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Fermentation medium optimization of *Streptomyces* sp. as an antifungal agent against the *Ganoderma boninensis* pathogen in oil palm

Syamsika Tahir[1,*], Widya Dwi Rukmi Putri[1], Agustin Krisna Wardani[1], Rofiq Sunaryanto[2]

[1]Department of Agricultural Product Technology, Faculty of Agricultural Technology, Brawijaya University, Jl. Veteran, Malang 65145, Indonesia

[2]LAPTIAB, Agency for the Assessment and Application of Technology, Jl. Raya Puspiptek, Tangerang 15314, Indonesia

*Corresponding author: syamsika.tahir@gmail.com

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ABSTRACT *Ganoderma boninensis* is the most common fungus which attacks oil palm trees. However, a significant percentage of inhibition to the problem is found through the use of *Streptomyces* sp. The optimization of the *Streptomyces* sp. fermentation medium growth factors affects the secondary metabolites production. This study aimed to identify the best formulation of carbon and nitrogen sources and the optimum concentration of *Streptomyces* sp. fermentation medium for antifungal compound production. The results showed that the best sources of carbon and nitrogen were liquid glucose and monosodium glutamate in the inhibition zones of 16.7 mm and 6.3 mm, while the best concentration levels were 20 g/L and 14.19 g/L, respectively. The results of the first optimization showed an inhibition zone response and area (%) of the optimum high-performance liquid chromatography (HPLC) chromatogram of 24.39 mm and 62.68 percent, respectively. Taking the suggestion of the first optimization, the second optimization produced 15.2 g/L and 8.3 g/L. The predicted value of the inhibition zone was 21.47 mm, and the area (%) of the HPLC chromatogram was 53.44 percent. The validation results showed an inhibition zone response of 22.01 mm and an HPLC chromatogram area (%) of 54.86 percent. The difference between the predicted and validation values was less than 5 percent; the validation value was thus in line with the value predicted by Design Expert 10.0.7. The chemical formula of the probable active compound is that of the cyclo(phenylalanyl-prolyl) compound.

KEYWORDS *Ganoderma boninensis*; Optimization of fermentation media; Secondary metabolites; *Streptomyces* sp.

1. Introduction

As the primary source of vegetable oil, palm oil is a viable export commodity that thrives in tropical regions, including Indonesia, which in 2014 was home to the largest oil palm plantation area in the world, totaling around 10.6 million hectares (Sari et al. 2021; Khatiwada et al. 2021). However, this figure is inversely proportional to the productivity value, which has declined, and Indonesian oil palm fields continue to produce less than Malaysian fields (Varkkey et al. 2018). The potential exists to produce 7–8 tons of palm oil per hectare per year, while Indonesian fields achieve an output of only around 2–3 tons per hectare per year (Sari et al. 2019). This is caused by various factors, notably the incursion of phytopathogenic microorganisms such as *Ganoderma*, *Phytophthora*, and *Fusarium* fungus (Ho and Tan 2015). Accounting for around 59 percent of cases, *Ganoderma boninensis* is one of the most prevalent fungi that attacks oil palm trees and is the primary cause of basal stem rot (BSR) (Khaled et al. 2018).

Previous research has investigated various measures for disease control and suppression, including the genetic engineering approach (Budiani et al. 2019), traditional cultural traditions of good sanitation processes, and the burning of infected oil palm trees. Furthermore, chemical pesticides such as hexaconazole, tradimefon, triadimenol, carboxin, benomyl, and cyproconazole are used. However, they have a severe environmental impact and are ineffectual or unsuitable for specific targeting (Najihah et al. 2015). In addition, hexaconazole has been reported to show only moderate persistence in soil and not achieve the maximum effect toward the target site. Meanwhile, repeated application may lead to accumulation, leaching, or impact soil microorganisms (Maznah et al. 2015).

Biological control based on microorganisms has grown in popularity (Pandit et al. 2022) due to its capacity to selectively target fungal infections while reducing negative environmental repercussions (Yen and Ali 2022). Bacteria from the phylum Actinomycetes, *Streptomyces*, displayed a significant percentage inhibition against *Ganoderma boninensis* (Budi et al. 2022). Actinomycetes iso-

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lated from *Ficus deltoidea* rhizosphere also demonstrated antifungal activity with *Streptomyces* sp. RTB 1 and RTB 34 exhibited the highest activity against all tested fungi, including *Candida albicans*, *Colletotrichum capsichi*, and *Fusarium oxysporum* (Janatiningrum and Lestari 2022). Due to the presence of ribostamycin, salinomycin, and landomycin B compounds, *Streptomyces* sp. isolates exhibited antifungal activity against *Ganoderma boninensis* (Lim et al. 2018). The presence of two leading compounds in *Streptomyces olivaceus*, sorbicillin and 3-methyl-N-(2′-phenethyl)-butyrylamide, were found to produce antifungal action against *Candida albicansin* the organism (Meng et al. 2019).

Through maximizing positive growth factors, such as internal and environmental variables, microorganisms can produce the maximum level of secondary metabolites. It is simpler to engineer external factors such as nutrition and the environment of microbial culture media. Carbon, nitrogen, and minerals (trace elements) are nutrients that bacteria need to grow (Fedorenko et al. 2015). However, their common production media remain relatively expensive (Singh et al. 2017). Thus, it is essential to adjust the media in such a way that renders them less costly at the same time as increasing the output of secondary metabolites. Therefore, to increase the production of secondary metabolites that act as antifungal pathogens for oil palm Ganoderma boninensis, a more in-depth study of the carbon and nitrogen sources required during fermentation and optimization of the growth media for *Streptomyces* sp. optimization using the response surface methodology (RSM) is needed.

This research is expected to identify the optimum conditions for the growth of *Streptomyces* sp. in producing secondary metabolites, which serve as antifungal compounds. Thus, the media can be used as a biocontrol, particularly against the pathogen *Ganoderma boninensis* that affects oil palms.

2. Materials and Methods

2.1. Pre-fermentation preparation

Streptomyces sp. was grown on yeast extract–malt extract (YEME) agar with a composition of yeast extract (Himedia, India) 3 g/L, malt extract (Himedia, India) 3 g/L, peptone (Himedia, India) 5 g/L, glucose (Merck, Germany) 15 g/L, and agar (Himedia, India) 16 g/L. Potato dextrose agar (PDA) (Himedia, India) medium was used to grow *Ganoderma boninensis*, which was then incubated at 28 °C for 3 d.

TABLE 1 Composition of standard fermentative media.

Media	g/L	Merk
Peptone	5	Himedia
Yeast extract	1	Himedia
Fe (III) citrate hydrate	0.3	Merck
Glucose	15	Merck
Aquades	1 liter	-

The bacterial cultures on the YEME agar were then transferred to vegetative media with the composition of yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 15 g/L, and aquadest. Fungus on PDA medium was transferred to potato dextrose broth media, then shaken at 150 rpm for 2 d at 30 °C (Sunaryanto et al. 2010).

2.2. Fermentation

Standard fermentative media were employed, the compositions of which are shown in Table 1. Each 100 mL fermentation medium received a 10% (v/v) seed culture inoculation under sterile conditions. The shaker was incubated at a speed of 150 rpm for 7 d at 30 °C (Sunaryanto et al. 2010).

2.3. Extraction of fermentation results

Ethyl acetate was used as the extraction solvent in two stages, with a 1:1 ratio between the solvent and microbial fermentation broth. The ethyl acetate and fermentation broth mixture was shaken horizontally for 15 m using a reciprocal shaker. It was then centrifuged (Suprema 5 TOMY, Japan) at 8,000 rpm for 15 m to separate the material into two layers. A rotating vacuum evaporator was used to remove and separate the organic solvent layer while a vacuum concentrator was used for concentration (Sakuma EC-2000, Japan) (Rao et al. 2017).

2.4. The selection of carbon and nitrogen sources

The cultivation media for selecting carbon sources consisted of 15 g/L carbon source, peptone 5 g/L, yeast extract 1 g/L, Fe (III) citrate hydrate 0.3 g/L, and aquadest. The carbon sources used were glucose, liquid glucose, sucrose, lactose, maltose, galactose, and fructose. The cultivation media for the selection of nitrogen sources with the weight of each source refers to the total nitrogen content (%) of standard fermentative media (control) in every 1 liter of medium to which was added 15 g/L glucose, Fe (III) citrate hydrate 0.3 g /L, and aquadest. The nitrogen sources used were peptone 5.76 g/L, ammonium sulfate 3.59 g/L, monosodium glutamate (MSG) 9.19 g/L, yeast extract 7.55 g/L, tofu waste 15 g/L, and urea 1.63 g/L. Shaken culture was used for the cultivation, which was performed in a 250 mL erlenmeyer with a 100 mL working volume for 7 d at 30 °C and 150 rpm. Following activity testing using the agar disk diffusion method, the crude extract of each medium was examined using a one-factor completely randomized design to identify the type of nutrient.

Column chromatography was used to fractionate the fermentation crude extract. The column used had a diameter of 2.5 cm and a height of 50 cm. The stationary phase material used was silica gel 60 F254 (Merck, Germany) (0.063–0.200 mm), while the mobile phase used 10 variations of a mixture of chloroform: methanol: aquadest to obtain different polarities. The HPLC chromatogram profile was then determined for 30 fractions of column chro-

TABLE 2 Independent variables in central composite research design 1.

Variable	Level code				
	-1.414	**-1**	**0**	**1**	**1.414**
Liquid glucose concentration, X1 (g/L)	585.786	10	20	30	341.421
MSG concentration, X2 (g/L)	0.0478644	4.19	14.19	24.19	283.321

matography using a Waters 2695, and the HPLC findings were compared. The agar disk diffusion method was then used to determine the activity of each fraction. The extracts were analyzed using LCHR-MS (Thermo Scientific Q Exactive, Germany) to predict the compounds that play a dominant role as antifungal pathogens of oil palm Ganoderma boninensis after it was confirmed that certain fractions contained antifungal compounds and actively inhibited the growth of pathogenic fungi of cocoa pods (Rahma et al. 2015).

2.5. *The selection of concentration levels of carbon and nitrogen sources*

At this stage, the research experiment used a one-factor completely randomized design within each process of determining the nutrient concentration as the midpoint for use in the optimization process.

The best carbon source that was identified for inhibiting pathogenic fungi would be sought at the best concentration level (g/L) for use during fermentation. The factors from the concentration level of carbon sources were 5, 10, 15, 20, 25, 30, 35, and 40.

Factors from the concentration level of the nitrogen source (g/L) were 9.19, 14.19, 19.19, 24.19, 29.19, and 34.19.

2.6. *Data analysis*

Analysis of variance (ANOVA) and Fisher's least significant difference (LSD) tests were used to analyze the data to determine the concentration level of the carbon and nitrogen sources. The data were analyzed with a 5 percent confidence interval. The optimal treatment was selected by examining the highest chromatogram area (%) value for each treatment and the inhibition zone created.

2.7. *Optimization of fermentation media composition*

The composition of the fermentation media was optimized using the Central Composite Design (CCD) experimental design with a combination of two treatment factors, namely the concentration level of the carbon source (X1, g/L) and nitrogen source (X1, g/L), which are presented in Table 2 as independent variables. This aimed to establish the optimum concentration of carbon and nitrogen sources required in *Streptomyces* sp. fermentation medium.

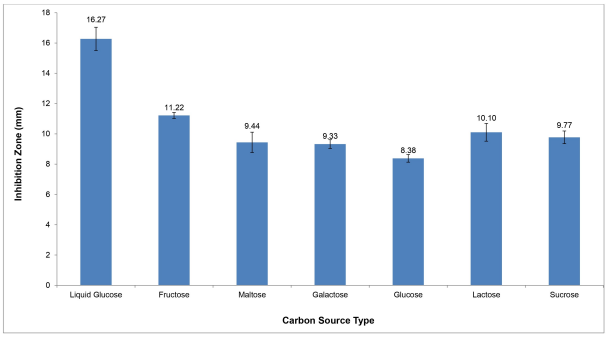
Observations were made on 13 treatments with three replications utilizing Design Expert D.X 10.0.7 software (Stat-Ease Inc., Minneapolis, MN, USA) on the area (%) of antifungal compounds readings from HPLC and agar disk diffusion method activity tests. Validation was subsequently performed in the lab following the recommendations made by the software. The optimization data were validated by contrasting the laboratory reaction values with the outcomes predicted by the Design Expert. This optimization was performed to determine the ideal concentration of carbon and nitrogen sources in the *Streptomyces* sp. fermentation medium.

3. Results and Discussion

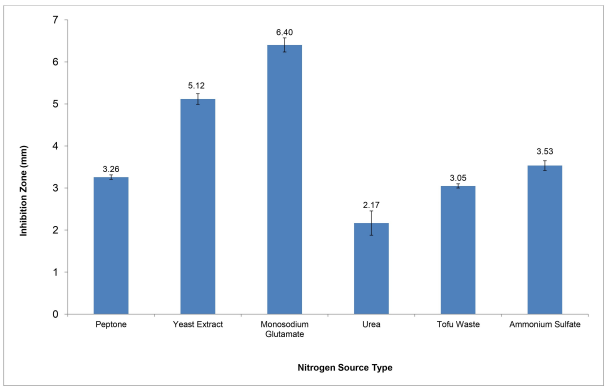
3.1. *Fermentation and activity test of Streptomyces sp. crude extract*

The liquid glucose inhibitory zone was compared to that of glucose (control), sucrose, lactose, maltose, galactose, and fructose.

In Figures 1a and 1b, various carbon and nitrogen sources are tested for antifungal compounds at the same concentration using disk diffusion testing, which is then continued using Fisher's test. The results of the Fisher's LSD test at a significance threshold of 0.05 revealed that



(a)



(b)

FIGURE 1 (a) Effect of the carbon source on the average inhibition zone of antifungal compounds. (b) Effect of the nitrogen source on the average inhibition zone of antifungal compounds.

liquid glucose and other carbon sources differed significantly from one another in subsequent analyses. According to Soraya et al. (2019), although glucose is readily metabolized directly, a higher glucose content will lead to rapid growth, while simultaneously suppressing the enzymatic reaction for the synthesis of secondary metabolites. As such, it is not recommended to perform this step when preparing cyclo(phenylalanyl-prolyl in the stationary phase. Consequently, liquid sugar formed from complex carbohydrates gives *Streptomyces* access to additional carbon sources after it has utilized all of the glucose. Given the need to convert additional complicated sugars into usable simpler compounds, the cell will become distracted from its primary goal of development and instead enter a stationary phase, during which secondary metabolites are created.

Figure 1b shows that MSG is the best nitrogen source in terms of the highest production of antifungal compounds with an average inhibition zone of 6.40 mm.

Additionally, according to Ju et al. (2018), glutamate plays a significant role in the antimicrobial action of *Streptomyces rimosus* AG-P1441. The antimicrobial activity is significantly enhanced by increasing the concentration of glutamate in the fermentation culture medium. Crude extract of *Streptomyces* sp. at a concentration of 10,000 ppm derived from a fermentative medium comprising the best forms of carbon and nitrogen sources inhibited *Ganoderma boninensis* and formed an inhibition zone diameter of 20.08 mm. These findings suggest that the modified fermentative media inhibits *Ganoderma boninensis* more effectively than the regular fermentative media, YEME broth, which forms an inhibition zone diameter of only 10.66 mm.

3.2. *Fractionation and characterization of active compounds*

The crude extract produced from bacterial fermentation was then fractionated to separate the fractions that were deemed to be active in order to determine the profile of the dominant constituent compounds. Column chromatography analysis generates a fraction with inhibitory power on *Streptomyces* sp. Fractions 7–10 show a thick color intensity and form an inhibition zone with a value range of 8.76–17.35 mm. In addition, the antifungal compounds assumed to be active appeared within the 41 m retention time, while the number of antifungal compounds (as measured by the area (%) of antifungal compounds) increased between the 7^{th} and 8^{th} fraction, which had the highest concentration. The concentration then began to decrease from fraction 9 onward. The active compounds from the column chromatography of fraction 8 were analyzed using LC-HRMS. The compound suspected of being active was that which appeared predominantly and had the largest peak of 24,006,707,30 peak area unit (PAU) at a retention time of 6.88 m. The results of the alleged LC-HRMS were then matched with an internet database (mzCloud MS/MS Library) to predict the type of compound.

The compound that appeared at the 6.88 m retention time had a molecular weight of 244.12 grams.mol^{-1} based on the internet database (mzCloud MS/MS Library). These results showed an 85.9 percent similarity to compounds with the molecular formula $C_{14}H_{16}N_2O_2$, which are known to be cyclo(phenylalanyl-prolyl) compounds.

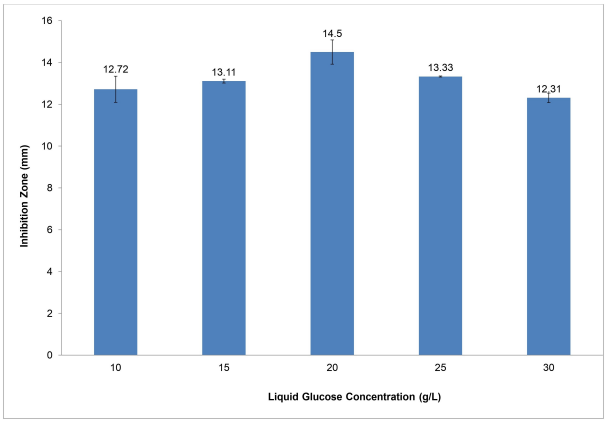


FIGURE 2 The average inhibition zone of antifungal compounds from various liquid glucose concentrations.

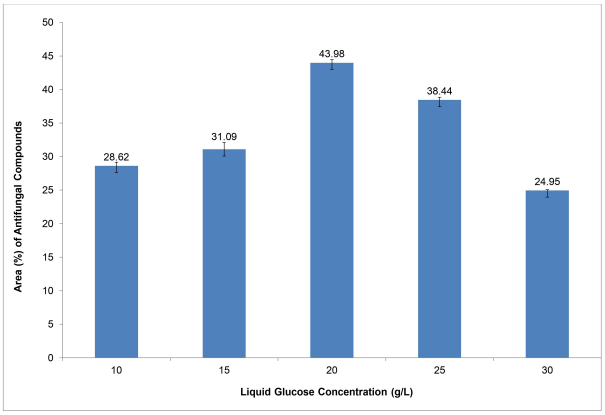


FIGURE 3 The average area (%) of antifungal compounds from various liquid glucose concentrations.

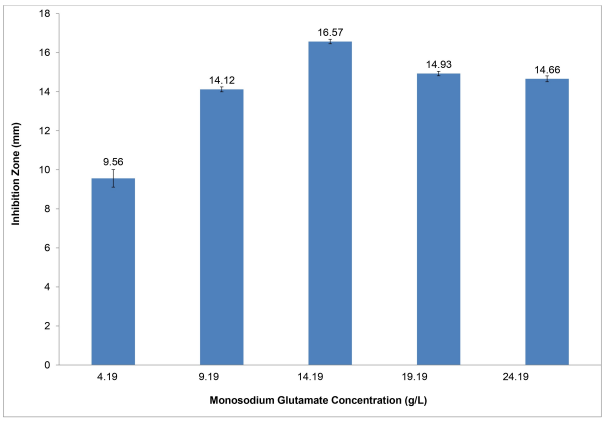


FIGURE 4 The average inhibition zone of antifungal compounds from various monosodium glutamate concentrations.

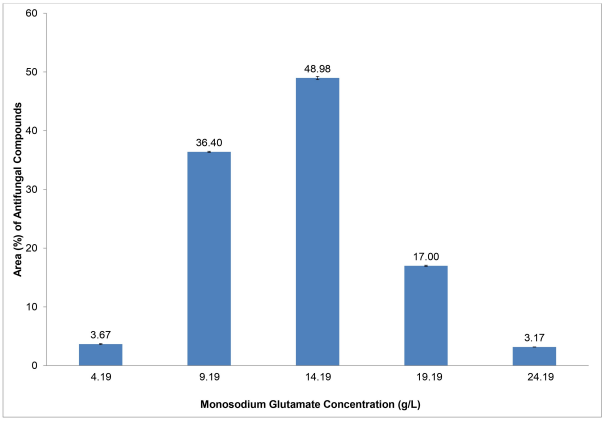


FIGURE 5 The average area (%) of antifungal compounds of antifungal compounds from various monosodium glutamate concentrations.

3.3. Selection of the best carbon source concentration level

The average inhibition zone of antifungal compounds from various liquid glucose concentrations was shown in Figure 2, with the highest data obtained at a concentration of 20 g/L. This is in direct proportion to the antifungal area data, which increased from 10 g/L liquid glucose (28.86 percent) to its maximum with the addition of glucose 20 g/L (44.44 percent). The area of antifungal chemicals decreased with the addition of glucose up to 25 g/L and so on at higher concentrations (Figure 3). Therefore, the addition of glucose up to 20 g/L was selected as the optimal concentration. This demonstrates that liquid glucose can be used as both a medium and carbon source for *Streptomyces* growth.

Based on the inhibition zone and area (%) of antifungal compounds, most antifungal compounds were produced with up to 14.19 g/L MSG, which then decreased with the addition of a higher peptone concentration (19.19 g/L) (Figure 4 and 5).

3.4. Optimization of Streptomyces sp. fermentation medium

CCD was used to optimize the fermentation medium for *Streptomyces* sp. with a combination of two factors, namely the concentration level of liquid glucose (X1, g/L) and MSG (X2, g/L) as independent variables, the inhibition zone (Y1, %), and the antifungal area (%) (Y2, %) as the control variable.

Based on the results of the first optimization, it is possible to conclude that the addition of carbon and nitrogen to the *Streptomyces* sp. fermentation medium in small or excessive amounts will reduce the effectiveness of *Streptomyces* sp. in producing antifungal compounds against *Ganoderma boninensis*, which are suspected to include cyclo(phenylalanyl-prolyl). The midpoint of the second optimization used the suggestions from the Design Expert D.X. 10.0.7 software (Table 3).

The suggestion from the first optimization was taken as the midpoint value in the second optimization. The latter comprised 13 treatments from Design Expert D.X. 10.0.7., which produced response data for the inhibition zone and the area (%) of antifungal compounds (Table 4). The data in Table 4 show the response of the inhibition zone and area (%) of the highest antifungal compound in the treatment center point design, specifically 20.06 g/L and 15.20 g/L, respectively.

3.5. Modeling and analysis of inhibitory zone response and area (%) of antifungal compounds

The results from the program analysis on the response of the inhibition zone and the area (%) of antifungal compounds revealed that the quadratic model was the suggested model. The mathematical equation model for the response of the inhibition zone (Y1) and the area (%) of

TABLE 3 Response data of inhibition zone and area (%) of antifungal compound based on first optimization design.

No	Real Variables		Coded Variables		Response	
	Carbon (g/L)	Nitrogen (g/L)	X1	X2	Y1 Inhibition zone (mm)	Y2 Area (%) antifungal
1	10.00	4.19	-1	-1	14.76	38.67
2	30.00	4.19	1	-1	18.23	41.64
3	10.00	24.19	-1	1	19.92	44.00
4	30.00	24.19	1	1	17.72	39.14
5	585.786	14.19	-1.414	0	16.50	30.12
6	341.421	14.19	1.414	0	15.00	38.77
7	20.00	0.0478644	0	-1.414	18.00	42.69
8	20.00	283.321	0	1.414	20.00	44.23
9	20.00	14.19	0	0	24.95	60.60
10	20.00	14.19	0	0	25.00	59.09
11	20.00	14.19	0	0	24.50	60.96
12	20.,00	14.19	0	0	25.23	61.19
13	20.00	14.19	0	0	24.39	62.68

TABLE 4 Response data of inhibition zone and area (%) of antifungal compound from second optimization design.

No	Real Variables		Coded Variables		Response	
	Carbon (g/L)	Nitrogen (g/L)	X1	X2	Y1 Inhibition zone (mm)	Y2 Area (%) antifungal
1	10.06	5.20	-1	-1	13.30	33.00
2	30.06	5.20	1	-1	17.20	36.31
3	10.06	25.20	-1	1	18.90	38.00
4	30.06	25.20	1	1	16.10	34.50
5	5.91786	15.20	-1.414	0	14.88	31.00
6	34.2021	15.20	1.414	0	13.38	30.32
7	20.06	1.05786	0	-1.414	16.38	38.60
8	20.06	29.3421	0	1.414	18.38	39.80
9	20.06	15.20	0	0	22.83	59.98
10	20.06	15.20	0	0	23.00	60.29
11	20.06	15.20	0	0	23.17	60.20
12	20.06	15.20	0	0	23.34	60.58
13	20.06	15.20	0	0	23.50	60.42

TABLE 5 Mathematical equation model for inhibition zone response and area (%) of antifungal compounds.

Response	Model	Math/Equation	Significant	Lack of Fit Test	R2
Inhibition Zone	quadratic	Y=23.17-0.25X1+0.79X2-1.93X1X2-4.43X12-2.80X22	< 0.0001	0.0909	0.9936
Area (%) of Antifungal Compounds	quadratic	Y=60.29-0.14X1+0.61X2-1.70X1X2-14.69X12-10.42X22	< 0.0001	0.0605	0.9995

the antifungal compound (Y2) are shown in Table 5.

The quadratic equation model shows that when the concentration of MSG increases, it is accompanied by a rise in the antifungal compounds of the inhibition zone and an increase in the response area (%), as indicated by a positive constant in the model. The antifungal compound inhibition zone and response area (%) decrease as the glucose concentration increases, as does the interaction between liquid glucose concentration and MSG concentration, the quadratic interaction of liquid glucose concentration, and the quadratic interaction of MSG concentration.

3.6. Inhibitory zone response and area (%) of antifungal compounds

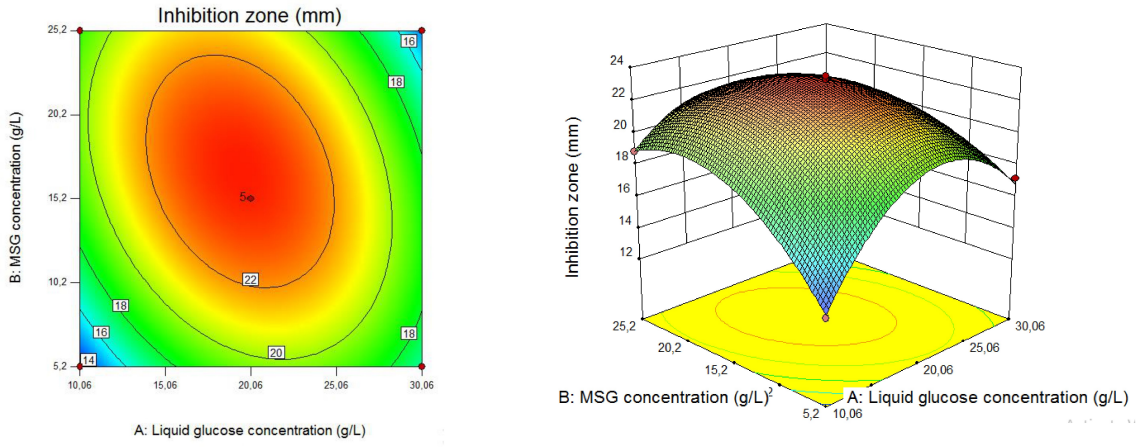
A response graph can be used to describe the effect of an independent variable on the response. Since this study employs two independent variables, two response surface graphs depict the interaction of the two independent factors and their effect on each response.

A contour plot (Figure 6a) and a 3D response surface plot (Figure 6b) show that the concentrations of liquid glucose and MSG affect the inhibition response. The area of the inhibition zone increased continuously while the concentration of liquid glucose increased from 15.05 g/L to 20.05 g/L and that of MSG increased from 10.20 g/L to 15.20 g/L. However, after the optimum point had been reached, further increasing the concentrations of liquid glucose and MSG decreased the area of the inhibitory zone. A fall in the inhibitory zone area was seen at a concentration of liquid glucose greater than 20.05 g/L and of MSG greater than 15.20 g/L, which may have been caused by *Streptomyces* sp. exhausting its carbon and nitrogen sources.

In Figure 7a, a contour plot of the liquid glucose and MSG concentrations to the response area (%) of antifungal compounds is displayed. The recommended optimization solution for a single response area (%) of antifungal compounds is X_1 = 20.05 g/L and X_2 = 15.20 g/L, while the area (%) of antifungal compounds predicted at that point is 60.88%. In Figure 7b, the curve of the 3D response surface resembles an inverted parabola and represents the highest reaction. The area (%) of antifungal compounds increased up to a liquid glucose concentration of 20.05 g/L and MSG 15.20 g/L. However, the area (%) of antifungal compounds then fell to a certain point, as shown by the minimum points, after the optimal concentration had been reached. The antifungal compound area (%) increased in proportion to the increase in liquid glucose and MSG concentration until it reached the optimal point.

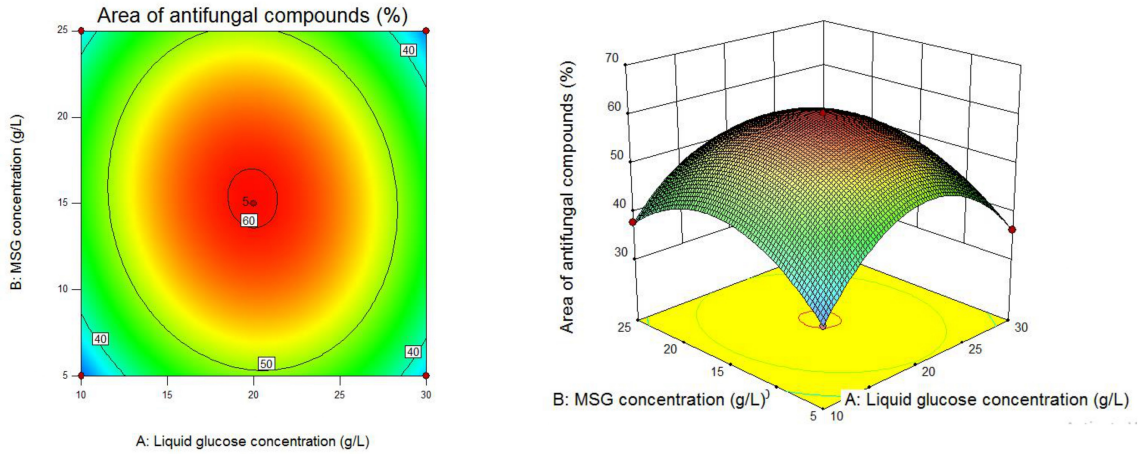
3.7. Validation of optimum conditions for model prediction results

The composition of the carbon source from liquid glucose at 19.78 g/L and the optimum nitrogen source from MSG at 16.05 g/L with a desirability value of 0.981 are shown in Table 6. The average inhibition zone was 23.22 mm, and the HPLC chromatogram area (%) was 60.26 percent, as predicted by the Design Expert program d.x. 10.0.7. The



(a) (b)

FIGURE 6 (a) Contour plot of liquid glucose and monosodium glutamate concentrations affect the inhibition zone response. (b) 3D response surface plot of liquid glucose and monosodium glutamate concentrations affect the inhibition zone response.



(a) (b)

FIGURE 7 (a) Contour plot of liquid glucose and monosodium glutamate concentrations affect the area (%) of antifungal compounds response. (b) 3D response surface plot of liquid glucose and monosodium glutamate concentrations affect the area (%) of antifungal compounds response.

TABLE 6 Suggestion results from second optimization.

Number	Liquid Glucose	Monosodium Glutamate (MSG)	Inhibition Zone	Antifungal Compound	R2	
1	19.78 g/L	16.05 g/L	23.22 mm	60.26%	0.981	selected

laboratory validation yielded an average inhibitory zone response of 23.76 mm and an HPLC chromatogram area (%) of 61.64 percent. According to these findings, the difference in the inhibition zone response value between the validation and projected results is 2.45 percent. The findings of the comparison revealed a difference between the prediction and validation values of less than 5 percent, indicating that the validation value is consistent with the predicted value. This confirms that the average inhibition zone of the test results corresponds to the program predictions. There was a difference of 2.58 percent between the prediction and validation of the HPLC chromatogram area response value (%). This is less than 5 percent, which indicates that the validation value is consistent with the value predicted by the Design Expert D.X 10.0.7 software.

4. Conclusions

MSG is the finest nitrogen source, while liquid glucose is the best carbon source. The optimal conditions for *Streptomyces* sp. fermentation medium comprise a liquid glucose concentration of 19.78 g/L and an MSG content of 16.05 g/L. The average inhibitory zone is validated to be 23.76 mm, and the % area of the HPLC chromatogram is 61.64 percent. The chemical formula of the likely active compound is $C_{14}H_{16}N_2O_2$ or the cyclo(phenylalanyl-prolyl) compound.

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Authors' contributions

ST, WDRP, AKW, RS designed the study. ST, RS carried out the laboratory work. ST, WDRP, AKW, RS analyzed the data. ST wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors of this article declare no competing interests.

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