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Factorial Optimization of Media Composition and Fermentation Conditions for Improved Novel Antimicrobial Compound Production by *Geotrichum candidum* OMON-1

S. O. Omeike[1*]

[1] *Department of Microbiology & Biotechnology, McPherson University, Seriki Sotayo, Ogun State, Nigeria.*

*Corresponding author: Tel: +2348166036372; E-mail: omeikeso@mcu.edu.ng

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This study investigated the optimization of fermentation conditions to enhance the production of the antimicrobial compound GP-2B by *Geotrichum candidum* OMON-1 using a two-level factorial design. A resolution IV 1/8th factorial design was employed to evaluate the effects of sodium chloride, agitation, tryptone, pH, dextrose, dipotassium hydrogen phosphate, peptone, and fermenter volume on GP-2B production. Randomized experiments were conducted, and antimicrobial activity was determined using the agar well-diffusion method against *Staphylococcus aureus*. The highest antimicrobial activity (21 mm) was achieved under optimized conditions: low pH (5.5), dextrose (2 g/L), dipotassium hydrogen phosphate (2 g/L), fermenter volume (40%), and high peptone (8 g/L), tryptone (25 g/L), sodium chloride (25 g/L), and agitation (150 rpm). Analysis of variance (ANOVA) and a half-normal plot identified tryptone (48.04%), tryptone-agitation interaction (19.85%), dextrose (-1.75; 12.01%), and pH (8.82%) as significant factors influencing fermentation. Tryptone had the most positive effect (+3.5), while dextrose had the most detrimental effect (-1.75). A curved 3D response surface plot was generated to visualize the optimization process. GP-2B produced under optimized conditions exhibited higher antimicrobial activity (28 mm) compared to non-optimized conditions (19.5 mm). This study demonstrates the effectiveness of a two-factorial design in improving GP-2B production and identifying critical factors in the fermentation process. The findings highlight the potential of statistical optimization for enhancing antimicrobial compound production in industrial applications.

Keywords: Antimicrobial compound, Design Expert software, Fermentation, *Geotrichum candidum* OMON-1, GP-2B, Two-factorial design.

INTRODUCTION

Geotrichum candidum is an acid-tolerant filamentous yeast that has numerous uses in food industries where it contributes to brewing, winemaking, production of enzymes, organic acids, and pharmaceuticals. The fungus is also known to produce bioactive secondary metabolites that possess antimicrobial properties against various pathogenic and spoilage bacteria. Compounds such as phenyllactic acid (PLA), indoleacetic acid (ILA), and phenylethyl alcohol (PEA), which are active against *L. monocytogenes*, have been produced by strains of the fungus. Recent studies have also described bioactivity of compounds from strains of the species against pathogens. Compounds showing broad-spectrum activity against stored grain pathogens are being recommended for biological fumigation, and another strain was reported to produce a novel GP-2B tripeptide from the fungus with bioactivity against bacteria. However, there is a need to produce these bioactive molecules in large quantities in consideration of their future industrial importance. This is achieved through fermentation, an industrial process that leads to mass production of the desired product, and the optimization of the process. According to Chai et al. (2022), this is important for industries and ensures both profitable income sustainability and product quality maintenance. Traditionally, fermentation optimization is carried out using the one factor at a time (OFAT) method, but this is considered labour-intensive, generates unreliable outcomes, does not reflect the effect of all interacting components during fermentation, and necessitates the need for more efficient techniques.

Statistical tools, including factorial model, Plackett-Burman design (PBD), and response surface methodology (RSM), effectively tackle OFAT limitations and are widely accepted for fermentation optimization. They provide better economic substitutes and promote the study of interactions between factors. They also lead to increased product yields, reduced process variability, reduced time, and overall costs. This study reports two-factorial optimization of medium composition and fermentation conditions to enhance antimicrobial compound GP-2B production by *Geotrichum candidum* OMON-1.

MATERIALS AND METHODS

Producer Strain and Clinical Isolate

Producer strain *Geotrichum candidum* OMON-1 and clinical strain *Staphylococcus aureus* were retrieved from the culture collection facility of the Institute of Microbial Technology, India. Both were sub-cultured on appropriate media prior to fermentation and antimicrobial sensitivity testing, respectively.

Fermentation of Geotrichum candidum OMON-1

Modified Tryptone Soya Broth (TSB) medium (25 g/L NaCl) was used as the antibiotic production medium and made up 60% (vol/vol) of the fermentation flask. An overnight *G. candidum* OMON-1 (1%) culture was inoculated and incubated in a shaker incubator at 30 °C and 150 rpm for 10 days.

Optimization of Fermentation Conditions and Antimicrobial Assay

Increased antibacterial compound production was investigated by varying media composition and environmental conditions using the two-level factorial in the Design Expert (DE) Software version 10.0.1. Eight nutritional and environmental factors were optimized at minimum/low (-1) and maximum/high (+1) levels without blocking. These included tryptone (15 and 25 g/L), pH (5.5 and 8.5), peptone (3 and 8 g/L), sodium chloride (NaCl; 10 and 25 g/L), dextrose (10 and 25 g/L), agitation (120 and 150 rpm), fermenter volume (40 and 60% v/v), and dipotassium hydrogen phosphate (K_2HPO_4; 2 and 4 g/L). Using a trade-off table at 1/8th factorial and design resolution four (IV), optimization was reduced to sixteen (16) randomized experimental runs. Nutritional factors were weighed and mixed appropriately, all runs were prepared simultaneously in separate flasks, and fermentation proceeded at appropriate environmental conditions for 10 days.

Following fermentation, bioactive fractions of experimental runs were extracted, and the zone of inhibition (Response 1) was determined via antimicrobial assay using the standard agar well-diffusion method. Fermentation also proceeded at determined optimized conditions in modified TSB broth. GP-2B was extracted and purified on RP-HPLC as previously described, and the peak height of the purified compound under normal and optimized conditions was compared to determine changes in compound concentration.

Preparation of Bioactive Fungal Extracts and Purification of GP-2B

Bioactive extracts were prepared using modified methods of Petit et al. (2009) and Okudoh and Wallis (2012). After fermentation, cultures were centrifuged at 9072 ×g for 15 minutes, and the cell-free supernatant was extracted using the solid-liquid extraction method with Diaion HP-20 resin beads to bind the active component. Elution was performed using an optimized three-solvent system of methanol/isopropanol/acetone (6:3:1 v/v) solution for 30 minutes at room temperature. The antimicrobial compound GP-2B was purified as previously described, with the bioactive extract initially separated on a CM-Sepharose CL-B6 (45–165 m; Pharmacia, Sweden) ion-exchange column and eluted with 50% (v/v) ammonium acetate-buffered 1 M NaCl (pH 5.0). The eluent was purified on a reverse-phase HPLC (Agilent Technologies, USA) with a C-18 column, UV detector, and acetonitrile and water buffered with 0.05% v/v trifluoroacetic acid (TFA) acting as mobile phase eluents. TFA was removed by further elution of the pure compound (GP-2B) with methanol/isopropanol (70:30 vol/vol), and it was lyophilized (SCANOVA) overnight to obtain GP-2B in powder form. GP-2B was reconstituted for antimicrobial assay by dissolving in 5 mL dimethyl sulfoxide (DMSO).

Antimicrobial Susceptibility Testing

Antimicrobial activity of the purified compound obtained following optimizations was determined against *S. aureus* via the agar well-diffusion technique as previously described. Briefly, 1 mL of *S. aureus* adjusted to 0.5 McFarland's standard was seeded onto Muller-Hinton agar medium. Wells were bored on plates using a cork borer (6 mm), and 1 mL of GP-2B purified from both fermentations, respectively, was added as appropriate. Seeded plates were incubated at 37 °C for 24 hours, and zones of inhibition were determined.

RESULTS

Table 1 presents the antimicrobial activity responses for each run in the two-factorial, eight-factor optimization process. The highest antimicrobial activity (21 mm) was achieved at Run 13, while Runs 5, 9, 10, and 12 showed the lowest activity (12 mm). Optimal conditions included high levels of tryptone (25 g/L), peptone (8 g/L), NaCl (25 g/L), and agitation (150 rpm), and low levels: pH (5.5), dextrose (2 g/L), K_2HPO_4 (2 g/L), and fermenter volume (40%).

Table 2 describes the effects of singular and interacting factors on fermentation. Key observations include that tryptone (factor A) had the highest positive effect (+3.5), dextrose (factor E) had a significant negative effect (-1.75), and the tryptone-agitation interaction (factor AF) showed a positive effect (+2.25), while pH, fermenter volume, and K_2HPO_4 had negative effects.

Furthermore, tryptone contributed the most (48.04%) to the fermentation process, followed by the tryptone–agitation interaction (19.85%), dextrose (12.01%), and pH (8.82%), as shown in Table 3. The ANOVA model, along with the factors tryptone, pH, dextrose, and the tryptone–agitation interaction, exhibited high F-values and statistically significant P-values ($P < 0.05$).

Diagnostic plots (Figures 1–4) revealed significant deviations for tryptone, the tryptone–agitation interaction, dextrose, and agitation speed in the Half-normal plot. The Normal Plot of Residuals showed a linear distribution with most values along the straight line; the Residuals vs. Predicted Values plot indicated most values clustered around the median line; and the Box-Cox plot yielded a lambda value of $\lambda = 1$, suggesting no further transformation of the response data (ZI) is required.

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Table 1 Real value experimental design of the 8-factor optimization process for increased antibiotics production by *Geotrichum candidum* OMON-1

Std	Run	Block	Factor 1 A: Tryptone ()	Factor 2 B: pH	Factor 3 C: Peptone ()	Factor 4 D: NaCl ()	Factor 5 E: Dextrose ()	Factor 6 F: Agitation ()	Factor 7 G: Fermentor (%)	Factor 8 H: K_2HPO_4 ()	Response 1 Inhibition Zone ()
10	1	Block 1	25.00	5.50	3.00	25.00	5.00	120.00	60.00	2.00	15.00
4	2	Block 1	25.00	8.50	3.00	10.00	5.00	150.00	40.00	2.00	17.00
1	3	Block 1	15.00	5.50	3.00	10.00	2.00	120.00	40.00	2.00	16.00
15	4	Block 1	15.00	8.50	8.00	25.00	5.00	120.00	40.00	2.00	14.00
11	5	Block 1	15.00	8.50	3.00	25.00	2.00	150.00	60.00	2.00	12.00
3	6	Block 1	15.00	8.50	3.00	10.00	5.00	120.00	60.00	4.00	13.00
6	7	Block 1	25.00	5.50	8.00	10.00	5.00	120.00	40.00	4.00	15.00
13	8	Block 1	15.00	5.50	8.00	25.00	2.00	120.00	60.00	4.00	16.00
9	9	Block 1	15.00	5.50	3.00	25.00	5.00	150.00	40.00	4.00	12.00
7	10	Block 1	15.00	8.50	8.00	10.00	2.00	150.00	40.00	4.00	12.00
8	11	Block 1	25.00	8.50	8.00	10.00	2.00	120.00	60.00	2.00	16.00
5	12	Block 1	15.00	5.50	8.00	10.00	5.00	150.00	60.00	2.00	12.00
14	13	Block 1	25.00	5.50	8.00	25.00	2.00	150.00	40.00	2.00	21.00
12	14	Block 1	25.00	8.50	3.00	25.00	2.00	120.00	40.00	4.00	16.00
2	15	Block 1	25.00	5.50	3.00	10.00	2.00	150.00	60.00	4.00	19.00
16	16	Block 1	25.00	8.50	8.00	25.00	5.00	150.00	60.00	4.00	14.00

the straight line; the Residuals vs. Predicted Values plot indicated most values clustered around the median line; and the Box-Cox plot yielded a lambda value of $\lambda = 1$, suggesting no further transformation of the response data (ZI) is required.

Description of interaction plots (Figures 1-2) shows intersecting linear lines between tryptone and agitation, and parallel linear lines between tryptone-pH interactions. Also, the Response Surface Plot (Figure 5) shows a curved response surface with the highest activity at 25 g/L tryptone and 150 rpm agitation.

Also, the Response Surface Plot (Figure 5) shows a curved response surface with the highest activity at optimized conditions. Lastly, comparison of purified compound concentration showed that GP-2B purified from optimized fermentation exhibited a higher peak area and antimicrobial activity (28 mm) compared to normal fermentation (19 mm).

DISCUSSION

Fermentation optimization is crucial in industrial settings to identify optimal parameters for product formation and reduce costs. The process efficiently identifies influential variables and their settings to optimize responses. Design of Experiment (DOE) statistical tools, particularly factorial models, are widely accepted for optimization. These tools analyze the effects of factors singly and in interaction, determining significant factors and their impacts on responses. A factorial model was applied to optimize GP-2B production in fermentation broths, and results showed that tryptone, peptone, NaCl, and agitation significantly influenced antimicrobial activity.

Optimal conditions included high levels of tryptone, peptone, and NaCl, low pH, and low dextrose concentration. Tryptone's positive effect, both singly and in interaction with agitation, was notable. Conversely, increasing dextrose concentration adversely affected product formation. Tryptone, a nitrogen source, was essential for GP-2B production. As a rich source of amino acids, tryptone can lead to increased production of the tripeptide antibacterial compound, and previous studies have reported its significance in antimicrobial compound production. Similarly, peptone provides amino acids and nitrogen compounds for the growth of fungi and yeasts, and increasing this substrate in fermentation medium was reported to positively contribute to a 15% increase in bioactive compound production by the endophytic fungus *Athelia rolfsii* following optimization. Agitation is an important industrial factor due to its potential influence on aeration, affecting the amount of dissolved oxygen in the broth, cell growth, and possibly metabolite production. Furthermore, higher agitation speed breaks up mycelial aggregates and enhances oxygen diffusion. However, the producer strain *Geotrichum candidum* OMON-1, a filamentous yeast, exhibits reduced mycelial aggregation, thus confirming the need for moderate agitation observed during optimization.

Analysis of Variance (ANOVA) statistics, indicated by high F-values, confirmed the factorial model's fitness, with significant Prob $> F$ values ($P < 0.05$) indicating the model's adequacy. Diagnostic plots verified that the response values fit the model, with the prominence of tryptone as the most critical factor ($> 90\%$ probability) aligning with optimized values. Residual plots also described insignificant error in the model, while the

Table 2 ANOVA parameters of the factorial design used for optimization of GP-2B production by *Geotrichum candidum* OMON-1

	Standardized Effects	Sum of Squares	% Contribution
Factors			
A: Tryptone	3.50	49.00	48.04
B: pH	-1.50	9.00	8.82
C: Peptone	0.00	0.00	0.00
D: NaCl	-0.25	0.25	0.25
E: Dextrose	-1.75	12.25	12.01
F: Agitation	-0.25	0.25	0.25
G: Fermentor Volume	-1.00	4.00	3.92
H: K_2HPO_4	-0.75	2.25	2.21
AB: Tryptone-pH	-0.50	1.00	0.98
AC: Tryptone-Peptone	0.00	0.00	0.00
AD: Tryptone-NaCl	-0.25	0.25	0.25
AE: Tryptone-Dextrose	-0.75	2.25	2.21
AF: Tryptone-Agitation	2.25	20.25	19.85
AG: Tryptone-Fermentor Volume	-0.50	1.00	0.98
AH: Tryptone-K_2HPO_4	-0.25	0.25	0.25

Key: A = Tryptone; B = pH; C = Peptone; D = NaCl; E = Dextrose; F = Agitation; G = Fermentor Volume; H = K_2HPO_4; AB = Tryptone-pH; AC = Tryptone-Peptone; AD = Tryptone-NaCl; AE = Tryptone-Dextrose; AF = Tryptone-Agitation; AG = Tryptone-Fermentor Volume; AH = Tryptone-K_2HPO_4

Table 3 Statistical parameters showing significant and non-significant values in the factorial model used for GP-2B optimization by *Geotrichum candidum* OMON-1

	Sum of Squares	Mean Square	F-Value	Prob ¿ F
Source/Factors				
Model	91.00	15.17	12.41	0.0007
A: Tryptone	49.00	49.00	40.09	0.0001
B: pH	9.00	9.00	7.36	0.0239
D: NaCl	0.25	0.25	0.20	0.6618
E: Dextrose	12.25	12.25	10.02	0.0114
F: Agitation	0.25	0.25	0.20	0.6618
AF: Tryptone-Agitation	20.25	20.25	16.57	0.0028

Key: A = Tryptone; B = pH; D = NaCl; E = Dextrose; F = Agitation; AF = Tryptone-Agitation

Box-Cox plot ($\lambda = 1$) indicated no need for response value transformation. The distribution of values around the median line in the residual plot further highlights factor interactions, reinforcing the significance of the tryptone–agitation interaction.

CONCLUSION

The two-factorial experimental design successfully optimized GP-2B production. Tryptone and the tryptone–agitation interaction exerted significant positive effects on fermentation, while dextrose exhibited the most pronounced negative effect. Statistical analyses further corroborated the optimization outcomes, demonstrating that GP-2B produced under optimized conditions displayed enhanced antimicrobial activity. This study identified key factors for formulating an optimized fermentation medium, which should be considered in future investigations.

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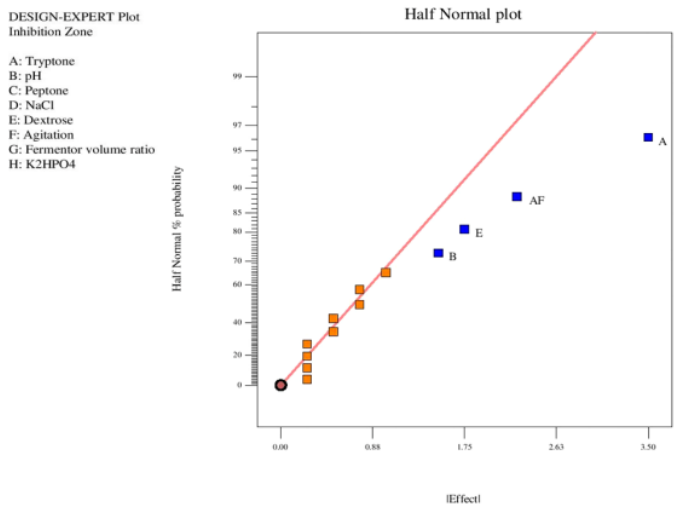

Figure 1 Half-normal plot of a two-factorial optimization system for increased antibiotic compound production based on zone of inhibition

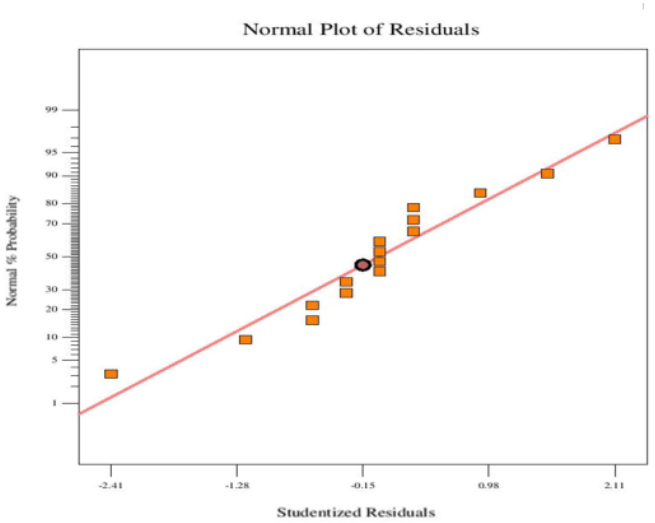

Figure 2 Percentage probability plot diagnostic of the factorial design, showing correlation between normalized residuals and studentized residuals

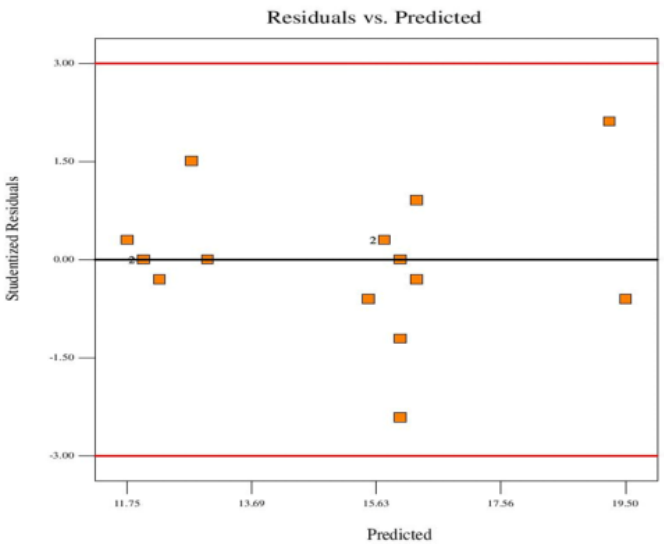

Figure 3 Diagnostic plot of the optimization model based on correlation between actual and predicted response

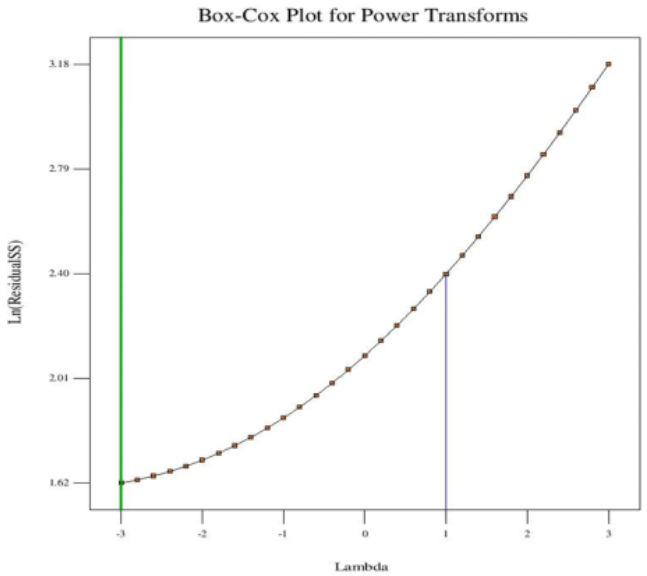

Figure 4 Box-Cox plot diagnosis of the two-factorial model to determine if transformation of response data is necessary. The blue line ($\lambda = 1$) indicates that the response data (ZI) requires no further transformation

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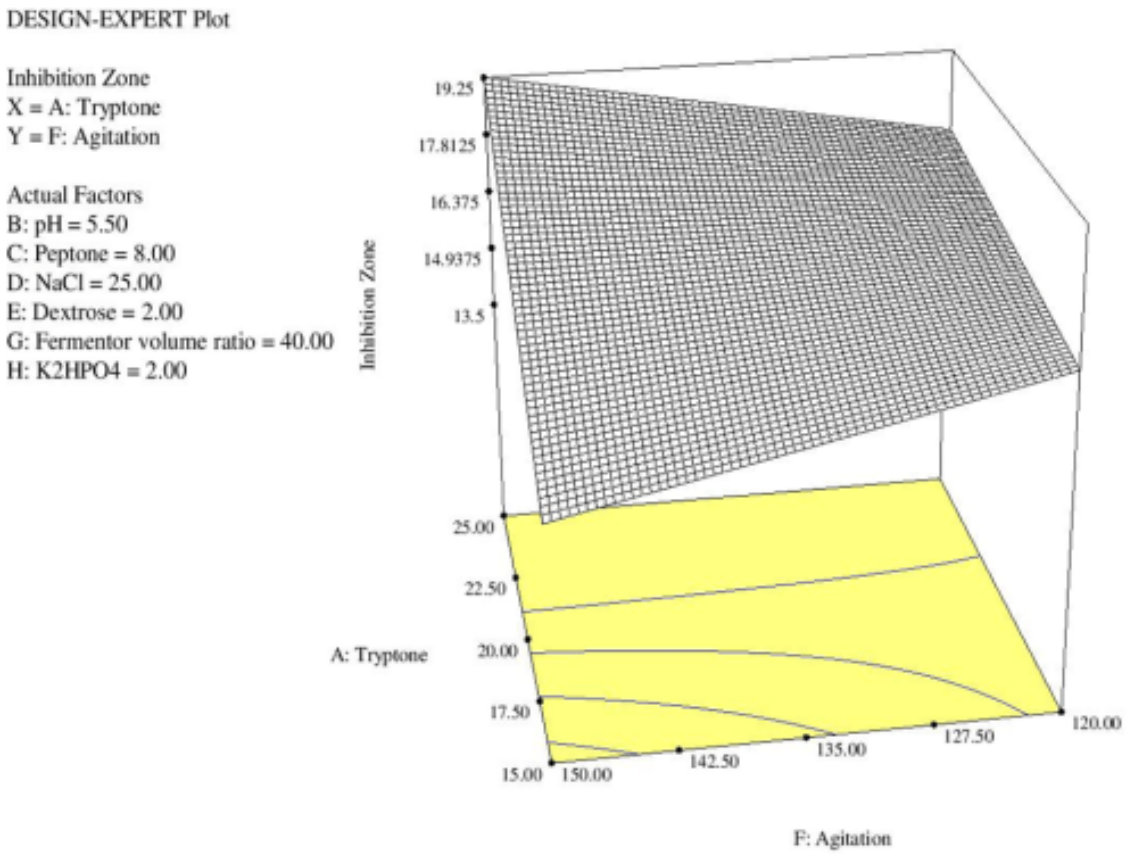

Figure 5 A curved response surface plot of optimized fermentation conditions

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