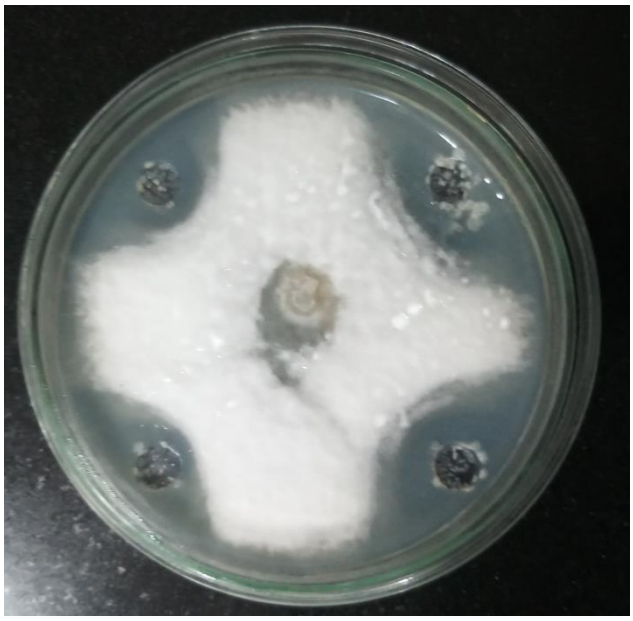
Chapter 4

Statistical optimization of Streptomyces production for antifungal activity



Chapter 4

4 Statistical optimization of Streptomyces production for antifungal activity

4.1 Introduction

Streptomyces are the primary bioactive chemical manufacturers for the biotechnology industry. This unique genus belonging to the order Actinomycetales has yielded many clinically significant antibiotics and commonly employed medicines against common ailments (Medema et al., 2011). Thousands of bioactive compounds have been identified and characterized, and many of them have been turned into medications for the treatment of a wide variety of human, veterinary, and agricultural diseases (Castillo et al., 2002; El-Shatoury et al., 2009; Singh et al., 2003). Therefore, actinomycetes are the most potent source of secondary metabolites, antibiotics, and other bioactive substances. It is generally established that each actinomycete strain has the genetic capability to create between 10 and 20 secondary metabolites (Bentley et al., 2002; Sosio et al., 2000). Actinomycetes produce 75 % of all known antibiotics, and Streptomyces plays a unique function in the manufacture of antibiotics (Nolan & Cross, 1988; Thakur et al., 2009). Streptomyces produced numerous therapeutic agents, including antibacterial agents such as tetracyclines, antifungal agents such as amphotericin, and anticancer medications such as Adriamycin and immunosuppressant tacrolimus (Hopwood, 2007). It has been claimed that Streptomyces contributes about 70% of the metabolites mentioned under actinobacteria (Zengler et al., 2005). Streptomycetes and associated actinomycetes continue to be excellent sources of novel secondary metabolites with various biological functions that may eventually be used as anti-infectives, anticancer medicines, or other pharmaceutically relevant substances (Bibb, 2005).

The bioactive compound-producing ability of an actinomycete is not a static property. Enhancing the production of bioactive compounds from a producer strain is essential to acquiring adequate quantity to assess their potential for developing as a lead compound. As a result of the difficulty and expense of chemical synthesis procedures, fermentation is the most prevalent method for bioactive component generation for the vast majority of antibiotics, including those recently introduced to the market. Consequently, enhancing and improving microbial strain culture conditions continue to be the most effective methods for reducing production volumes and costs and ensuring the quality and reproducibility of drug manufacture (Marinelli et al., 2015).

Regarding secondary metabolite biosynthesis, strain and culture conditions have a significant impact (Xinxuan Wang et al., 2010). Cultural conditions (nature and concentration) have affected antibiotic synthesis, as reported by (Parekh et al., 2000). The metabolism of *Streptomyces* strains is highly dependent on carbon and nitrogen supplies and minerals for both growth and antibiotic production (Sanchez & Demain, 2002; Yu et al., 2008). Numerous studies have shown the necessity of optimizing metabolites production (Purama & Goyal, 2008; L.-L. Yuan et al., 2008). Another study found that modest changes in culture medium composition can alter metabolic profiles and secondary metabolite synthesis, according to Wang et al. (2011).

The optimization of secondary metabolite production experiments is usually carried out using non-statistical and statistical approaches. The nutritional requirement and physical parameters have been manipulated for several microorganisms by the successive non-statistical method like One-factor-at-a-Time Approach (OFAT) and Response Surface Methodology (RSM). One factor at a time (OFAT) approach was the principal used procedure, which is time-consuming and claims many experiments (Kanmani et al., 2013). Consequently, the statistical experimental design approach and response surface methodology (RSM) are widely applied to determine the most significant factors and their optimal levels affecting the production (Kanmani et al., 2013; Wang et al., 2011). RSM with Central Composite Design (CCD) (Kanmani et al., 2013; Wang et al., 2010; Wang et al., 2011) and Box-Behnken Design (BBD) experiments (Kanmani et al., 2013) are used for selected factors optimization.

In the classical medium optimization technique, one-factor-at-a-time (OFAT) experiments, just one variable or factor is varied at a time while other variables are held constant. The concentrations of the chosen medium components were then altered within a predetermined range (Singh et al., 2017). Due to its simplicity and practicality, the OFAT has been the instrument of choice among researchers for developing the medium composition and usage in the earliest stages of various fields (Gonzalez et al., 1995). This technique is still employed during the first stages of medium formulation to manufacture a new metabolite or known molecule from an unknown source (Singh et al., 2017). OFAT is further subdivided into removal, supplementation, replacement experiments and physical parameters based on the methodology employed (Singh et al., 2017).

All medium components are withdrawn one by one from the production medium in the removal experiment. After an appropriate length of incubation, their effects on the production of secondary metabolite or the product of interest are observed in terms of suitable parameters (Singh et al., 2017). It has been previously demonstrated that removing soybean meal, glycerol, or NaCl from the fermentation medium decreased the yield of antifungal compounds produced by *Streptomyces capoamus* by 20–40 % (Singh et al., 2008). Generally, supplementation experiments are conducted to determine the impact of different carbon and nitrogen additions on metabolite synthesis. During the research of antifungal production by *Streptomyces violaceusniger*, supplementing the production medium with xylose, sorbitol, and hydroxyl proline increased the output by 70–90% (Tripathi et al., 2004). Similarly, glycerol and peptone were identified as the best carbon and nitrogen sources for synthesizing antifungal and antibacterial metabolites by *Streptomyces rimosus* during submerged fermentation (Singh & Rai, 2012). Carbon/nitrogen sources that have shown a positive effect in supplementation trials on target metabolite production are often employed as a full carbon/nitrogen source in the medium formulation in replacement experiments (Singh et al., 2017). Many researchers employed OFAT studies to standardize the physical parameters of the fermentation process, such as pH, temperature, agitation, and aeration (Niwas et al., 2013).

There are both advantages and disadvantages of using the OFAT approach of medium optimization. With OFAT, a series of experiments can be conducted, and results analyzed using simple graphs without high-end statistical analysis/programs. In OFAT, it is difficult to estimate "interactions" because the experiments are a hit-and-miss scattershot sequence (Gupte & Kulkarni, 2003). OFAT approaches' key drawbacks, according to Vaidya et al. (2003), are the time and money required to analyze a large number of variables. As a result of this methodology, many trials are needed to discover the optimum level, which is complex, time-consuming, and uneconomical (Gupte & Kulkarni, 2003). Despite this, the OFAT approach can be an excellent screening tool when nothing is known about the media (Singh et al., 2017).

Response surface methodology (RSM) is a valuable experimental technique for optimising the parameters of fermentation processes by applying mathematics and statistics (Kavitha et al., 2016; Kong et al., 2014). Utilizing RSM to optimise fermentation conditions is predicated primarily on the following investigations (Qiu et al., 2012): 1) utilizing the Plackett-Burman

design to identify factors that significantly influence the fermentation process; 2) determining the path of steepest ascent to determine the approximate range of the best fermentation conditions using the key factors; and 3) utilizing the Box-Behnken test design to establish the fermentation model and determine the optimal fermentation conditions. RSM has been used extensively to optimize microbial fermentation processes, and it can assess the influence of numerous parameters (Nawaz et al., 2016). Moreover, RSM can be used to optimize fermentation conditions to fulfill the nutritional requirements of a particular microbe, hence eliminating the addition of extra superfluous components to the culture medium (Jiayu Feng et al., 2017). RSM requires fewer trials than other optimization methods to calculate the multiple variables and their interactions (Kavitha et al., 2016). Several Streptomyces species, such as *Streptomyces sp.*HJC-D1 (Kong et al., 2014), *Streptomyces nogalater* NIISTA30 (Jacob et al., 2017), *Streptomyces sp.* SY-BS5 (Souagui et al., 2015), and *Streptomyces sp*. SYYLWHS-1-4 (R.-F. Li et al., 2016) have been subjected to RSM strategy to enhance the synthesis of their antibacterial compounds. Therefore, RSM techniques are required to enhance bioactive metabolite production (Managamuri et al., 2017).

Hence, the proposed study is an effort to optimize the antifungal compound produced by *Streptomyces* isolated from the rhizospheric soil of *Cajanus cajan*. To optimize the medium for the production of antifungal compounds, experimental methods were designed.

4.2 Materials and Methods

4.2.1 Seed stock preparation

The spore suspension of *Streptomyces* sp. added to sterile seed medium containing 5.0 g of casein enzymatic hydrolysate and 3.0 g of yeast extract powder in 1liter distilled water and 10^8 spores/50 mL medium. Inoculated seed medium was incubated in a shaker at 150 rpm for 4 days at 30°C and used as seed stock.

4.2.2 Mycelial biomass determination

Growth of *Streptomyces* sp. in fermentation media was determined by estimation of mycelia biomass. The mycelial pellets were collected by centrifugation of Fermentation media at 10,000

rpm for 15 min and dried at 60°C. The dry biomass was weighed and expressed as g/L of the culture medium.

4.2.3 Determination of antifungal activity by agar well diffusion method

Streptomyces sp. was grown on a fermentation medium at 28.0°C in a rotary shaker (150 rpm) for 10 days. The culture broth was centrifuged at 10,000 rpm for 15 min to obtain the cell-free supernatant. The supernatant was filter sterilized. The wells (6.0 mm diameter, 4.0 cm apart) were made using a sterile cork borer, 100.0 µl of clear supernatant was loaded into one well, and a fungus plug from 7 to 8 days old fungus PDA plate was put into another well for the assay of antagonistic activity against *Fusarium udum.* The antifungal activity was estimated by determining the diameter of zones of inhibition using the following equation. Negative controls included only liquid culture media (Yun et al., 2018).

$$Inhibition\ (\%) = \frac{[\mathrm{A}] - [\mathrm{B}]}{[\mathrm{A}]} \times 100$$

Where [A] = Growth diameter in untreated Control; [B] = Growth Diameter in Treatment

4.2.4 Selection of basal nutrient medium

The comparison research utilised two distinct media, ISP1 and TSB, to identify the ideal nutrient medium for subsequent development (Table 4.1). 2% seed culture was added into 150 mL of the sterile media in 250 mL flasks. The flasks were incubated at 30°C on a rotary shaker at 150 rpm for ten days. After incubation, the biomass determination was carried out as described previously in triplicate to select an appropriate basal medium.

Table 4.1: Media used for selection of Basal medium

Ingredients	**Quantity/Value**
Tryptone Soya Broth (TSB)	
Pancreatic digest of casein (g/L)	17
Papaic digest of soyabean meal (g/L)	3

Sodium chloride (g/L)	5
Dextrose (g/L)	2.5
Dibasic potassium phosphate (g/L)	2.5
pH	7.3±0.5
Tryptone yeast extract broth (ISP-1)	
Casein enzymic hydrolysate (g/L)	5
Yeast extract (g/L)	3
pH	7.2±0.5

Using the one variable at a time strategy, the optimal carbon and nitrogen sources with the highest antifungal activity were determined.Nitrogen and carbon sources (tyrosine, glycine, soybean meal, and peptone) were each provided in the same baseline medium (ISP 1 + NaCl + salts) at a rate of 2g% (Vijayakumar et al., 2012). The concentrations of the remaining components were kept constant. The antifungal activity and biomass were determined after ten days of incubation at 30°C in a rotary shaker with 120 rpm.

4.2.5 Optimization of selected media components by Response surface methodology

Response surface methodology (RSM) was used with central composite design (CCD) to optimize the media components selected according to OFAT-based screening experiments. The Design-Expert software (version 7) was used for the experimental design and the regression analysis of the data. The media components (independent variables) considered are mannitol and glycine. The concentration range given for mannitol and glycine is 0.2 to 2.0 g%. The experiment was carried out in 13 trials (Tables 4.2& 4.3) with five replicates at the center point. All 13 experimental trials were run in triplicates.

Table 4.2: CCD experimental trials

Run No.	Factor 1 A: Mannitol gm (gm %)	Factor 2 B: Glycine (gm %)
1	0.46	0.46
2	1.74	0.46
3	0.46	1.74
4	1.74	1.74
5	0.20	1.10
6	2.00	1.10
7	1.10	0.20
8	1.10	2.00
9	1.10	1.10
10	1.10	1.10
11	1.10	1.10
12	1.10	1.10
13	1.10	1.10

Table 4.3: CCD experimental trials in terms of volume

Std	Mannitol (mL) (10g %)	Glycine (mL) (10g%)	Salts (mL)*	Basal medium (mL)*	Total volume (mL
1	6.9	6.9	4.5	131.7	150.0
2	26.1	6.9	4.5	112.5	150.0
3	6.9	26.1	4.5	112.5	150.0
4	26.1	26.1	4.5	93.3	150.0
5	3.0	16.5	4.5	126.0	150.0
6	30.0	16.5	4.5	99.0	150.0
7	16.5	3.0	4.5	126.0	150.0
8	16.5	30.0	4.5	99.0	150.0
9	16.5	16.5	4.5	112.5	150.0
10	16.5	16.5	4.5	112.5	150.0
11	16.5	16.5	4.5	112.5	150.0
12	16.5	16.5	4.5	112.5	150.0
13	16.5	16.5	4.5	112.5	150.0

*1.5 mL of each of 3 salt stocks (as mentioned in table 2) was added.

4.3 Results

4.3.1 Statistical optimization of culture medium

4.3.1.1 Selection of basal nutrient medium

Streptomyces sp. was treated to biomass generation in two distinct media, TSB and ISP-1, in order to determine the optimal medium. ISP-1 demonstrated the highest bio-mass output (13.4 g/L) (Figure 4.1). Consequently, ISP-1 was used to identify acceptable carbon and nitrogen sources and enhance antifungal chemical production in batch cultures by *Streptomyces* sp.

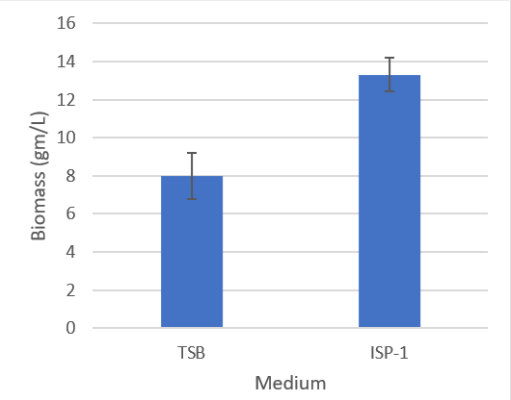


Figure 4.1: Biomass in gm/L for selection of basal medium

4.3.1.2 Effect of salt formulation

Mycelial biomass produced by *Streptomyces* in the production media containing various salt compositions were given in Figure 4.2. ISP-1+NaCl+salts supported the highest biomass, which was used in further experiments.

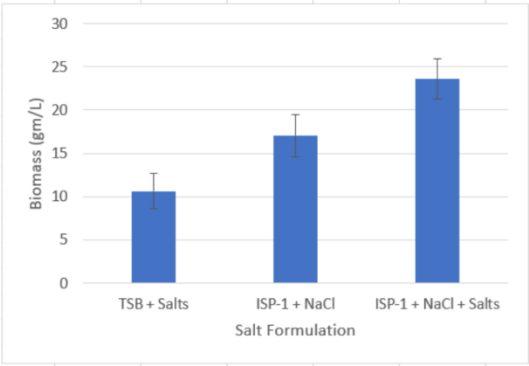


Figure 4.2: Effect of salt formulation on mycelial biomass

4.3.1.3 Selection of carbon and nitrogen sources by OFAT method

On the selected ISP-1 medium, the effect of various carbon and nitrogen sources on the antifungal activity of *Streptomyces sp.* was investigated. Among the different carbon sources studied, *Streptomyces* sp. S-9 showed maximum bio-mass production in mannitol (30.5g/L) (Figure 4.3) and anti-fungal activity in mannitol (32.7%) was the most profound among the carbon sources (Figure 4.4 & 4.5). Among the various nitrogen sources tested, higher bio-mass (47g/L) (Figure 4.6) and antifungal activity (37%) (Figure 4.7 & 4.8) were observed with glycine. Thus, mannitol and glycine were selected as the carbon source and nitrogen source for further experiments, respectively. The plates of the 13 experimental trialsafter culturing on optimized media are presented in Figure 4.9.

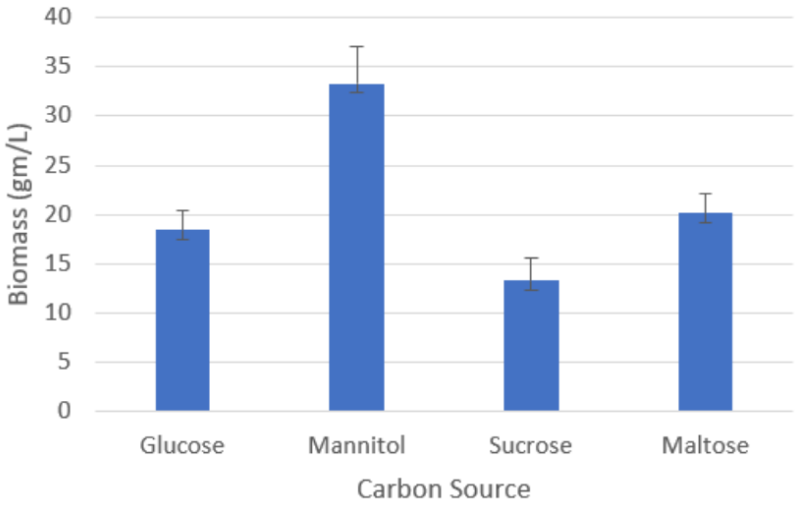


Figure 4.3: Biomass (gm/L) in different carbon sources

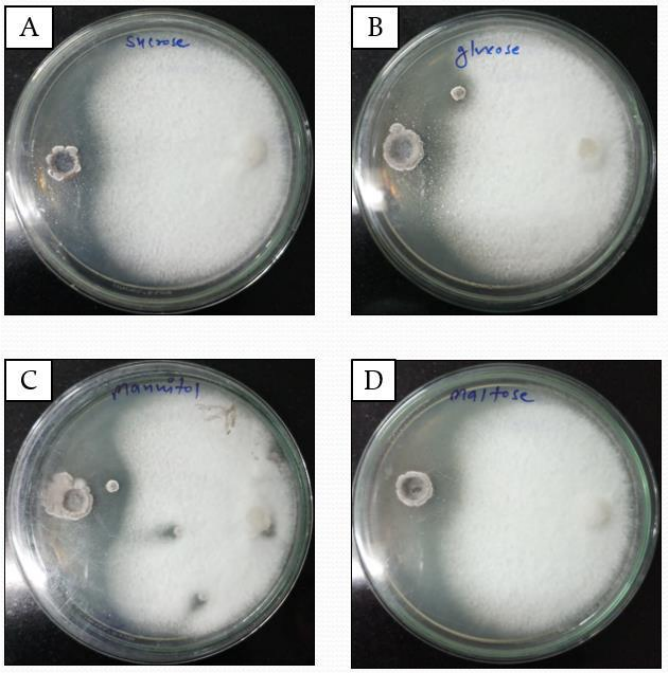


Figure 4.4: Antifungal activity on medium containing different carbon source (A) Sucrose, (B) Glucose, (C) Mannitol, (D) Maltose

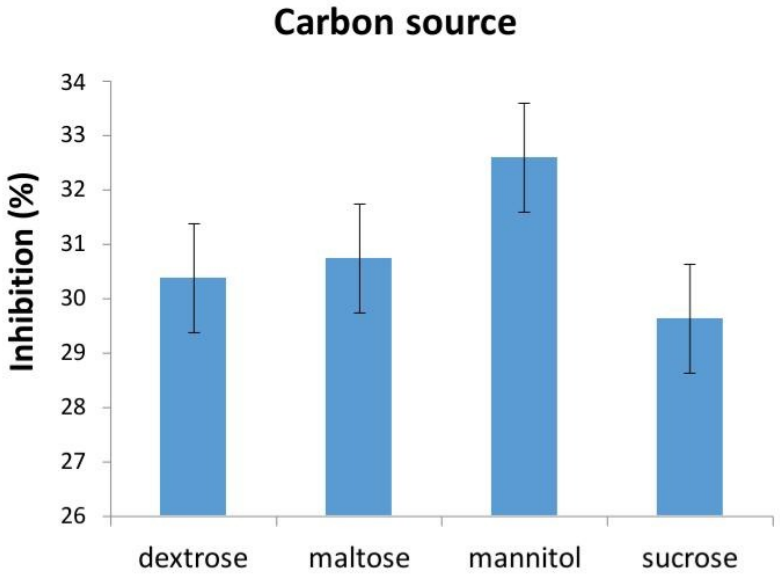


Figure 4.5: Antifungal activity % in different carbon sources

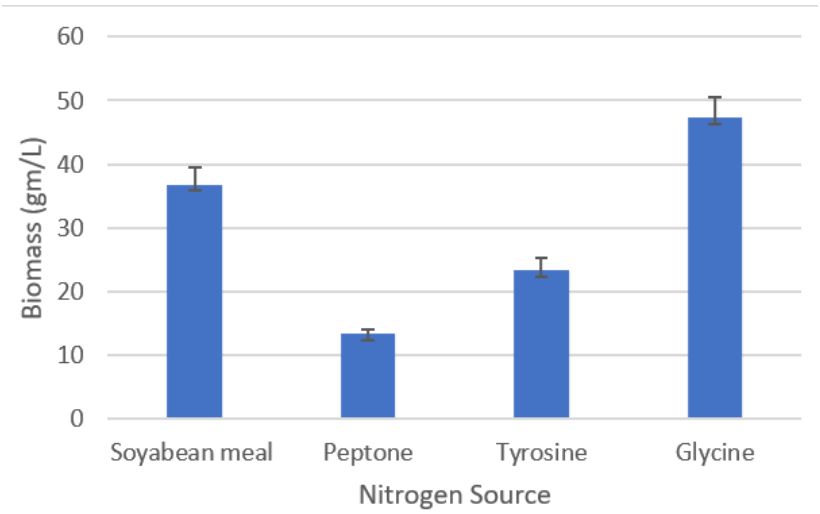


Figure 4.6: Biomass (gm/L) in different nitrogen sources

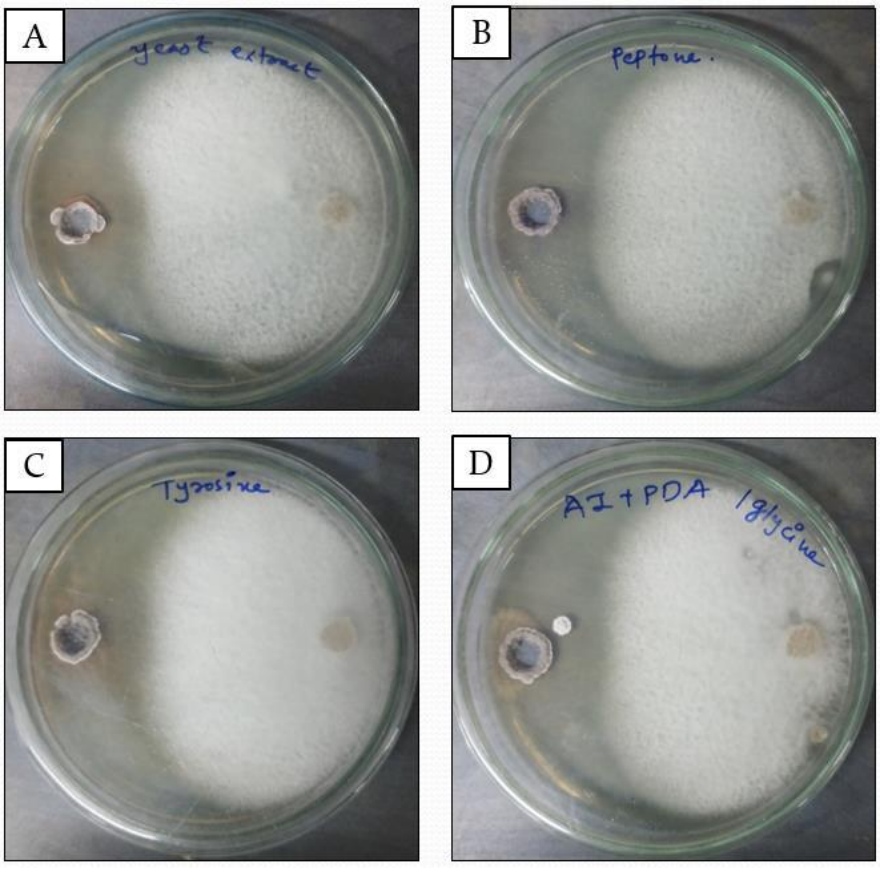


Figure 4.7: Antifungal activity on medium containing different nitrogen source; (A) Soyabean meal, (B) Peptone, (C) Tyrosine, (D) Glycine

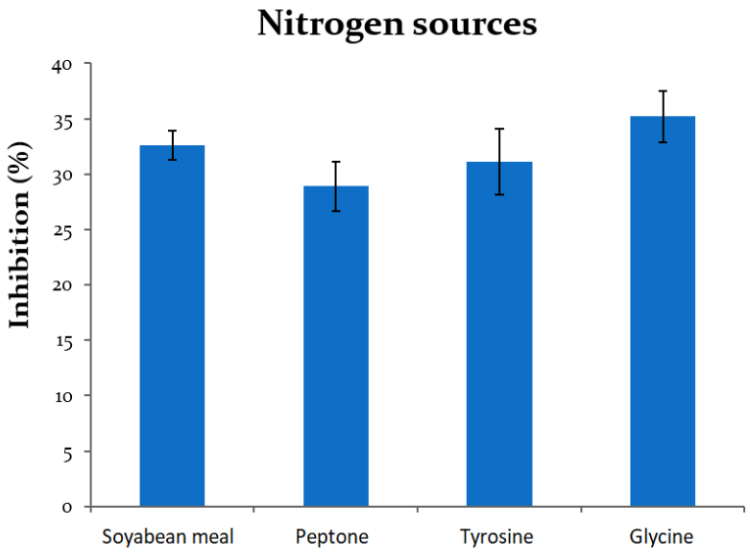


Figure 4.8: Antifungal activity % in different nitrogen sources

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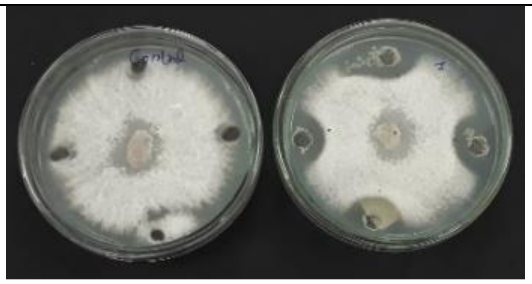
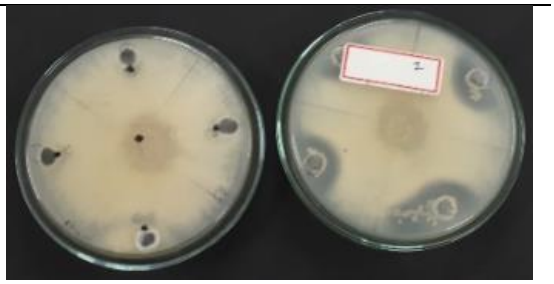
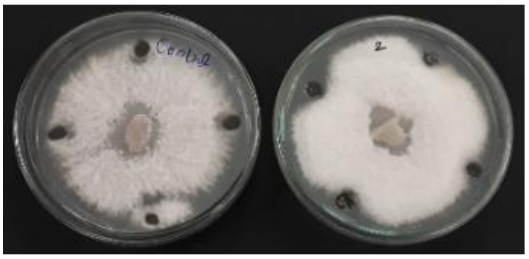
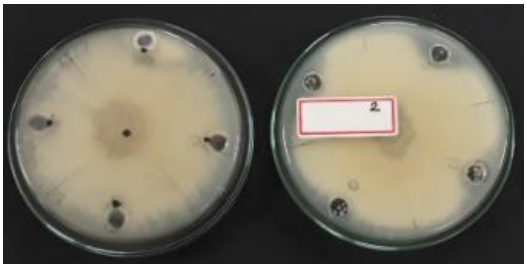
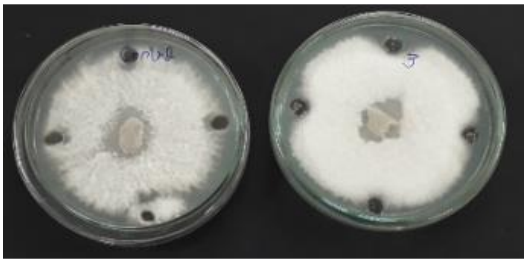
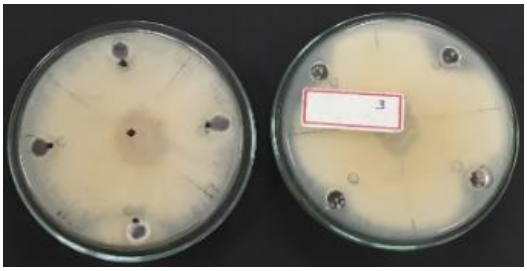
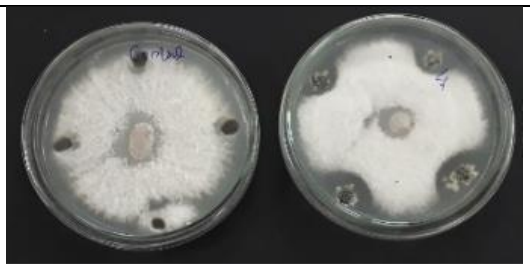
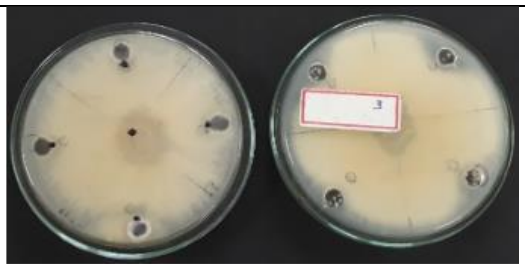
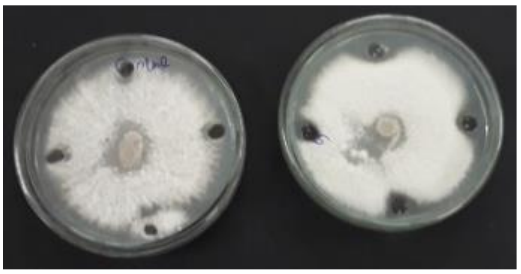
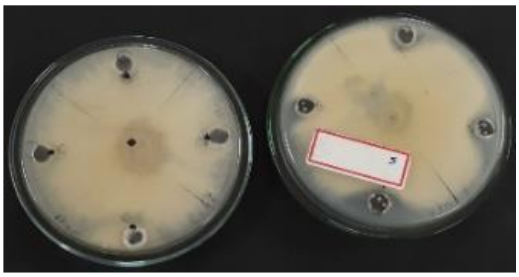
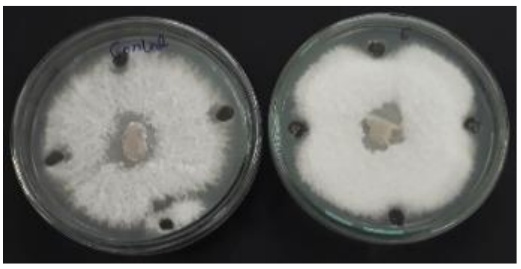
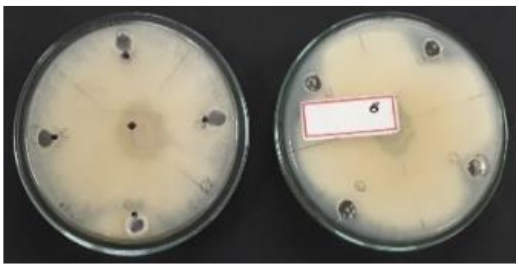
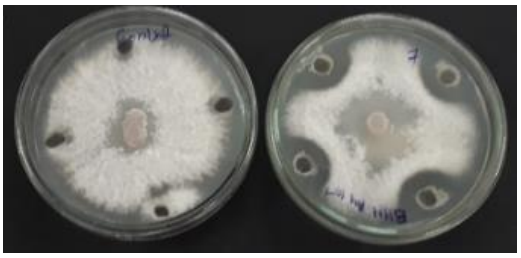
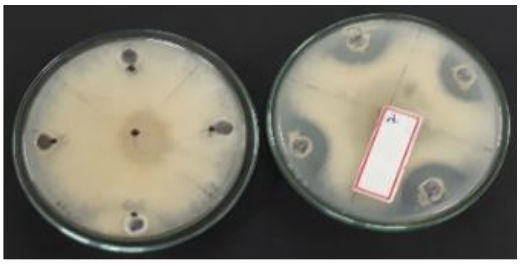
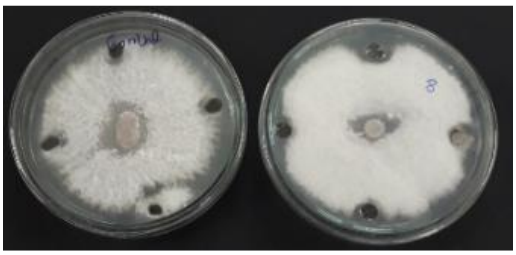
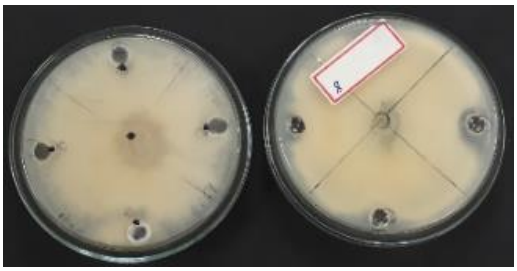
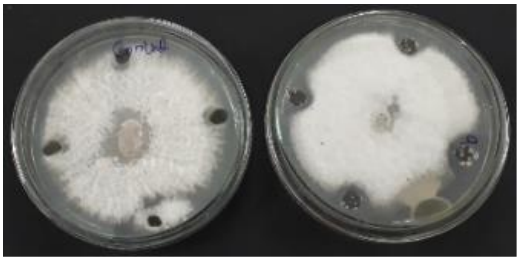
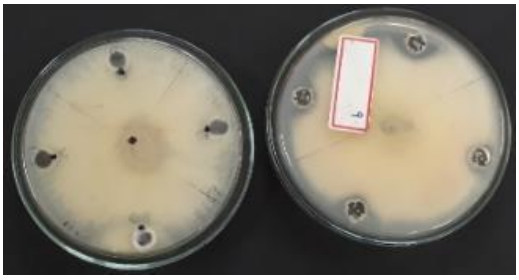
1		
2		
3		

4		
5		
6		
7		
8		
9		

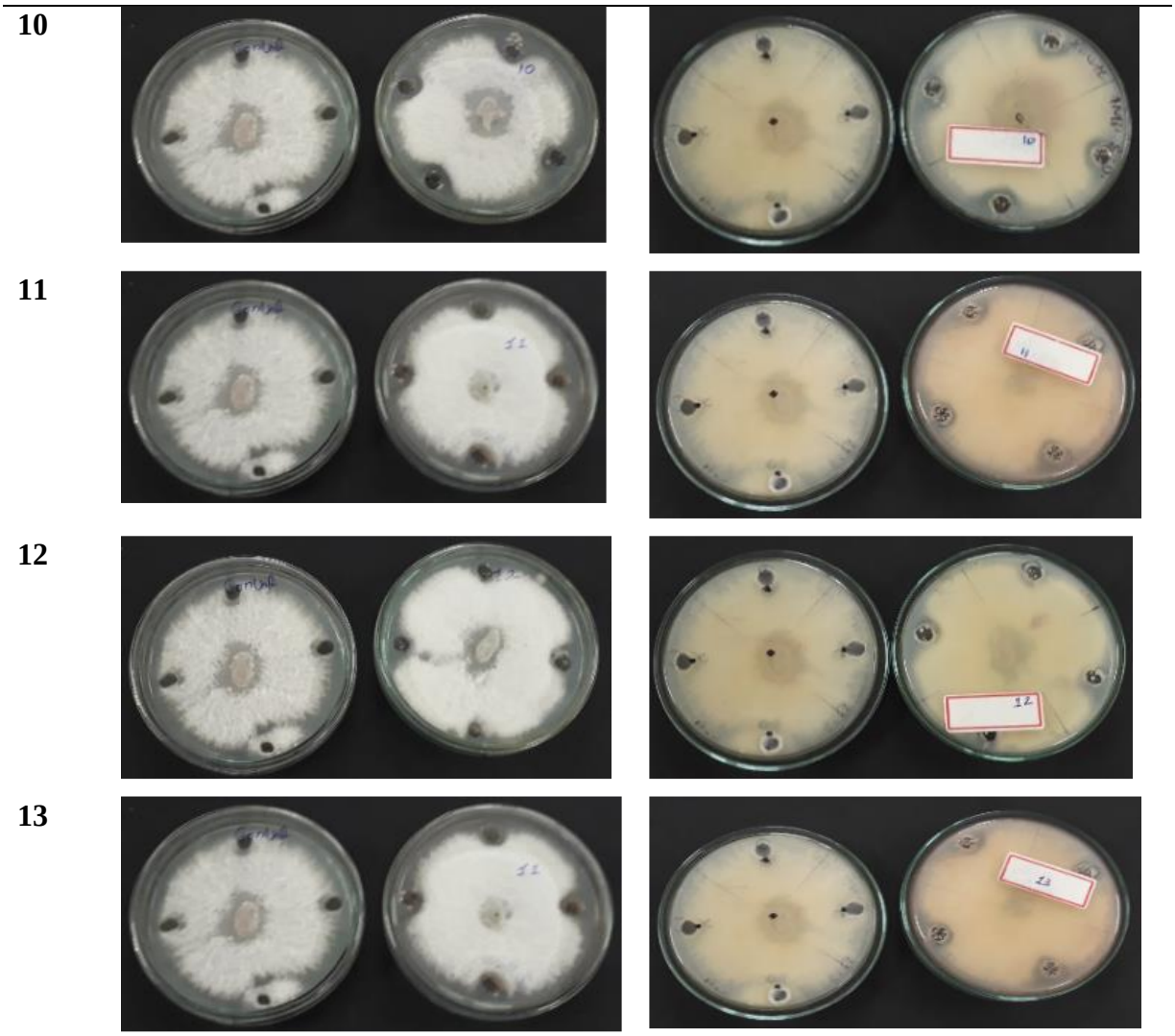


Figure 4.9: Antifungal activity shown by 13 experimental trials

4.3.2 Optimization of selected media components by Response surface methodology

RSM with Central Composite Design was applied to determine the optimal levels of the two selected variables such as glycine and mannitol. Batches of experiments were conducted using CCD and the results were given to determine the effect of independent factors on the response (Table 4.4). The following equation was obtained for the two-factor system by evaluating experimental results.

Y (Anti-fungal activity) = $66.67382 – 24.87603A – 55.87727B + 21.00309AB – 0.052315A^2 + 12.81188B^2$

Where Y was the predicted response (antifungal activity), A and B were the coded values of mannitol and glycine respectively.

Table 4.4: Central Composite Design matrix used in RSM studies along with the experimental response in terms of antifungal activity

Run No	**Factor 1 A: Mannitol (gm %)**	**Factor 2 B: Glycine (gm %)**	**Response: Antifungal Activity (%)**
1	0.46	0.46	37.50
2	1.74	0.46	16.66
3	0.46	1.74	16.66
4	1.74	1.74	29.85
5	0.20	1.10	18.05
6	2.00	1.10	16.66
7	1.10	0.20	33.33
8	1.10	2.00	22.22
9	1.10	1.10	24.07
10	1.10	1.10	23.61
11	1.10	1.10	18.05
12	1.10	1.10	13.19
13	1.10	1.10	14.57

The results of the present study showed that the model is highly significant, as evident from the Fisher's *F*-test with a low *P*-value (0.04). The significance of the model was further supported by a statistically insignificant lack of fit, as was evident from the lower calculated F-value (0.34) (Figure 4.10). Analysis of Variance (ANOVA) was applied to verify the quadratic regression model statistically and the results were presented in Figure 4.11.

Response 1 Antifungal Act Transform: None

***** WARNING: The Cubic Model is Aliased! *****

Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs Total	6222.45	1	6222.45			
Linear vs Mean	79.74	2	39.87	0.66	0.5393	
2FI vs Linear	289.43	1	289.43	8.21	0.0186	
Quadratic vs 2FI	190.74	2	95.37	5.28	0.0400	Sugge
Cubic vs Quadra	12.17	2	6.08	0.27	0.7766	Ali
Residual	114.32	5	22.86			
Total	6908.84	13	531.45			

*"Sequential Model Sum of Squares [Type I]": Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Linear	505.87	6	84.31	3.35	0.1312	
2FI	216.44	5	43.29	1.72	0.3101	
Quadratic	25.70	3	8.57	0.34	0.7989	Sugge
Cubic	13.53	1	13.53	0.54	0.5043	Ali
Pure Error	100.78	4	25.20			

Figure 4.10: Fit summary analysis of the quadratic model

Response 1 Antifungal Activity

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	559.91	5	111.98	6.20	0.0165	significant
A-Mannitol	*11.55*	*1*	*11.55*	*0.64*	*0.4503*	
B-Glycine	*68.19*	*1*	*68.19*	*3.77*	*0.0932*	
AB	*289.43*	*1*	*289.43*	*16.02*	*0.0052*	
A^2	*3.123E-003*	*1*	*3.123E-003*	*1.728E-004*	*0.9899*	
B^2	*187.30*	*1*	*187.30*	*10.37*	*0.0147*	
Residual	126.48	7	18.07			
Lack of Fit	*25.70*	*3*	*8.57*	*0.34*	*0.7989*	*not significant*
Pure Error	*100.78*	*4*	*25.20*			
Cor Total	686.39	12				

The Model F-value of 6.20 implies the model is significant. There is only a 1.65% chance that a "Model F-Value" this large could occur due to noise.

Figure 4.11: ANOVA of quadratic model and significance test

Diagnostic plots were drawn to judge the model adequacy and clarify the signs of any problems in the experimental data. Plot of observed response (antibiotic activity) versus predicted reponse is shown in Figure 4.12. In this case, predicted values were in agreement with observed ones in the range of the operating variables. Three-dimensional (3D) response surface plots assist understanding of the main as well as the interaction effects of two factors, such as mannitol and glycine. The plots obviously showed that the higher and lower concentration of mannitol and glycine favor the higher antifungal activity production in *Streptomyces* sp. S-9 However, the antifungal activity was significantly suppressed with intermediate range of mannitol and glycine concentration (Figure 4.13).

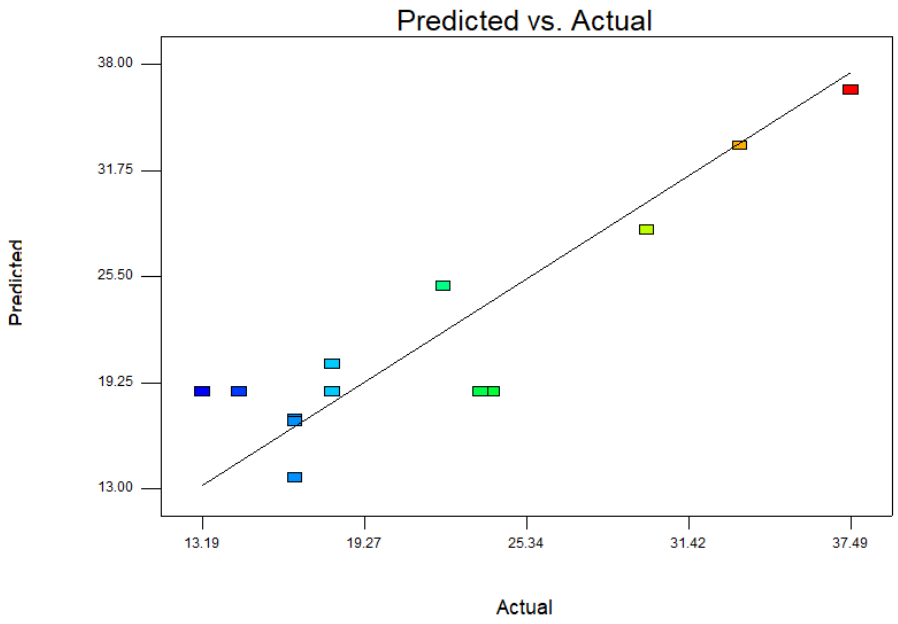


Figure 4.12: Residual diagnostic plot of quadratic model. Observed verses predicted response plot

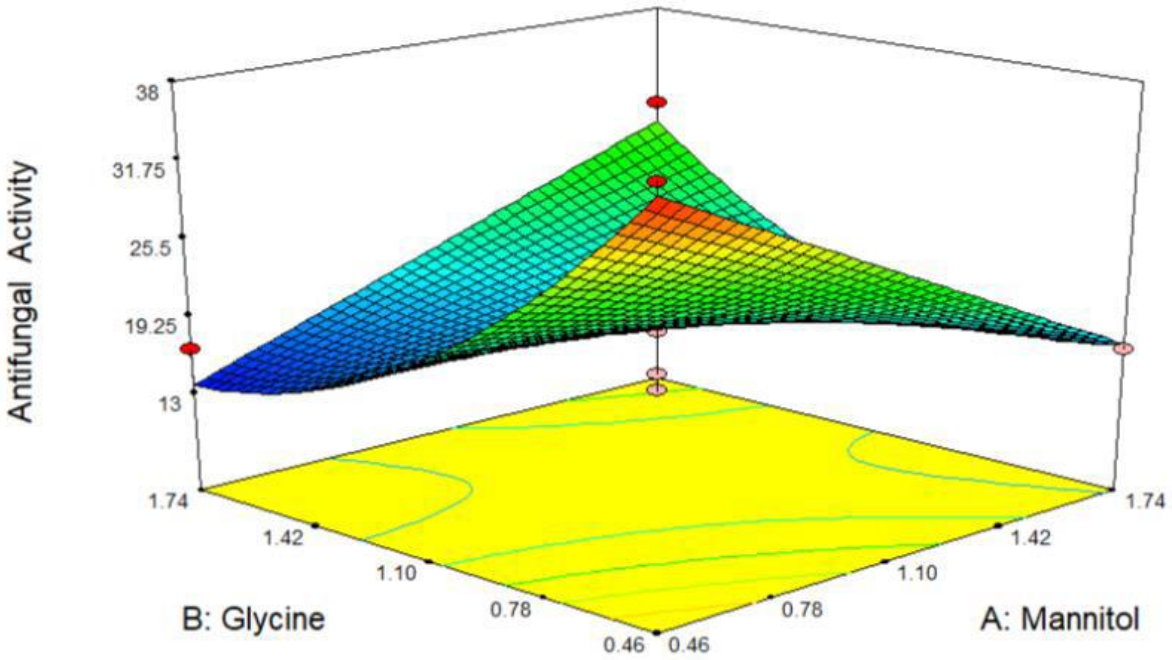


Figure 4.13: 3D Response surface plot showing interactive effects of variables on antifungal activity by *Streptomyces* sp. S-9

4.3.3 Optimization and Experimental Validation

On the basis of numerical optimization, the quadratic model predicted that the maximum antifungal activity was 37%, when the concentration of mannitol and glycine is 0.46g% for both. Experiments in shake-flasks with a predicted ideal medium were done to verify the statistical results. In the laboratory, an antifungal activity of 36% (Table 4.5) was obtained, which is in line with the highest projected value of 37%. (Figure 4.14). This result suggested that the model is adequate for prediction of antifungal activity in *Streptomyces* sp. S-9. The final optimized medium contained 5.0g of tryptone, 3.0g of yeast extract, 5.0g of NaCl, salt formulation (as mentioned previously-table 4.4), 4.6g of mannitol, and 4.6g of glycine in 1L of distilled water. Antifungal activity shown on different optimized and unoptimized media was presented in Figure 4.15.

Table 4.5: Experimental validation of the combined effect of variables under unoptimized and optimized levels on the antifungal activity of *Streptomyces* sp. *S-9*

	Glycine (g %)	**Mannitol (g %)**	**Antifungal activity (%)**	**Predicted Antifungal activity (%)**
Flask 1	2.0	2.0	36.11	37.49
Flask 2	0.46	0.46	35.00	37.49
Basal ISP 1flask	-	-	20.83	-

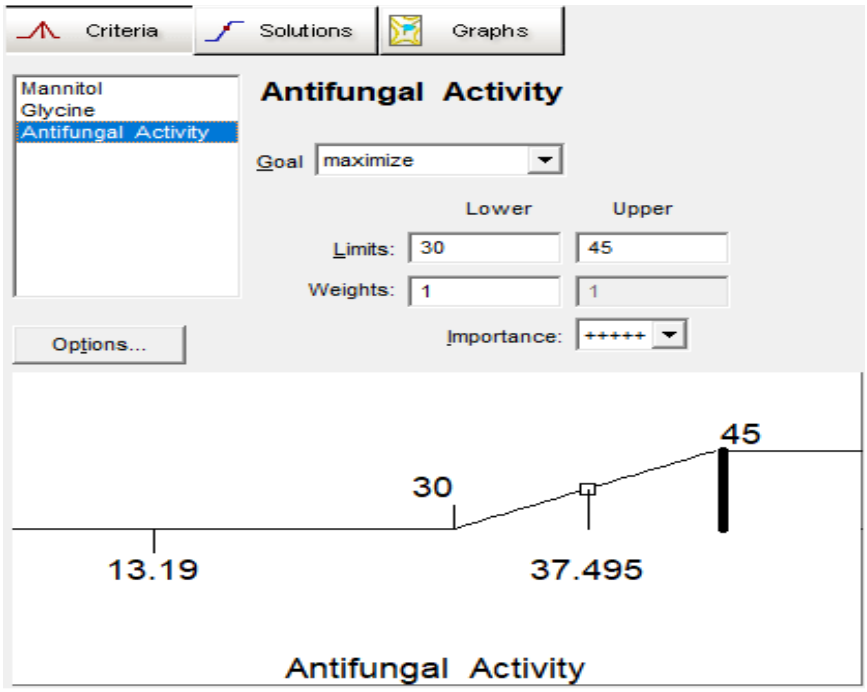


Figure 4.14: Summary of criteria set for optimization run

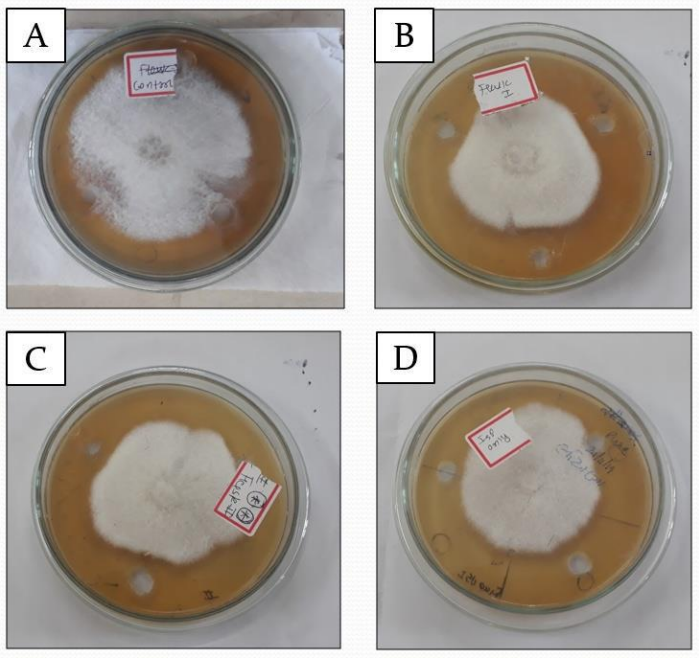


Figure 4.15: Antifungal activity shown on different media (A) Control (B) 2 g% mannitol and glycine (C) optimized 0.46 g% mannitol and glycine (D) non-optimized basal medium in the absence of mannitol and glycine

4.4 Discussion

Streptomyces are potential producers of antibiotic secondary metabolites (Jose & Jebakumar, 2012; Mohamed & Galal, 2005; Satheeja & Jebakumar, 2011). Their ability to produce antibiotics is not a fixed characteristic, and it can be considerably influenced by medium components (Farid et al., 2000; Rateb et al., 2014). The majorities of Streptomyces' metabolites are extracellular and possess powerful antibacterial properties (Singh et al., 2014). Fermentation by microorganisms is a complex, non-linear, unstructured process. Minor modifications in the content and conditions of the fermentation medium can affect the yields of specific chemicals and alter the metabolic profile of a strain (Kaur et al., 2014; Scherlach & Hertweck, 2009).

This study was primarily concerned with optimizing *Streptomyces* sp. S-9 antibiotic production as a function of amounts of components in the production medium. Minor alterations to the composition of the culture medium can have a considerable impact on the production of secondary metabolites in microorganisms (Chaston et al., 2011; Wang et al., 2011). It is challenging to determine the appropriate fermentation conditions. To acquire the optimal fermentation conditions, it is required to construct an experiment using a reasonable experimental method. RSM can be used to optimise the composition of the fermentation medium and growth conditions to enhance the generation of secondary metabolites, hence facilitating the discovery of novel natural active components (Arul Jose & Jebakumar, 2014; Mazarei et al., 2017; Wang et al., 2011). Several researchers engaged in antibiotics discovery projects have utilized RSM as a statistical method to identify, control, and optimize influencing medium elements and have documented an increase in antibiotic output. For example, Wang et al. (2011) used the RSM technique to optimize the medium for antibiotic synthesis by *Xenorhabdus bovienii* and observed a 37.8% increase in antibiotic activity. Chen et al. (2013) also reported a 2.7-fold increase in antibiotic production by *Bacillus sp.*ZJUIBE-076 utilizing the RSM method.

In the present study, antifungal production was affected by nutrients sources. To enhance this production, the optimal concentration of mannitol and glycine must be increased to 4.6g/L. Mannitol and glycine were found to be the most influencing factors for antifungal activity by *Streptomyces* sp. S-9 as determined by OFAT non statistical method. There was 16% increase in the antifungal activity with optimized medium compared with non-optimized medium (absence

of mannitol and glycine) as determined by CCD statistical design. Nonetheless, some researchers scientists have documented an increase in *Streptomyces* sp. 19 G-317's antifungal productions in maltose-enhanced medium (Feng et al., 2011). According to reports, various carbon sources affect the formation of secondary metabolites. Catabolic suppression or the "glucose effect" inhibits biosynthesis in the majority of antibiotic-producing microorganisms (Larpent & Larpent-Gourgaud, 1990). A difficult-to-metabolize carbon source, such as polysaccharides (starch, dextrins), is frequently preferable. An example of catabolic repression of secondary metabolism in actinomycetes is the suppression of actinomycin production by *S. antibioticus* when additional glucose is given to the medium (Singh et al., 2009).

Utilizing a central composite design, a total of 13 runs were done to examine the interactions between the two selected variables (mannitol and glycine) and to establish how they affected the metabolite production. We utilized ANOVA to evaluate the significance of the design by gaining a better grasp of the causes of variance (Mourabet et al., 2017). The Fisher's test compares the square mean of the model and residual error variance sources (Kasiri et al., 2013). The F-value of 6.20 indicated that the model created in the current study was significant and may be utilized to explain the variation in the investigated response (antifungal production). Alternately, the loss of fitness should be insignificant for the model to suit the experimental design adequately. In our results, the lack of fit was not statistically significant (P-value>0.05), indicating that the model was adequate for this investigation. P-values were used to indicate the significance of each factor. As the P-value decreases and the total of squares increases, the associated factor becomes more significant (Wang & Liu, 2008; Zhou et al., 2020). The predicted model was shown as 3D-plots and contour plots to better comprehend the variables' effects on antifungal metabolite synthesis and their interactions. As a result, their optimal values can be precisely identified using 3D plots, which can show the reaction as a function of the various levels of each of the components. On the other hand, 2D contour plots can show the importance of an interaction between any two elements: an elliptical or saddle contour suggests that the interaction between the two factors is considerable, while a circular contour indicates that the interaction is weak (Wang et al., 2015; Wang & Liu, 2008; Zhou et al., 2020).