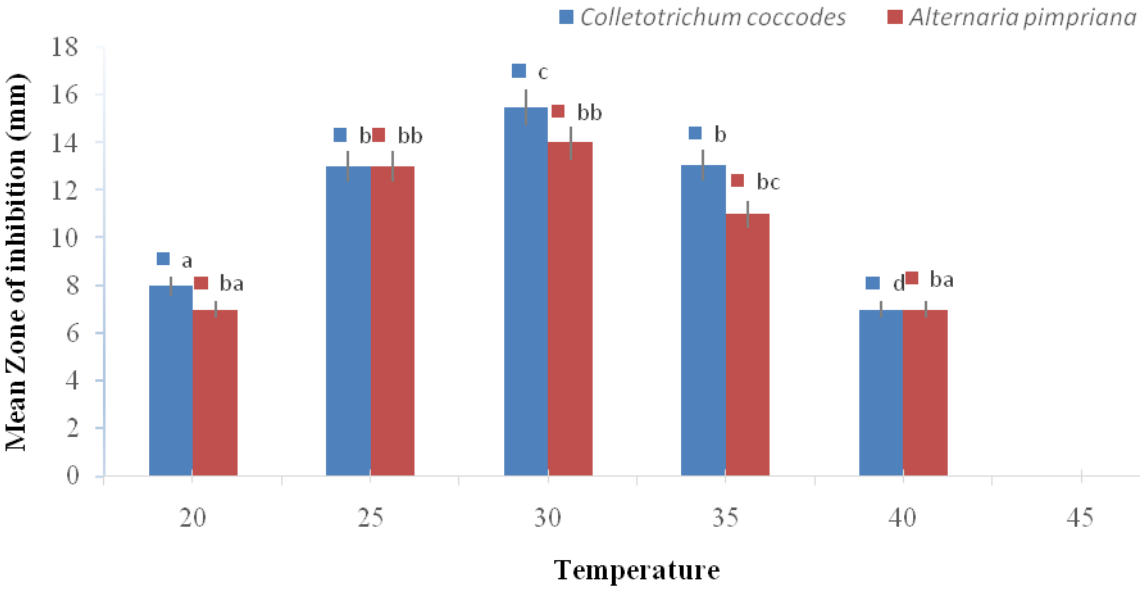


Fig. 2: Effect of incubation temperature on antifungal metabolite production by *Janibacter* sp. RC18

Bars represent mean ±SE of three replicates. Different letters on bars indicates significant difference between treatments, Using Duncan's multiple range test ($P \leq 0.05$).

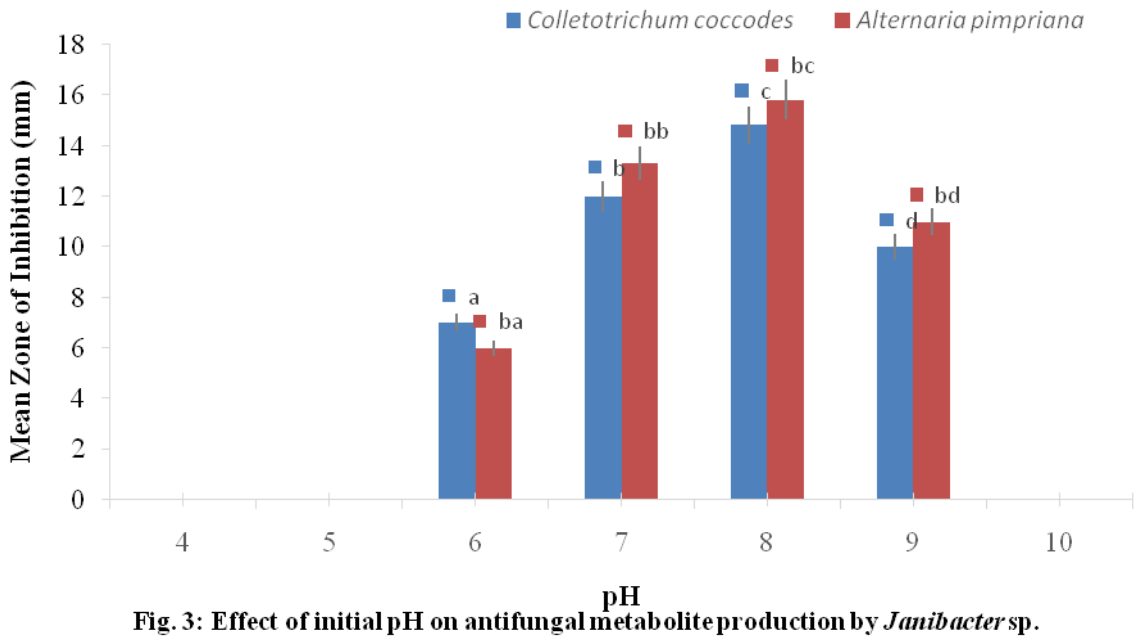


Fig. 3: Effect of initial pH on antifungal metabolite production by *Janibacter* sp. RC18

Bars represent mean ± SE of three replicates. Different letters on bars indicates significant difference between treatments, Using Duncan's multiple range test ($P \leq 0.05$).

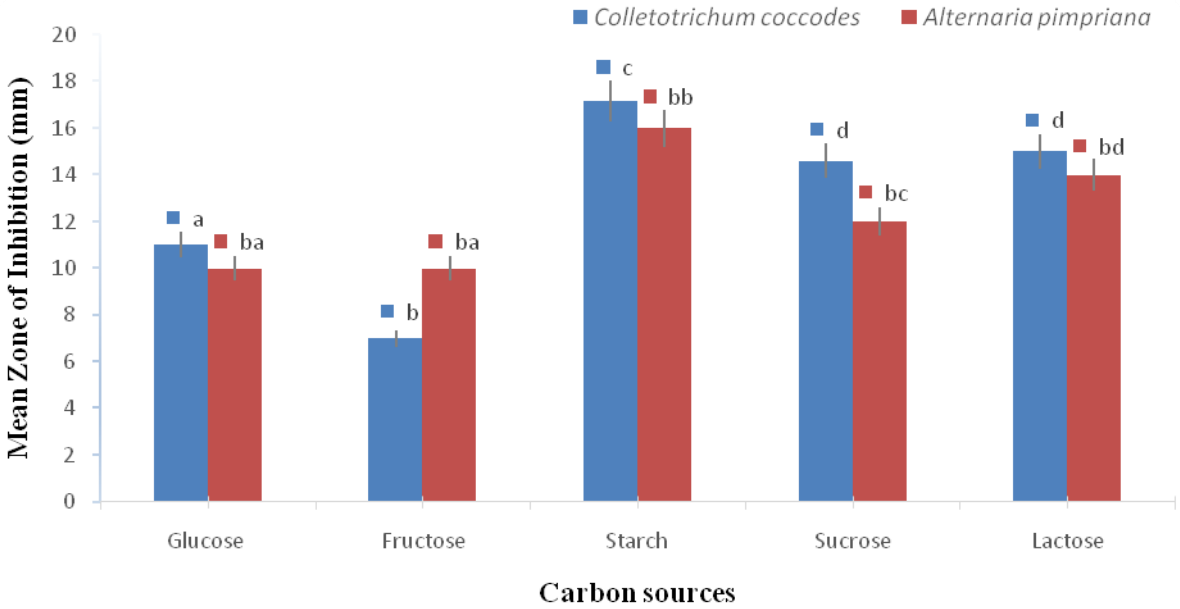


Fig. 4: Effect of different carbon sources on antifungal metabolite production by *Janibacter* sp. RC18

Bars represent mean ±SE of three replicates. Different letters on bars indicates significant difference between treatments, Using Duncan's multiple range test ($P \leq 0.05$).

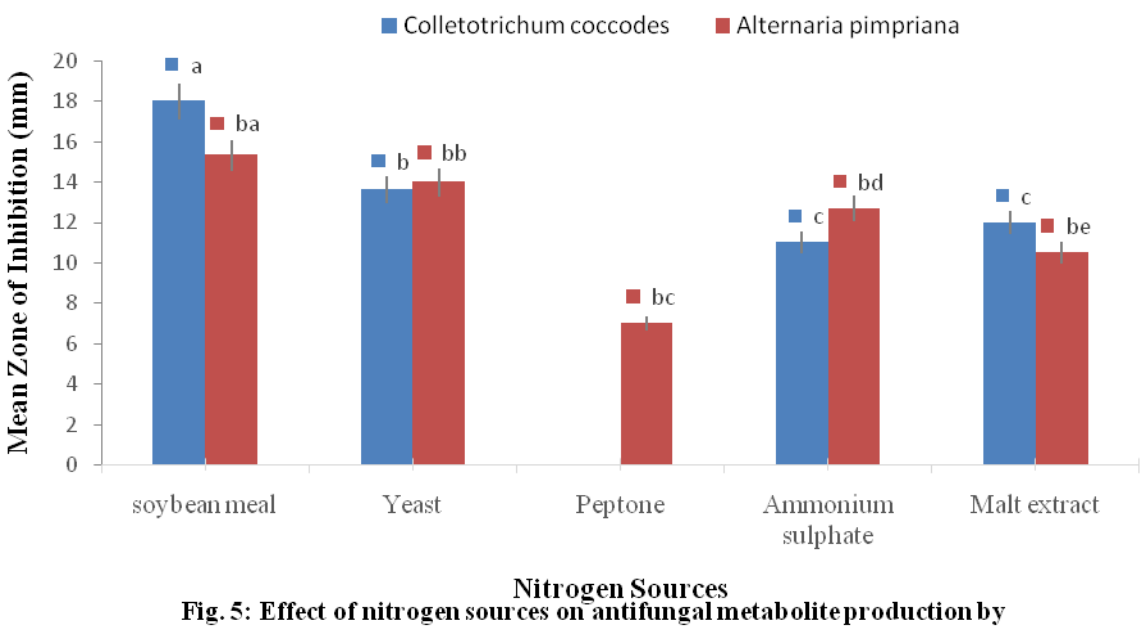


Fig. 5: Effect of nitrogen sources on antifungal metabolite production by *Janibacter* sp. RC18

Bars represent mean ± SE of three replicates. Different letters on bars indicates significant difference between treatments, Using Duncan's multiple range test ($P \leq 0.05$).

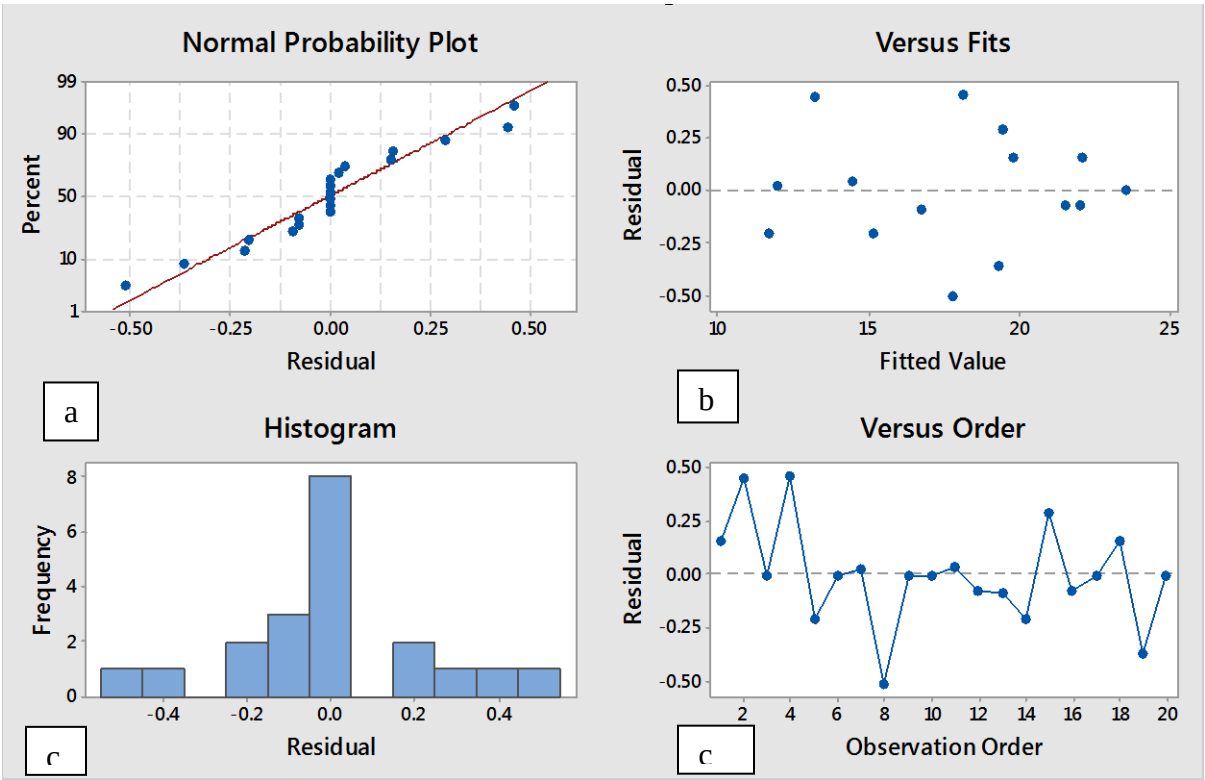
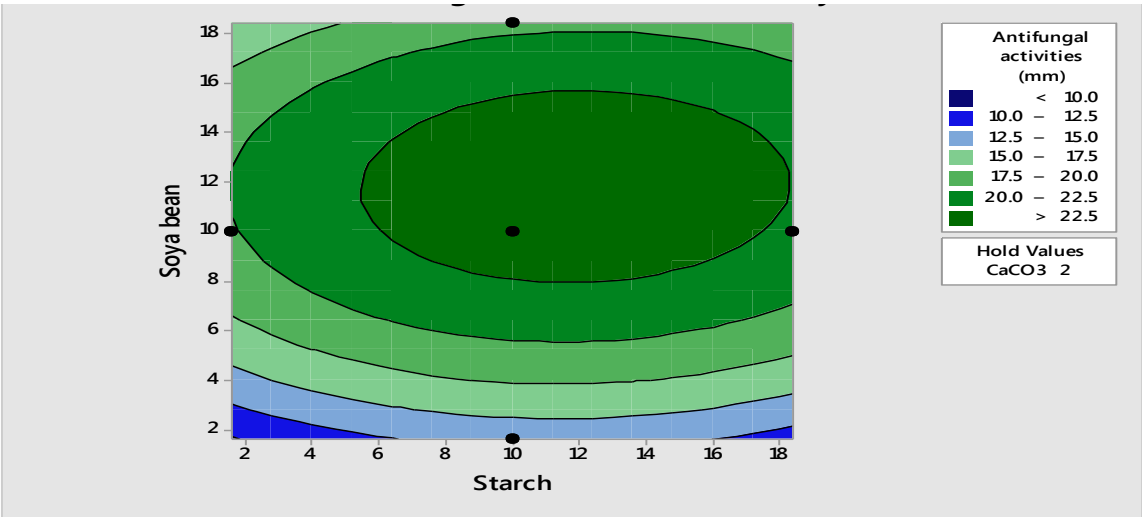
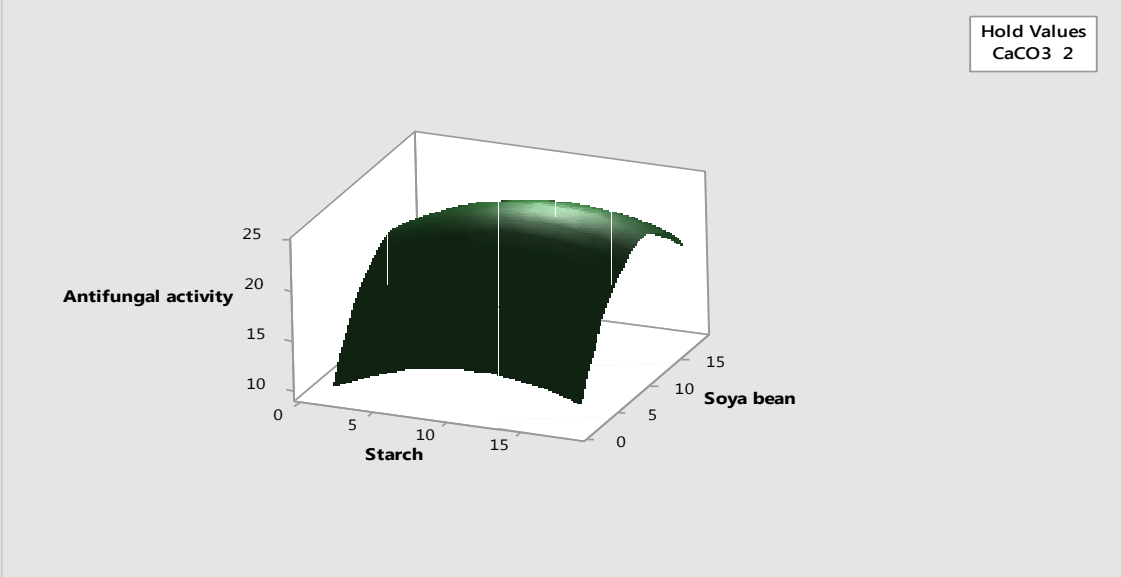


Fig.6 Residual plot for Antifungal activity (mm)

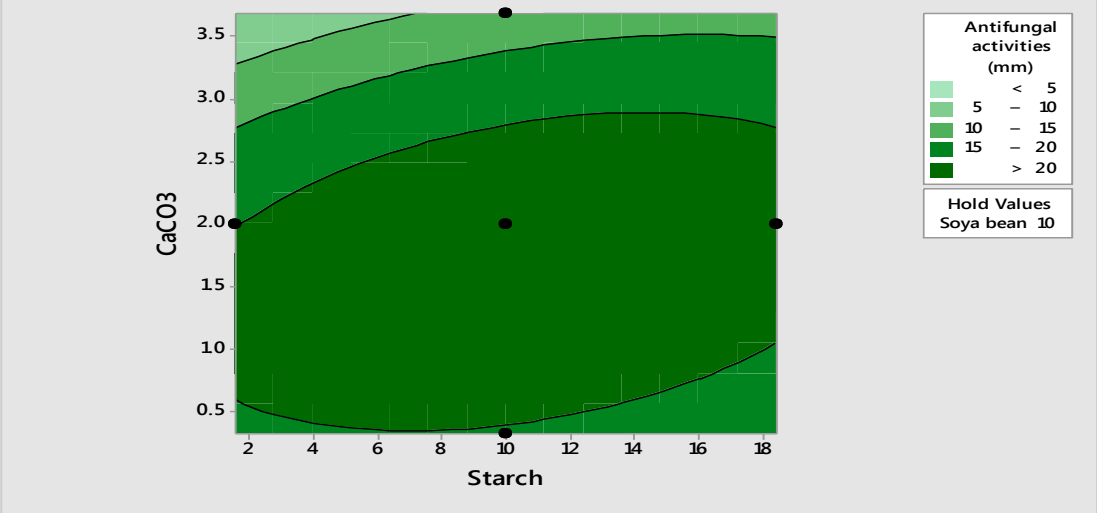
a) Normal probability plot. (b) plot of residual versus fitted value (c) histogram of residual (d) Residual versus observed order.


Soya bean
Starch
Antifungal activities (mm)
< 10.0
10.0 – 12.5
12.5 – 15.0
15.0 – 17.5
17.5 – 20.0
20.0 – 22.5
> 22.5
Hold Values
CaCO3 2

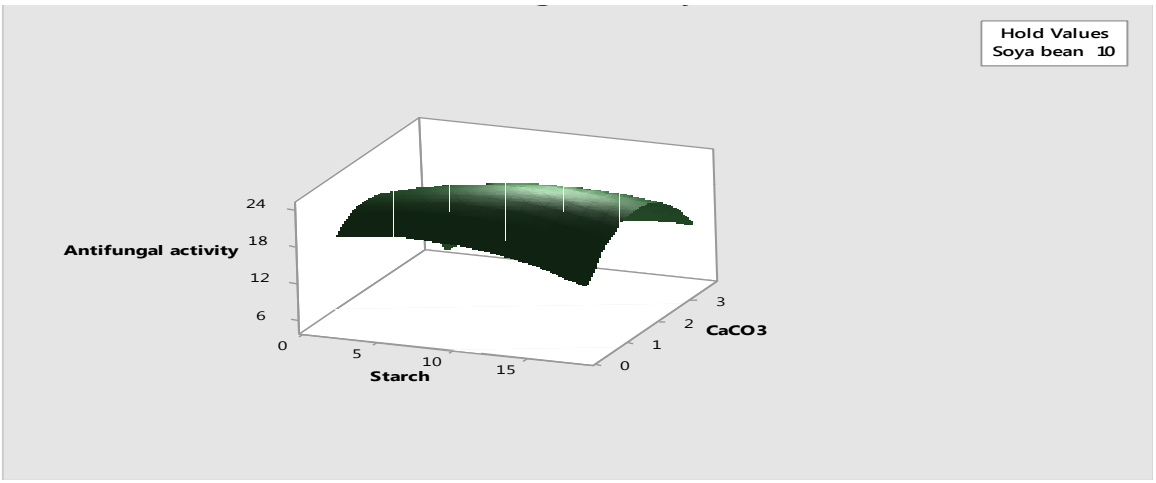
(a)


Hold Values
CaCO3 2
Antifungal activity
Starch
Soya bean

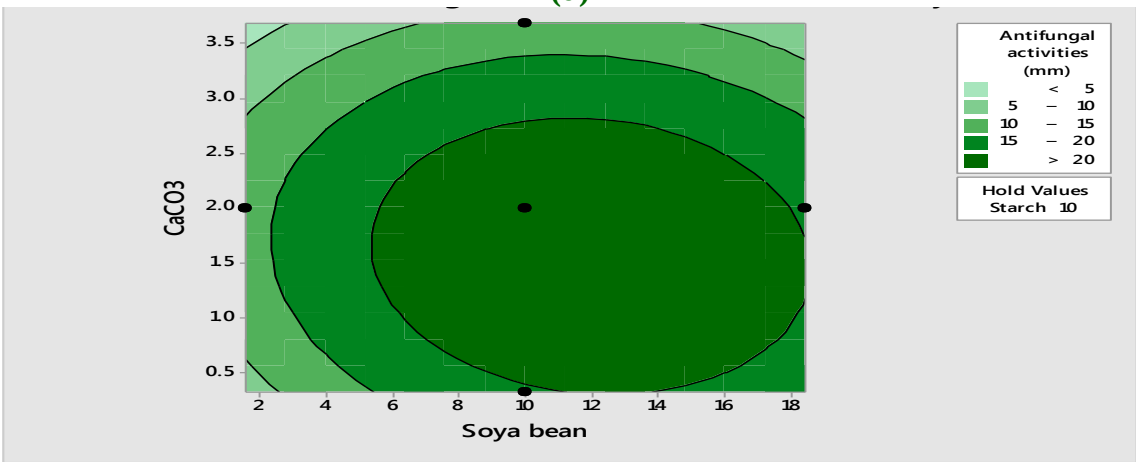
(b)


CaCO3
Starch
Antifungal activities (mm)
< 5
5 – 10
10 – 15
15 – 20
> 20
Hold Values
Soya bean 10

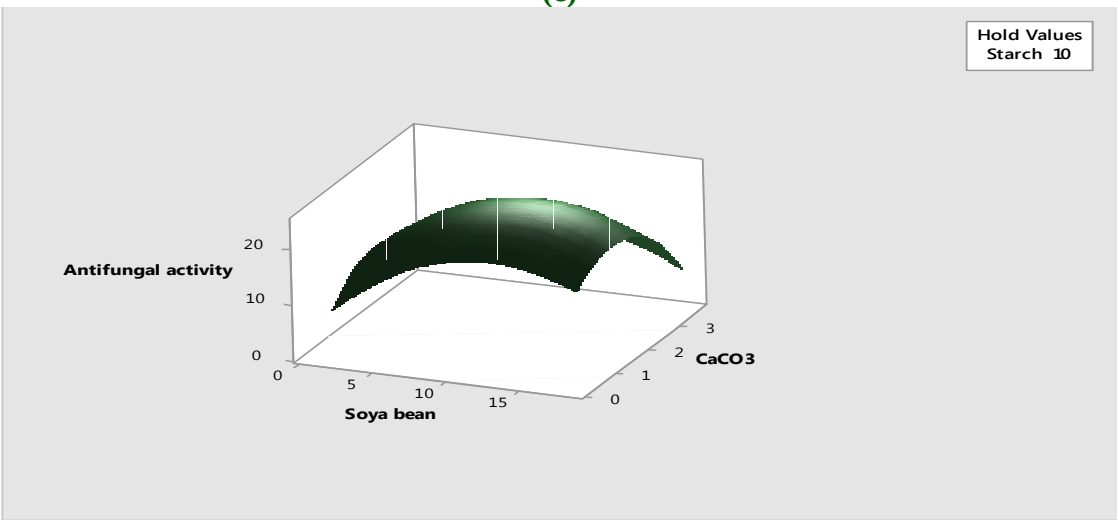
(c)



(d)



(e)



(f)

Fig.7 Response surface plots (3D) and contour plots (2D) showing the individual and interactive effect of variables on the antifungal activity of *Janibacter* sp. RC18 against *C. coccodes*. (a,b) effect of soybean meal and starch. (c,d) effect of starch and $CaCO_3$ (e, f) effect of soybean and $CaCO_3$)

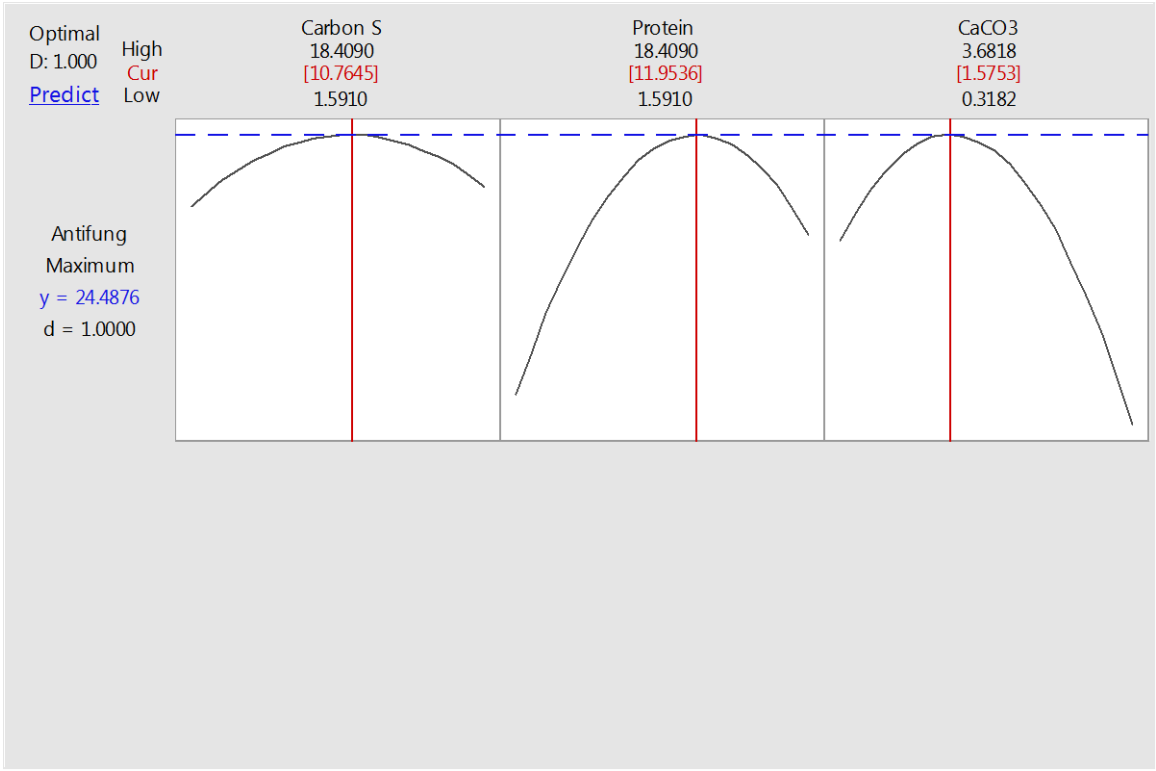


Fig.8 Optimal plot

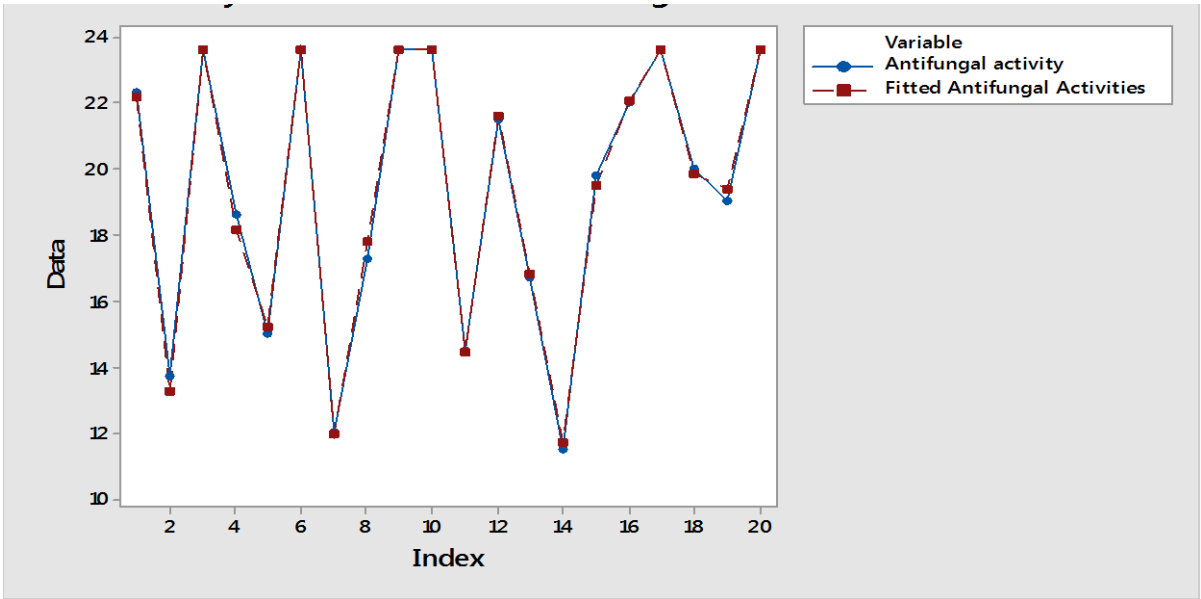


Fig.9 Parity plot of the observed and predicted antifungal activity

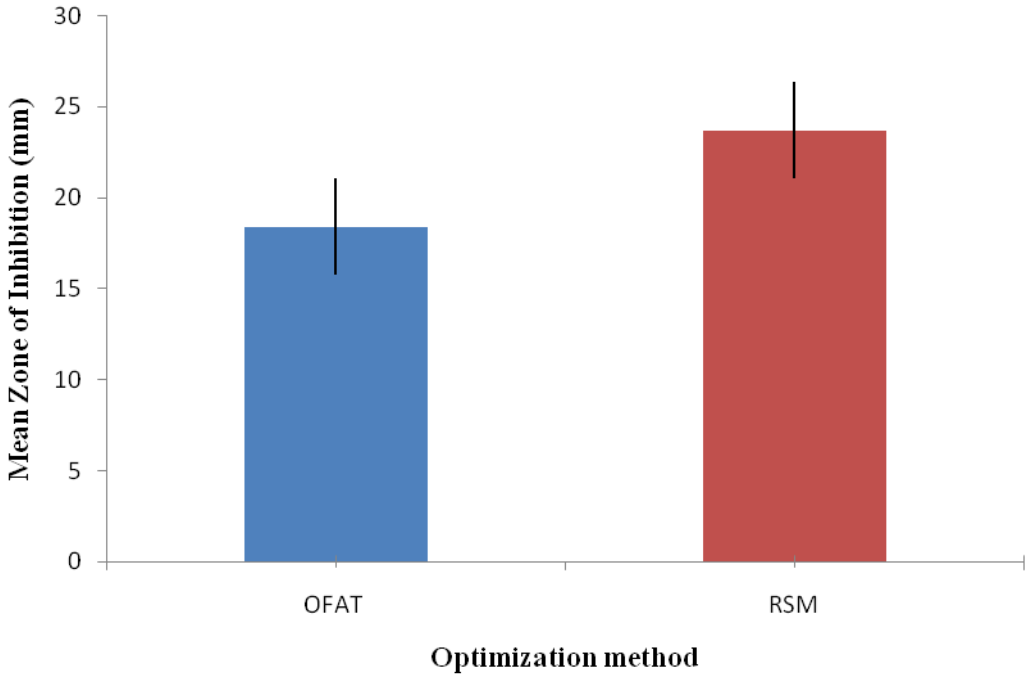


Fig. 10: Comperative medium optimization for anifungal production by *Janibacter* sp. Strain RC18 using OFAT and RSM

Thus, the RSM model was validated with good correlation as the predicted results match well with the experimental results obtained using RSM optimized value.

Validation of the experiment

To test for the validity of the models in predicting the optimal response, validation experiments were carried out at the predicted optimal value.

The result showed that the maximum antifungal activity against *C. coccodes* was 23.7 ± 0.09 mm which was close to the predicted response value 24.49 mm. The result of RSM optimization revealed that antifungal metabolite production was enhanced by 22.33% when compared to the OFAT SS medium (Soybean meal Starch broth) 18.4 ± 0.06 mm as shown in Fig.10.

Antifungal bioactive metabolite production is a complex process, it does not only depend on a potent strain but also on media composition and physical fermentation conditions such as (pH, temperature and time). Minor modification of the fermentation media composition does not only enhance production of bioactive compounds but also leads to changes in metabolic profile of the strain.

Fermentation time is an important factor that affects the antifungal metabolite production, the result obtained showed a gradual increase in antifungal production from day 3 incubation period to day 7 which was recorded as the optimum beyond this fermentation period was a decline in antimetabolite production. This might be explained by activities taking place at different fermentation phases according to Wang (2011), the first phase pre-fermentation phase (growth phase), the second phase of rapid production of secondary metabolite accumulation, and the third phase (post fermentation) phase of slow accumulation of metabolite. This result is in agreement with investigations of Sharon *et al.,* (2014) which

recorded incubation day 7 as optimum for antimetabolite production in *Streptomyces* sp. KOD10. Khattab *et al.,* (2016) and Aliero *et al.,* (2018) also reported optimum antifungal production for *Streptomyces* sp. at incubation day 7.

On the other hand, maximum antifungal production was obtained at temperature 30^{o}C for *Janibacter* sp RC18, the decline afterward up to 45^{o}C where antifungal metabolite production was not recorded. Similarly, Bindu *et al.,* (2018) showed that temperature 30^{o}C was optimum for secondary metabolism in *Streptomyces lavenducolor* VHB-9, Singh and Roymon (2014) also recorded that optimum temperature for antimicrobial metabolite production in *Streptomyces* sp. isolated from soil was 30^{o}C.

The maximum antifungal production by *Janibacter* sp. RC18 was at an initial pH 8, high acidity (pH 3 and 4) and high alkalinity (pH 10) inhibited antifungal metabolite production in the studied strain. Most actinomycetes are neutrophilic, therefore have optimum pH range between 7 – 8 (Augustine, 2005).

Exploring the effect of carbon source on antifungal metabolite production in *Janibacter* sp. RC18 showed that minimal production of antifungal metabolite production was obtained with glucose and fructose which are readily utilizable carbon that are preferable for rapid growth of most microorganisms while the best carbon source for the studied strain was recorded to be starch a complex carbon source. Singh *et al.,* (2017) reported starch as the best carbon source for enhancement of antifungal metabolite production in *Streptomyces* sp. Also in agreement with the result is the investigations of Abdelwahed *et al.,* (2012) showed that starch was the best carbon source for *Streptomycescyaneus* DN37.

In a similar way, investigations on the effect of nitrogen sources on regulation of antifungal metabolite production on the studied strain revealed that soybean meal was the best nitrogen source at which maximum biosynthesis of antifungal metabolite production was attained. In concordance with this result is the report of Bhavana *et al.,* (2014) which reported soybean meal as the most excellent nitrogen source for *Streptomyces carpaticus*. The reason might be that soybean meal as a complex nitrogen source is not easily utilizable and also contains many amino acids which might act as precursor in secondary metabolism (Augustine, 2005).

Optimization by response surface methodology using central composite design

In the statistical optimization the focus was on the nutritional factors whereby soybean meal (nitrogen source) and starch (carbon source) which were found significant from the result of preliminary optimization using OFAT, $CaCO_3$ which enhances the activity of rare and slow growing actinomycetes can inhibit secondary metabolism at high concentration (Yi *et al*., 2015). CCD was used by RSM, the analysis helped to find out interaction between variables and optimum combination of the three nutritional variables starch, soybean meal and $CaCO_3$.

Response surface quadratic model

In the regression analysis, the significance of every coefficient that showed the interaction among the independent variables (starch, soybean meal and $CaCO_3$) were determined by *F*- test and p values of the Fisher's test. The linear regression coefficient terms of starch and soybean meal showed great impact on antifungal metabolite production, they had positive and significant (p = 0.000) effect,

which implies that production increased as parameter values increased. While the linear term of $CaCO_3$ had negative and insignificant (p = 0.349) effect which means that antifungal production decreased as parameter values increased. This is supported by the fact that $CaCO_3$ inhibits secondary metabolisms (Yi *et al*., 2015).

Also the quadratic terms of coefficient were all negatively significant which implies that increase in concentration of starch, soybean and $CaCO_3$ beyond a certain concentration decreases the antifungal metabolite production. For the interactive terms of coefficient, the interaction between starch and $CaCO_3$ (X_1X_3), soybean and $CaCO_3$ (X_2X_3) were significant while interaction between starch and soybean (X_1X_2) were not significant which implies that increase in parameters result in decrease in antifungal metabolite production.

The ANOVA result described the statistical analysis for significances of all factors, the adequacy of the model was analysed by the R^2 coefficient, correlation and model significance (F – value). The quality of the fit of the equation is described by R^2, a good R^2 should be 80% and above (Kocheki *et al*., 2009). The result obtained R^2 for antifungal metabolite production was 0.9968, this is an indication that the model could explain about 99.68% of the variability and it was attributed to independent variables.

The good of fit was determined by R^2 adj, in this study R^2 adj of 0.994 is an indication of agreement of a good model among the obtained and predicted values for response output (Danbaba *et al*., 2015). Model significance (F- value) is a measure of variation of data around the mean. The probability value of (P_{model}>F) of less than 0.05 implies that the model is statistically significant, which means present model can serve as good prediction of experimental result (Yun *et al*., 2018; Wang *et al*., 2018).

However, the coefficient of variation low level (CV = 3.24 %) suggested that these experiment were reliable and precise. Axial Points with coded value (0) were repeated six times in order to estimate the pure error for the lack of fit (LOF) tested. Insignificant LOF is the most desirable and can be used for predictions. This model produced LOF that is not significant (P_{model}>F) at 0.0509.

Residual plot

The residual plot is the most analytical tool for the model, the linearity in the error pattern of the normal probability plot is an indication that there were no signs of problems in the data, this verified the normality regression model. The plots of residual versus fitted value implies homoscendasiticity (constant variance), the randomly scattered points spread around 0 without obvious shape being made by this points indicates assumption of the error having zero mean and equal variance. Furthermore, the dumbbell shape of the histogram plot of frequency versus residual suggested normality of the design also the plot of residual versus observation order shows that the residual is symmetric and have a constant variance. This implies that the result from this model can be used for inferential purpose following the limit theorem (Montgomery, 1997; Tsai *et al*., 1998).

Response analysis

The result of 3D indicates that concentration of the three nutritional variables should not exceed a certain concentration so as not repress antifungal production. The elliptical shaped 2D contour plot of interactive terms of starch and $CaCO_3$, and soybean and starch depicts that their interactive terms were

significant, the circular shape of the soybean and $CaCO_3$ means insignificant interactive term. These results are in agreement with the results obtained in Table 4.

Validation of experiment

Validation of the result was carried out using the optimal nutritional variables condition as follows starch 10.76 g/l, soybean meal 11.95 g/l and $CaCO_3$ 1.57 g/l in a shake flask condition. The maximum antifungal activity obtained experimentally was 23.7 ± 0.09 mm diameter of inhibition as against 24.49 mm predicted. This is in agreement with the model prediction, hence, the model developed was considered to be reliable and precise for predicting the enhanced antifungal metabolite production in *Janibacter* sp strain RC18. Also in comparison with OFAT optimized medium there was about 22.33 % increase in antifungal metabolite production which means RSM is a good tool for optimization.

Conclusion of the study is as follows:

To enhance the antifungal metabolite production in *Janibacter* sp. RC18, preliminary optimization was carried out using OFAT to select significant nutritional variables. Thereafter, RSM using CCD was adopted to optimize the selected significant fermentation media components (starch, soybean meal and $CaCO_3$). The statistical optimization resulted in antifungal activity of 23.7 ± 0.09 mm against *C. coccodes* which was 22.33 % higher than that of OFAT optimized medium (18.4 ± 0.06 mm). The predicted values were in excellent correlation with experimental values hence validating the experiment and confirming accuracy of the model.

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