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Research article

Medium optimization using response surface methodology to produce antifungal substance from *Streptomyces samsunensis* RB-4 against *Rhizoctonia solani*

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Abstract

Importance of the work: Actinomycetes exhibit promising antimicrobial activity against pathogens causing plant diseases. To date, statistical optimization of medium components has not been addressed to enhance the production of an antifungal substance from *Streptomyces samsunensis*, a prolific source of antibiotics and other useful compounds.

Objectives: To optimize the medium composition using a statistics-based experimental design to produce an antifungal substance from the newly isolated *S. samsunensis* strain RB-4 against the fungal pathogen *Rhizoctonia solani*. Response surface methodology (RSM) was applied to determine the effects of medium components (yeast extract, malt extract and glucose) on the biocontrol activity of *S. samsunensis* RB-4 against *R. solani*.

Materials & Methods: Morphological characteristics, diaminopimelic acid isomer (A_2pm) in whole-cell hydrolysate and 16S rRNA gene of *S. samsunensis* RB-4 were examined. Selection of suitable medium for antifungal substance production was performed. RSM based on CCD was chosen to optimize the interactive effects of factors on antifungal substance production. The antifungal activity was screened using the agar-well diffusion method.

Results: The optimal concentrations of the tested medium components for maximum antifungal substance production were 3.62 g/L yeast extract, 18.9 g/L malt extract and 6.74 g/L glucose. An inhibition zone of 15 mm (60% inhibition) was observed with the optimized medium, which was in agreement with the predicted value (14.17 mm).

Main finding: The results proved that RSM could be used as an appropriate and dependable tool to optimize the medium composition to produce an antifungal substance against *R. solani* from *S. samsunensis*.

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Introduction

Rhizoctonia solani, a soil-borne plant pathogenic fungus that was first described as the causative agent of a fungal disease in potatoes in 1858, is a major problem in agriculture, with a broad spectrum of hosts and global spread, causing plant diseases such as damping-off, stem lesions, stem rot, root rot, brown patches and sheath blight (Shanmugasundaram, 1991). Its propensity to generate sclerotia improves its chances of survival in infected areas (Ainsworth, 1981; Shanmugasundaram, 1991). *R. solani* usually infects soybean, wheat, rice, sugar beet, potato and tomato, causing root rot and damping-off (Goudjal et al., 2014).

In the last 20 yr, the use of secondary metabolites from actinomycetes has been described as an alternative strategy for the biocontrol of plant diseases and to reduce the use of chemical agents (Dhanasekaran et al., 2005; Huang et al., 2011; Priya et al., 2012). Actinomycetes such as *Streptomyces griseoviridis* have been used to control plant pathogenic fungi, including *Fusarium* spp. and *Alternaria* spp. (Goudjal et al., 2014).

Streptomyces samsunensis, a member of the *Streptomyces violaceusniger* clade, was first isolated from the rhizosphere of *Robinia pseudoacacia* (Sazak et al., 2011). This species can produce three macrolides, efomycins M and G and oxohygrolidin, as well as two polyethers, abierixin and 29-O-methylabierixin (Lu and Shen, 2004; Barth and Mulzer, 2008; Supong et al., 2016). Additionally, *S. samsunensis* is the only natural source for efomycin M production (Bader et al., 2007; Barth and Mulzer, 2008; Supong et al., 2016). Furthermore, all these compounds exhibit biological activity against many cell types and microorganisms (Dramae et al., 2013; Supong et al., 2016). However, no studies have addressed the statistical optimization of medium components to enhance the production of antifungal compounds from *S. samsunensis* (Feng et al., 2011; Souagui et al., 2019), which is necessary to develop these compounds for biocontrol applications. The present study examined the production of an antifungal substance from the newly isolated *S. samsunensis* strain RB-4 under laboratory cultivation using response surface methodology (RSM), a strong strategic experimental tool for optimizing conditions using a multivariable approach (Khuri and Cornell, 2018). RSM involves the use of factorial design and regression analysis to evaluate and identify the effective factors using mathematical models, analyzing the experimental data and predicting the relationships between the responses and variables, with the ultimately aim of maximizing the responses, such as biomass or product yields (Kenari et al., 2021).

The nutritional requirements of *S. samsunensis* RB-4 were investigated in the present study, using International Streptomyces Project-2 (ISP2) medium as the base medium to maximize the antagonistic activity against *R. solani*. The most important variables were optimized using RSM, with a central composite design (CCD) and a mathematical model that expressed the correlations between the zone of inhibition and levels of important medium components glucose, yeast extract and malt extract. The results from this study provide information pertaining to the interaction between medium components and antifungal activity in actinomycetes and prove that statistical optimization using RSM could be used as a valuable tool for the optimization of antifungal activity of *S. samsunensis*.

Materials and Methods

Microorganisms

S. samsunensis RB-4 (MT879618.1) was isolated from a soil sample obtained from Rayong, Thailand and screened for antifungal activity against *R. solani* using a dual culture technique (Sadfi et al., 2001). The strain RB-4 was grown on ISP3 (oatmeal medium) containing 60 g/L oat meal and 12 g/L agar at pH 7.0 (Shirling and Gottlieb, 1966) and ISP2 agar plates containing 4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose and 20 g/L agar at pH 7.0 (Shirling and Gottlieb, 1966) for 14 d. Then morphological characteristics were examined using a scanning electron microscope (SEM; JSM 5600 LV; JEOL; Tokyo, Japan). The presence of diaminopimelic acid isomer (A_2pm) in whole-cell hydrolysate was determined according to the methods described by Becker et al. (1964). Genomic DNA from the strain RB-4 was extracted and purified according to the method of Kieser et al. (2000). The 16S rRNA gene was amplified via polymerase chain reaction (PCR) using the universal bacterial primers 27f (5'AGAGTTTGATCMTGGCTCAG 3') and 1525r (5'AAGGAGGTGWTCCARCC 3'). Amplification was performed on a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA) according to the program: initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Then, the PCR products were purified and sequenced. The obtained 16S rRNA gene sequence

was aligned with the corresponding sequences of *Streptomyces* type strains retrieved from the EMBL/GenBank database, using the CLUSTAL_X and PHYDIT programs. An unrooted phylogenetic tree was constructed using a neighbor-joining algorithm. The resultant tree topology was evaluated using bootstrap analyses based on 1,000 resamplings. All analyses were performed using the TREECON software.

S. samsunensis RB-4 was grown on glucose-yeast extract agar containing 2 g/L glucose, 5 g/L yeast extract, 0.2 g/L $CaCl.2H_2O$ and 15 g/L agar at pH 7.0 and 30 °C. The organism was subcultured on a fresh plate every month and stored at 4 ± 1 °C. The plant pathogen *R. solani* was obtained from the Plant Protection Research and Development Office, Thailand and maintained on glucose-malt extract-peptone (GMP) agar plates containing 15 g/L glucose, 6 g/L peptone, 3 g/L malt extract, 3 g/L yeast extract, 5 g/L NaCl, 20 g/L agar and 0.25 g/L $MgSO_4.7H_2O$.

Inoculum preparation

S. samsunensis RB-4 was grown on ISP2 agar plates for 7 d. Three loopfuls of spores were used to inoculate 100 mL of ISP2 broth in a 250 mL Erlenmeyer flask and incubated overnight at 37 °C with 200 revolutions per minute (rpm) shaking (Bioshaker BR-43FM-MR; Taitec Corporation; Saitama, Japan) for use as the primary inoculum.

Selection of medium for antifungal substance production

S. samsunensis RB-4 (3% v/v) was used to inoculate 100 mL of one of the following six media in separate 250 mL Erlenmeyer flasks. All media were performed in triplicate.

1) Starch-casein broth (SCB) containing 10 g/L soluble starch, 0.3 g/L casein, 2 g/L KNO_3, 2 g/L NaCl, 0.05 g/L $MgSO_4.7H_2O$, 0.02 g/L $CaCO_3$, 0.01 g/L $FeSO_4.7H_2O$ and 2 g/L K_2HPO_4 at pH 7.0 (Küster and Williams, 1964)

2) ISP2 broth containing 4 g/L yeast extract, 10 g/L malt extract and 4 g/L glucose at pH 7.0 (Shirling and Gottlieb, 1966)

3) Yeast extract-malt extract (YM) broth containing 3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone and 10 g/L glucose at pH 7.0 (Gouliamova et al., 2012)

4) Basal medium 1 containing 10 g/L rice bran, 1 g/L K_2HPO_4, 2 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4.7H_2O$ and 1 g/L NaCl at pH 7.0

5) Basal medium 2 containing 2 g/L rice bran, 5 g/L grist, 1 g/L K_2HPO_4, 2 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4.7H_2O$ and 1 g/L NaCl at pH 7.0

6) Basal medium 3 containing 5 g/L rice bran, 1 g/L K_2HPO_4, 2 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4.7H_2O$ and 1 g/L NaCl at pH 7.0

All flasks were incubated at 30 °C with 150 rpm shaking for 6 d. The cultures were centrifuged (10,000×*g*) and filtered (Whatman® cellulose filter paper; ø 0.45 µm) to obtain the cell-free supernatant containing antifungal compounds.

Mathematical model experiments

Yeast extract, malt extract and glucose were considered the significant medium components affecting the production of antifungal substance from *S. samsunensis* (Souagui et al., 2015). RSM based on CCD was chosen as a suitable tool to optimize the interactive effects of these factors on antifungal substance production (Messis et al., 2014; Souagui et al., 2015). The experimental factors were determined using a two-level factorial design that consisted of yeast extract (0.64–7.36 g/L), malt extract (11.59–28.41 g/L) and glucose (2.64–9.36 g/L) using a 2^3 full factorial design experiment with six star points ($\alpha = \pm 1.68$) and three replicates at the center point (Table 3). The second-order model predicting antifungal substance production was expressed as a regression relationship, as shown in Equation 1:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 \quad (1)$$

where Y is the predicted response (production of antifungal substance, indicated by the inhibition zone size in millimeters); a_0 is a constant; a_1, a_2, and a_3 are linear terms; a_{11}, a_{22}, and a_{33} are quadratic terms; a_{12}, a_{13}, and a_{23} are interaction terms; and X_1, X_2, and X_3 are the test variables studied. The data were analyzed using the Design-Expert software version 13 (Stat Ease Inc.; Minneapolis, MA, USA).

Analysis of antifungal activity

The antifungal activity was screened using the agar-well diffusion method (Valgas et al., 2007). Fungal disks (5 mm diameter) were cut from the edge of mycelia of *R. solani* aged 7 d and placed on the GMP agar surface. After 48 hr incubation, four wells (5 mm diameter) were made at the center of the agar plates, with a spacing of 25 mm in each well and 50 µL of cell-free supernatant were placed in each well. The inhibition zone size was determined as R1–R2 after three days incubation at 30 °C, where R1 is the farthest radial distance (in millimeters)

of *R. solani* growth on GMP plates (control) after 5 d and R2 is the radial distance (in millimeters) of fungal growth from the inoculation point to the colony margin in the direction of the antagonist (Sadfi et al., 2001; Magaldi et al., 2004).

Results and Discussion

Identification of S. samsunensis RB-4

The SEM analysis revealed that strain RB-4 formed a filamentous helix spore (Fig. 1). Whole-cell hydrolysates of the isolate were rich in *LL*-A_2pm. Molecular identification revealed that the 16S rRNA gene (1,418 bp) of the isolated strain shared 99.80% sequence similarity with that of *S. samsunensis* (EU077190). A phylogenetic tree generated based on the 16S rRNA gene sequences of 1,397 nucleotides from related actinomycetes also showed that the RB-4 strain was phylogenetically closely related to *S. samsunensis* species (Fig. 2).

Selection of medium for antifungal substance production

The best medium for production of the antifungal substance from *S. samsunensis* RB-4 was selected by determining the inhibition of *R. solani* growth (Fig. 3). *S. samsunensis* RB-4 grown in IPS2 broth showed maximal antifungal activity with 5.75% inhibition, followed by the culture grown in YM medium, with 5.25% inhibition. No inhibition of *R. solani* growth was observed when the RB-4 strain was grown in SCB or the basal media 1, 2, and 3, indicating the lack of antifungal activity.

Fig. 1 Scanning electron micrograph of spore chain of *Streptomyces samsunensis* RB-4, magnification 35000×, An electron microscope uses electrons accelerated by a voltage of 5 kV. Scale bar 1 um.

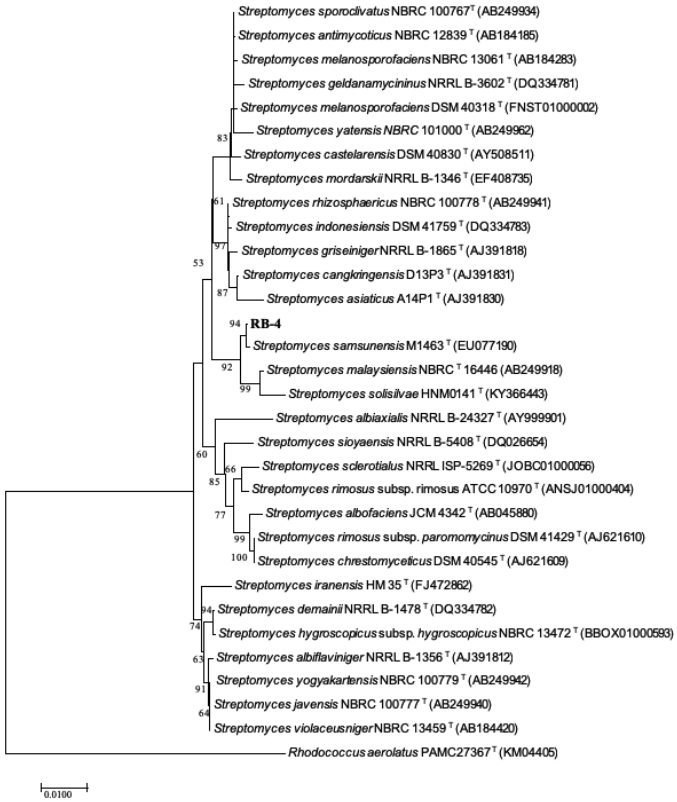

Fig. 2 Neighbor-joining tree based on 16S rDNA sequences showing phylogenetic relationship between strain RB-4 and related type strains of genus *Streptomyces*, where *Rhodococcus aerolatus* PAMC27367T (KM044053) was used as an outgroup, the numbers on branches indicate percentage bootstrap values of 1,000 replications (only values > 50% are indicated) and scale bar = 0.01 substitutions per nucleotide position

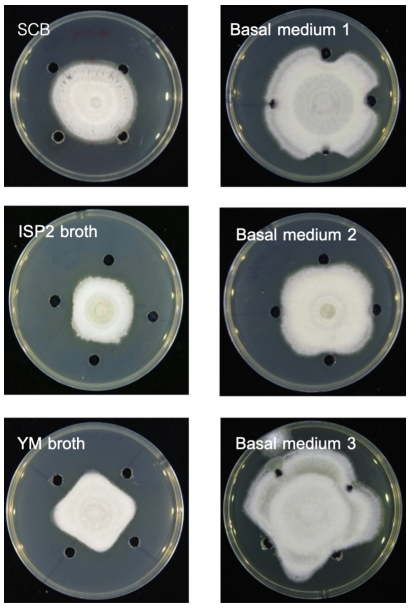

Fig. 3 Antifungal substance production efficiency of *Streptomyces samsunensis* RB-4 grown in six media against *Rhizoctonia solani*

Thus, *S. samsunensis* RB-4 was observed to exhibit activity against the pathogenic fungus *R. solani* in the present study. In another study, *S. samsunensis* suppressed the growth of different microorganisms: *Aspergillus niger*, *Bacillus licheniformis*, *Candida albicans*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptomyces murinus*, but it did not inhibit *Aspergillus flavus*, *Escherichia coli* and *Saccharomyces cerevisiae* (Sazak et al., 2011). It has also been demonstrated to act against *Mycobacterium tuberculosis*, *Bacillus cereus*, *Colletotrichum gloeosporioides* and *Colletotrichum capsici* and the malarial parasite *Plasmodium falciparum* (Supong et al., 2016); however, antifungal activity of *S. samsunensis* against *R. solani* has not been reported thus far.

Although *Streptomyces* spp. have been shown to thrive and produce antifungal substance in ISP2 medium, the nutrient composition of the cultivation medium required to achieve the maximum production of antifungal substance may vary for each strain and needs to be optimized (Augustine et al., 2005; Meklat et al., 2012; Kesavan and Hemalatha, 2015). The present study investigated antifungal substance production from the RB-4 isolate at the laboratory scale using shake flask cultivation. *S. samsunensis* RB-4 grew well on ISP2 formula 1.

Effect of medium composition on antifungal substance production

Yeast extract, malt extract and glucose concentrations were studied as the three independent variables affecting the production of antifungal substance using CCD with 20 experimental runs, as shown in Table 1. The results of the analysis of variance for the response surface of a quadratic model for antifungal substance production are presented in Table 2. Statistical analysis of the response (inhibition zone size in millimeters) revealed a regression-based determination coefficient (R^2) of 0.8144, which suggested a 81.44% fit between the developed model and experimental data, with the remaining 18.56% signifying the potential effects of other variables. The *F* value of 4.88 with a *p*-value (probability > F) of 0.0105 was indicative of the fit of the model to the experimental results and demonstrated that the model was statistically significant. This result indicated that the response equation was a suitable model for the relationship between the independent variables and the response (Ahsan et al., 2017). The regression equation for the antifungal substance production response in terms of the actual variables is shown in Equation 2:

$$Y = 13.87-0.55X_1-0.28X_2+1.19X_3-1.02X_1X_2-0.80X_1X_3-1.51X_2X_3+0.13X_1^2+0.50X_2^2-0.12X_3^2 \quad (2)$$

Table 1 Experimental design used in response surface methodology with three independent variables: yeast extract (X_1), malt extract (X_2) and glucose (X_3)

Run no.	Level			Actual level			Inhibition zone (mm)	
	X_1	X_2	X_3	X_1 (yeast extract, g/L)	X_2 (malt extract, g/L)	X_3 (glucose, g/L)	Observed	Predicted
1	−1	−1	−1	2	15	4	10	10.69
2	1	−1	−1	6	15	4	10	13.23
3	−1	1	−1	2	25	4	11	15.19
4	1	1	−1	6	25	4	8	13.65
5	−1	−1	1	2	15	8	12.75	17.69
6	1	−1	1	6	15	8	11.25	17.03
7	−1	1	1	2	25	8	9.75	16.15
8	1	1	1	6	25	8	12.25	11.41
9	−1.68	0	0	0.64	20	6	12.5	15.16
10	1.68	0	0	7.36	20	6	9.25	13.31
11	0	−1.68	0	4	11.59	6	11.75	15.75
12	0	1.68	0	4	28.41	6	11.25	14.81
13	0	0	−1.68	4	20	2.64	6.75	11.53
14	0	0	1.68	4	20	9.36	12.25	15.53
15	0	0	0	4	20	6	11.5	13.87
16	0	0	0	4	20	6	14.25	13.87
17	0	0	0	4	20	6	14	13.87
18	0	0	0	4	20	6	14.75	13.87
19	0	0	0	4	20	6	14.25	13.87
20	0	0	0	4	20	6	14.5	13.87

Table 2 Analysis of variance for fitted quadratic polynomial model for production of antifungal substance from *Streptomyces samsunensis* RB-4

Source	Sum of squares	Degree of freedom	Mean squares	*F* value	*p*-value
Model	75.17	9	8.35	4.88	0.0105
X_1	4.08	1	4.08	2.38	0.1537
X_2	1.08	1	1.08	0.63	0.4455
X_3	19.34	1	19.34	11.29	0.0072
X_1^2	14.97	1	14.97	8.74	0.7925
X_2^2	9.18	1	9.18	5.36	0.3052
X_3^2	32.65	1	32.65	19.06	0.7925
$X_1 X_2$	0.13	1	0.13	0.073	0.0144
$X_1 X_3$	2	1	2	1.17	0.0431
$X_2 X_3$	0.12	1	0.12	0.073	0.0014
Residual	17.13	10	1.71		
Lack of fit	10.03	5	2.01	1.41	0.3564
Total	92.30	19			

Coefficient of determination = 0.8144

Table 3 Experimental range and levels of independent variables used in central composite design

Variable code	Variable	Level				
		−α	−1	0	+1	+α
X_1	Yeast extract (g/L)	0.64	2	4	6	7.36
X_2	Malt extract (g/L)	11.59	15	20	25	28.41
X_3	Glucose (g/L)	2.64	4	6	8	9.36

where Y is the production of antifungal substances (measured as the inhibition zone size in millimeters) and X_1, X_2 and X_3 are the concentrations of yeast extract, malt extract and glucose, respectively, all in grams per liter.

The model coefficients generated via regression analysis for each variable are listed in Table 2. The results revealed that only glucose had a significant effect on the antifungal activity. The favorable effect of glucose can be attributed to its requirement as a substrate for the synthesis of a variety of biomolecules, especially in the glycolytic cycle. The Embden-Meyerhof-Parnas pathway is the primary mechanism through which actinomycetes break down glucose and convert it into metabolic intermediates, which are then converted into various end-products (Takahashi and Yamada, 1999). The quadratic terms of yeast extract (X_1^2), malt extract (X_2^2) and glucose (X_3^2) did not significantly ($p > 0.05$) affect the antifungal activity. The interaction relationships and optimal values of the variables were determined using response surface plots (Fig. 2). These factors (X_1X_2, X_1X_3 and X_2X_3) showed a significant interaction with the production of antifungal substance, as illustrated by the corresponding *p*-values ($p < 0.05$) in Table 2. Antifungal substance production increased with increasing concentrations of yeast and malt extracts. Specifically, antifungal substance production increased as the yeast extract concentration increased from 2 g/L to 4 g/L and the malt extract concentration from 15 g/L to 19 g/L and then decreased beyond these concentration ranges (Fig. 4A). It was evident that the production of antifungal substances increased when the yeast extract concentration was within the range 3.4–4.0 g/L and that of the malt extract was within the range 19–20 g/L.

On examining the effects of yeast extract and glucose on the production of antifungal substance (Fig. 4B), antifungal substance production similarly increased over the yeast extract concentration range 2.0–4.0 g/L and decreased at higher concentrations. In contrast, only a slight increase in antifungal activity occurred when the glucose concentration was increased from 2.0 g/L to 7.0 g/L. Thus, maximum antifungal substance production was achieved with 3.4–4.0 g/L yeast extract and 6.5–6.7 g/L glucose. Another study demonstrated that GlnR (central regulator of nitrogen metabolism) operates as a universal nitrogen metabolism regulator in actinomycetes while also regulating carbon source transportation (Görke and Stülke, 2008), which supports the synergistic interaction between the glucose and yeast extract observed in the present study.

Fig. 4C illustrates the interaction between malt extract and glucose concentrations. The antifungal substance production increased with increasing concentrations of malt extract. Antifungal activity also increased over the glucose concentration range 2.0–7.0 g/L and decreased at higher concentrations. From this response plot, malt extract and glucose concentrations of 19 g/L and 6.8 g/L, respectively, resulted in the maximal antifungal substance production.

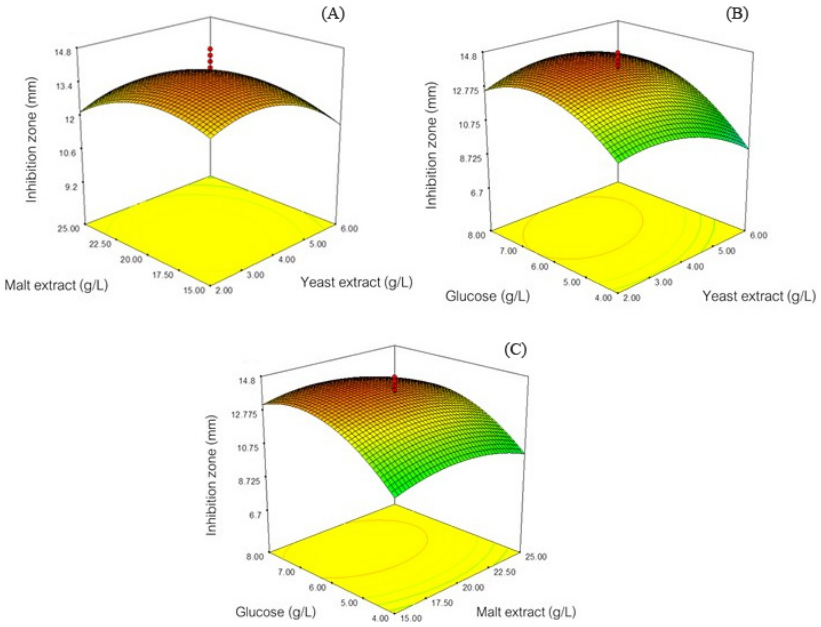

Fig. 4 Response plots of combined effects of three nutrients on production of antifungal substances from *Streptomyces samsunensis* RB-4: (A) yeast extract (X_1)

The Design-Expert software was used to optimize the regression equation to predict the response in the experimental regions (Singh, 2017). An optimum zone of inhibition size of 14.17 mm was predicted with yeast extract, malt extract and glucose concentrations of 3.62 g/L, 18.9 g/L and 6.74 g/L, respectively. Three experiments were conducted under optimal conditions to validate the model prediction. A zone of inhibition of 15 mm (60% inhibition) was observed, similar to the predicted value of 14.17 mm. Messis et al. (2014) conducted a similar study for the optimization of antifungal substance production from *Streptomyces* sp. TKJ2. The results of the present study were comparable to those reported by Zhao et al. (2017), in which RSM was used to optimize the medium for maximum production of antifungal active substance from *Streptomyces lydicus* E12 in flask cultivation. The optimum values of the tested variables were: starch 84.96 g/L, soybean cake powder 4.13 g/L, glucose 5 g/L, $MgSO_4 \cdot 7H_2O$ 1.23 g/L, $K_2HPO_4.3H_2O$ 2.14 g/L and NaCl 0.5 g/L. The test result of 67.44% antifungal inhibition agreed with the prediction and increased by 14.28% compared to the basal medium (Zhao et al., 2017).

In summary, the CCD used in this study generated a highly significant quadratic polynomial to determine the appropriate concentrations of elements with substantial impact on antifungal activity of *S. samsunensis* RB-4 (Mangamuri et al., 2014). This study also confirmed that RSM could be used to optimize the production of antifungal active substance from *S. samsunensis*.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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