Statistical optimization of media composition and fermentation conditions for improved production of novel antimicrobial compound by Geotrichum candidum OMON-1

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Research Article

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Abstract

Optimization of fermentation conditions is important for increased compound production and use of statistical designs could improve efficiency of the process. Optimization of nutritional and environmental requirements for increased GP-2B production by *Geotrichum candidum* OMON-1 in tryptose soy broth (TSB) at flask level using two-level factorial tool in Design Expert (DE) software was investigated. Using 1/8th factorial and design resolution IV, pre-determined low and high levels of tryptone, pH, peptone, sodium chloride, dextrose, agitation, fermenter volume and dipotassium hydrogen phosphate were considered in 16 randomized experimental runs. Antimicrobial activity (Response) of bioextract from each experimental run against *Staphylococcus aureus* was determined via agar well-diffusion. Fermentation proceeded in both DE-optimized and standard TSB medium and concentration (peak height) of HPLC-purified GP-2B compared. Highest antimicrobial activity (21 mm) was achieved at Run 13 (high tryptone (25 g/L), peptone (8 g/L), NaCl (25 g/L), agitation (150 rpm); low pH (5.5), dextrose (2 g/L), K_2HPO_4 (2 g/L), fermenter volume (40%)). Analysis of Variance (ANOVA) and Half-normal plot described tryptone (48.04%), tryptone-agitation interaction (19.85%), dextrose (-1.75; 12.01%) and pH (8.82%) as significant contributors to fermentation, while tryptone (+ 3.5) and dextrose (-1.75) had best and most detrimental influence respectively. A curved 3D response surface plot was obtained, with concentration and activity against *S. aureus* of GP-2B from optimized conditions (3.143×10^8; 21 mm) higher than at normal conditions (2.869×10^8; 19.5 mm). The two factorial design successfully improved fermentation requirements for increased GP-2B production by *G. candidum* OMON-1.

Introduction

Geotrichum candidum is a filamentous yeast and the commonest species in the genus (Kawtharani et al. 2020). It is an acid tolerant species and has been widely applied in cheese making where it confers proteolytic, lipolytic, amino acid catabolism as well as de-acidification activities in cheese, and traditional fermentation of milk. It also influences the characteristics appearance, taste and aroma of the cheese (Eliskase-Lechner et al. 2011). Aside its wide application in milk and cheese industry, *G. candidum* has been documented to have antibacterial activity, inhibiting pathogenic and spoilage bacteria including *Listeria monocytogenes, Salmonella typhi, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Providencia stuarti* and *Klebsiella oxytoca* (Dieuleveux et al. 1998a,1998b), indicating ability to produce bioactive secondary metabolites. Phenyllactic acid (PLA), indoleacetic acid (ILA) and phenylethyl alcohol (PEA) are three secondary metabolites that are produced from these species and have been documented as antimicrobial compounds. PLA and ILA initiate alterations in behavior and structure of *L. monocytogenes* thereby completely inhibiting its growth, while PEA on the other hand has been reported to enhance membrane damage and also inhibit RNA and protein synthesis (Kawtharani et al. 2020). Of all the metabolites, PLA has been documented as the most effective natural antibiotic (Dieuleveux et al. 1998a). Furthermore, our previous study has shown the production of tripeptide GP-2B by this species isolated from a chemical waste site.

Increased production of antimicrobial compounds has received much attention, especially towards optimization of media and production conditions (Wang et al. 2011). Enhanced optimization of media physical and nutritional parameters can improve the production of microbial secondary metabolites (Feng et al., 2011). The traditional method of optimizing media for antimicrobial production of one factor at a time (OFAT) involves changing one factor at a time while other factors are kept constant. Ahmed et al. (2018) reported in their study that this method is labour intensive, and reports have noted the generation of unreliable outcomes as another limiting factor while also stating that it does not reflect the effect of all the interactions of the components in the fermentation media (Bhagwat et al. 2015; Singh et al. 2016).

To avert these limitations, the use of statistical tools has been shown to have promising future in antimicrobial optimization. Consequently, statistical experimental design techniques especially Plackett-Burman design (PBD), response surface methodology (RSM) and two-level factorial are widely adopted to optimize media and fermentation conditions (Wang et al. 2011; Feng et al. 2017). Strategies based on statistical tools provide better economical substitutes in which interaction between factors can also be studied (Ahmed et al. 2018). These statistical experimental design procedures have been reported to increase product yields, reduced process variability, reduced time and overall costs, compared with traditional OFAT technique (Adinarayana et al. 2003; Wang et al. 2011). This study reports statistical optimization of medium composition and fermentation conditions to enhance production of antimicrobial compound GP-2B by *Geotrichum candidum* OMON-1 while confirming retention of antimicrobial activity.

Materials And Methods

Producer strain and clinical isolate

Both producer strain *G. candidum* OMON-1 and *S. aureus* clinical strain were retrieved from the culture collection facility in the Microbiology laboratory, McPherson University, Nigeria. Isolates were grown on fresh medium, as appropriate, prior to fermentation and antimicrobial sensitivity testing respectively.

Fermentation of *Geotrichumcandidum*OMON-1

Tryptone Soya Broth medium (TSB) was modified to include 25g/L of NaCl and used as antibiotic production medium as previously described (Omeike et al. 2020). Modified TSB Medium was weighed to make 60% vol/vol fermentation flask of 1500 mL capacity and sterilized by autoclaving at 121 °C for 15 min. *G. candidum* OMON-1 culture (12-24hrs old) was inoculated at 1% broth volume and incubated at 30 °C and 150 rpm for 10 days.

Optimization of fermentation conditions for improved antimicrobial compound production

Increased antibacterial compound production was investigated by varying factors including production medium composition and environmental conditions using the Two-level factorial design in the Design

Expert (DE) Software version 10.0.1. Eight (8) recognized factors which included both nutritional and environmental requirements were considered for optimization at two levels: minimum/low (-1) and maximum/high (+1) levels and without blocking to determine the best combination for increased production. Due to high number of factors, the trade-off table present in the two-factorial system (Table 1) was used to reduce experimental cost. Therefore, the $1/8^{th}$ factorial and design resolution four (IV) was chosen with a combination of factors aliasing the main factors, resulting in sixteen (16) experimental runs.

Insert Table 1

Factors and their minimum (-1) and maximum (+1) values considered are: 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) respectively. Low and high levels of each factor was inputted into the DE software and a possible randomized set of sixteen (16) experimental combinations were generated as described in Table 2 below. 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. After fermentation, each flask was extracted, and antimicrobial activity determined.

Insert Table 2

Antimicrobial activity of extract from each experimental run- which is also the outcome- was appropriately labeled ‘Response 1’ in DE and was measured based on zone of inhibition in susceptibility testing. Fermentation thereafter proceeded at optimized condition as determined in DE and in modified TSB broth appropriately after which GP-2B was extracted and purified on HPLC system. Height of purified peak on HPLC was compared to determine effect of optimization on product concentration.

Preparation of crude fungal extracts and purification of GP-2B

Crude fungal extracts were prepared using a modification of methods described by Petit et al. (2009) and Okudoh and Wallis (2012). After respective incubation periods, fermented cultures were centrifuged at 9000 rpm for 15 mins to remove cells and spores. Thereafter, cell-free supernatant was extracted using a solid-liquid extraction model by binding the antibacterial-active component to a resin, Diaion HP-20 beads. Bounded compounds were eluted using an optimized three-solvent system of methanol/isopropanol/acetone (6:3:1 v/v) solution for 30 min at room temperature for optimum recovery of active compounds from the bound resin.

Antimicrobial compound GP-2B was purified as previously described (Omeike et al. 2020). Bioactive extract was initially separated on an ion-exchange column of CM-Sepharose CL-B6 (45–165 µm; Pharmacia, Sweden) eluted with 50% (v/v) ammonium acetate-buffered 1 M NaCl (pH 5.0). This fraction was purified on a reverse-phase HPLC (Agilent Technologies, USA) with a C-18 column and UV detector, Acetonitrile and water buffered with 0.05% v/v trifluoroacetic acid (TFA) acting as mobile phase eluents.

TFA was removed by further elution of pure compound (GP-2B) with methanol/isopropanol (70:30 vol/vol).

Antimicrobial susceptibility testing

Retention of antimicrobial activity by bioextract and purified compound against *S. aureus* was determined via agar well-diffusion technique as previously described (CLSI 2012). Briefly, 1 mL of *S. aureus* adjusted to 0.5 McPharland's standard was seeded onto Muller-Hinton agar medium. Wells were bored on plates using cork borer (6 mm) and bioactive extract/GP-2B were added appropriately. Seeded plates were incubated at 37 °C for 24 h and zone of inhibition (ZI)

Results

Optimization of GP-2B production

Response (antimicrobial activity) for each run in the two-factorial eight-factor optimization process is described in Table 3. Optimum antimicrobial activity was achieved at Run 13 while four runs (Runs 5, 9, 10 and 12) had minimum activity of 12 mm. The experimental design of factors which led to optimum activity included tryptone (25 g/L), peptone (8 g/L), NaCl (25 g/L) and agitation (150 rpm) at high levels, while pH (5.5), dextrose (2 g/L), K_2HPO_4(2 g/L) and fermenter volume (40%) where at low levels. However, Runs with lowest antimicrobial activity had tryptone at low level (15 g/L) and agitation at high level (150 rpm) in common. Furthermore, both minimum and maximum antimicrobial activity had different tryptone concentration (15 g/L and 25 g/L respectively) but similar agitation speed (150 rpm).

Insert Table 3

Table 4 shows the effects of factors, singly and in interaction, on fermentation computed by the DE software. Tryptone (factor A) and dextrose (factor E) had the highest positive (+3.5) and negative (-1.75) effect on fermentation respectively. Also, increasing pH (factors B), K_2HPO_4 concentration (factor H) and tryptone-fermenter volume interaction (factor AG) had significant negative effects (-1.5, -0.75 and -0.25 respectively) on fermentation. However, NaCl (factor D), Agitation (factor F), tryptone and NaCl interaction (factor A-D) and tryptone-K_2HPO_4 interaction (factor A-H) had negligible negative effect on antibiotic production in fermentation (-0.25). Furthermore, tryptone had the highest contribution in the fermentation process (48.04%), while peptone (factor C) and tryptone-peptone interaction (factor AC) had no effect or contribution on the fermentation process (0.00).

Insert Table 4

Table 5 describes the summary of Analysis of Variance (ANOVA) parameters for the GP-2B fermentation optimization model, with the two-factorial model had an F-value of 12.41. Tryptone (A) had the highest F-value (40.09), while it was lowest for NaCl (D) and agitation (F) (0.2). Similarly, low Prob>F rate of 0.01%,

0.07%, 0.239% and 0.28% were determined for tryptone (A), Model, pH (B) and tryptone-agitation (AF) respectively, while NaCl and agitation had high Prob> F rate of 66.18%.

Insert Table 5

Fig. 1 represents the half-normal plot of responses obtained in the two-factorial model which shows the factors that are important in the fermentation process based on deviation, and it was observed that tryptone, tryptone-agitation interaction, dextrose and agitation speed respectively exhibited significant deviation from the linear plot.

Insert Fig. 1

Figs. 2-4 represent diagnostic activities of the two-factorial system to determine ifResponse (antimicrobial activity) plotted fits the model. The Normal Plot of Residuals (Fig. 2) described linear plot with most of the values along the straight line, Plot of Residuals vs Predicted values (Fig. 3) described most of the obtained values (over 80%) around the median line, while the Box-Cox plot for Power Transformation (Fig. 4) describes a Box-Cox value, $\lambda = 1$ (blue line).

Insert Figs. 2-4

Fig. 5A-B further describes both the 2D plot of interaction between tryptone and agitation, and tryptone and pH on antimicrobial activity. Intersecting linear lines were observed between tryptone and agitation, while parallel linear lines were observed between tryptone-pH interactions. Fig. 6 described the 3D response surface plot of interaction at optimum antimicrobial activity. A curved response surface was observed with highest activity at the upper left-hand corner of the box corresponding to 25 g/L of tryptone and 150 rpm agitation. Lowest bioactivity was observed at the lower left-hand side of the box which corresponds to 15 g/L and 150rpm tryptone and agitation respectively.

Insert Figs. 5 and 6

Fig. 7A-B described the concentration of pure compound obtained based on purified GP-2B peak height when fermentation proceeded at both predicted and optimum factor values respectively. It was observed that the peak area of GP-2B purified from optimum fermentation (3814 mAU; 3.143×10^8) was higher than that obtained for GP-2B purified from normal fermentation (3619 mAU; 2.869×10^8).

Insert Fig. 7

Discussion

Fermentation optimization is important in an industrial environment because it identifies the optimal parameters required for product formation while also reducing cost. Critically, optimization helps to identify, in an efficient way, influential variables as well as finding the correct settings to optimize the response (Ruttimann and Wegener, 2015). Furthermore, use of design of experiment (DOE) statistical tool

for optimization varies several factors simultaneously and is more efficient in studying two or more factors (Uy and Telford 2009; Bouchekara et al. 2011). DOE tools analyze the effect of experimental factors simultaneously and altering independent factors' values to determine their effect on the dependent response (Montgomery, 2005). It also aids in determining significant factors that the response, and how such factors affect the response (Wu and Hamada 2009; Montgomery 2005). The factorial model is a DOE tool is a widely accepted economic approach to optimization by various authors (Box et al. 2005; Myers et al. 2009; Wu and Hamada 2009). It can provide statistical information for identifying important factors and factor-interactions, and also predict optimal nutritional and environmental combinations for increased antibiotic production in fermentation broths (Jaynes et al. 2013).

The model F-value of 12.41 implies that the two-factorial model is significant because there is only a 0.07% chance that the model's F-value could be due to noise in the experiment (Montgomery 2005). Similarly, values of Prob > F less than 0.05 for factors A (tryptone), B (pH), E (dextrose) and AF (tryptone-agitation interaction) indicate that these model factors are significant in the production of GP-2B by *G. candidum* OMON-1. In the same vein, deviation of factors A, AF, E and B in the half-normal plot shows that they are the important factors in the fermentation system, with tryptone the most important factor (> 90% probability). Checking for errors in the two-factorial model used, observation of response values around the straight line in the normal plot of residuals means there is insignificant error in the model, while observation of over 80% of values around median line also describes a good model. The Box-Cox plot (λ value) indicates if transformation is needed in the response values processed, and λ value of 1 indicates that response values do not need to be measured on a different scale (e.g. log of the response) to further improve the model. Furthermore, concentration of GP-2B produced at optimized conditions higher than that produced under predicted condition agrees with the effectiveness of the model for optimization.

Conclusively, fermentation for GP-2B production was successfully optimized in this study using the two-factorial model in DE software. Important factors in the fermentation process were successfully determined while those with negative influence or no influence on fermentation were also identified. This eliminates unfavourable conditions while maximizing important positive factors towards formulation of an optimized fermentation medium which should be considered in future studies.

Declarations

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Competing Interest

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Omeike S.O. and Kareem S.O. contributed to study conception and design. Material preparation, data collection and analysis were performed by Omeike S.O. and Bamigbade G.B. The first draft of the manuscript was co-written by Omeike S.O. and Kareem S.O. All authors read, commented on draft and approved the final manuscript.

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Tables

Table 1: Schematic representation of the trade-off table used for process optimization

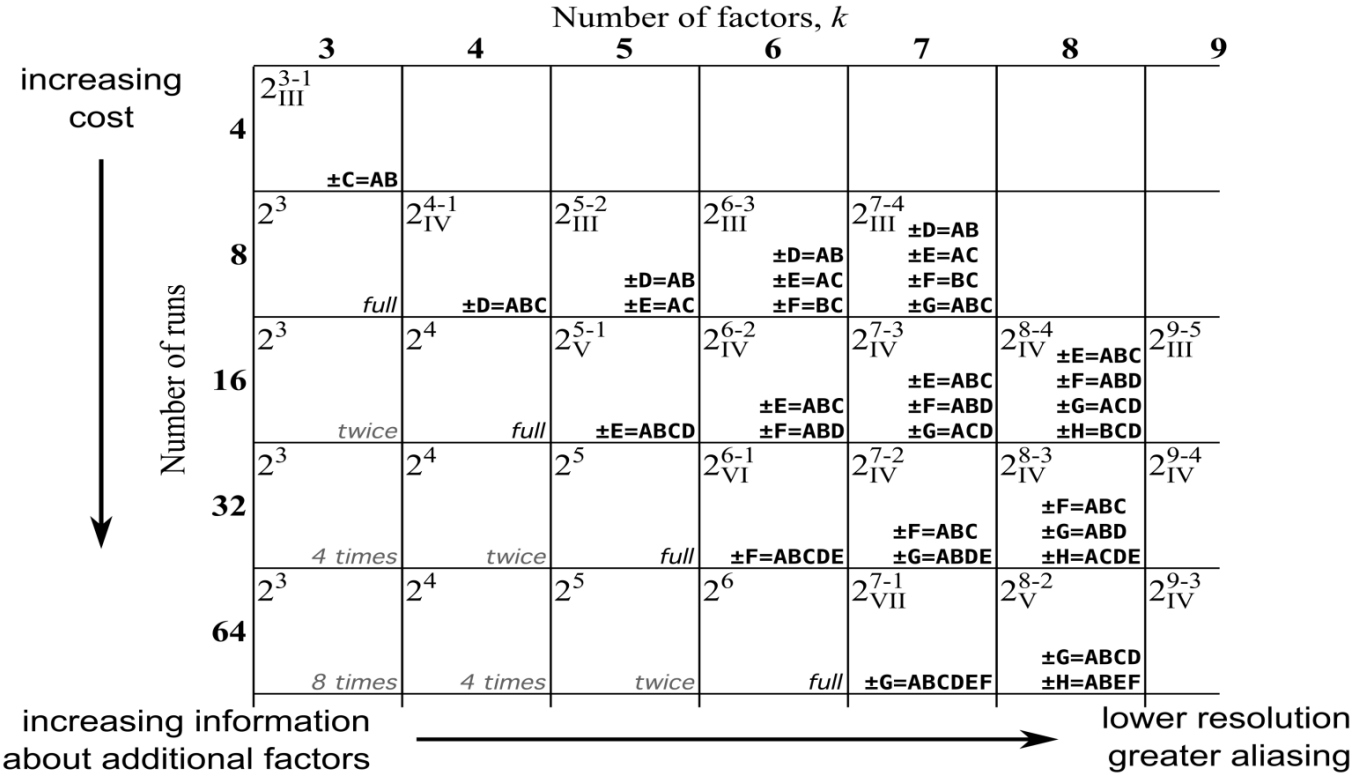


Table 2: Coded value Experimental design of the 8-factor optimization process for increased antibiotics production by *Geotrichumcandidum*OMON-1

Std	Run	Block	Factor 1 A:Tryptone g/L	Factor 2 B:pH	Factor 3 C:Peptone g/L	Factor 4 D:NaCl g/L	Factor 5 E:Dextrose g/L	Factor 6 F:Agitation rpm	Factor 7 G:Fermentor v %	Factor 8 H:K2HPO4 g/L	Response 1 Inhibition Zone mm
11	1	{ 1 }	-1.000	1.000	-1.000	1.000	-1.000	1.000	1.000	-1.000	
12	2	{ 1 }	1.000	1.000	-1.000	1.000	-1.000	-1.000	-1.000	1.000	
2	3	{ 1 }	1.000	-1.000	-1.000	-1.000	-1.000	1.000	1.000	1.000	
16	4	{ 1 }	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
10	5	{ 1 }	1.000	-1.000	-1.000	1.000	1.000	-1.000	1.000	-1.000	
7	6	{ 1 }	-1.000	1.000	1.000	-1.000	-1.000	1.000	-1.000	1.000	
6	7	{ 1 }	1.000	-1.000	1.000	-1.000	1.000	-1.000	-1.000	1.000	
5	8	{ 1 }	-1.000	-1.000	1.000	-1.000	1.000	1.000	1.000	-1.000	
15	9	{ 1 }	-1.000	1.000	1.000	1.000	1.000	-1.000	-1.000	-1.000	
4	10	{ 1 }	1.000	1.000	-1.000	-1.000	1.000	1.000	-1.000	-1.000	
8	11	{ 1 }	1.000	1.000	1.000	-1.000	-1.000	-1.000	1.000	-1.000	
9	12	{ 1 }	-1.000	-1.000	-1.000	1.000	1.000	1.000	-1.000	1.000	
3	13	{ 1 }	-1.000	1.000	-1.000	-1.000	1.000	-1.000	1.000	1.000	
1	14	{ 1 }	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	
14	15	{ 1 }	1.000	-1.000	1.000	1.000	-1.000	1.000	-1.000	-1.000	
13	16	{ 1 }	-1.000	-1.000	1.000	1.000	-1.000	-1.000	1.000	1.000	

Table 3: Real value Experimental design of the 8-factor optimization process for increased antibiotics production by *Geotrichumcandidum*OMON-1

	Std	Run	Block	Factor 1 A:Tryptone g/L	Factor 2 B:pH	Factor 3 C:Peptone g/L	Factor 4 D:NaCl g/L	Factor 5 E:Dextrose g/L	Factor 6 F:Agitation rpm	Factor 7 G:Fermentor v %	Factor 8 H:K2HPO4 g/L	Response 1 Inhibition Zon mm
	10	1	Block 1	25.00	5.50	3.00	25.00	5.00	120.00	60.00	2.00	15
	4	2	Block 1	25.00	8.50	3.00	10.00	5.00	150.00	40.00	2.00	17
	1	3	Block 1	15.00	5.50	3.00	10.00	2.00	120.00	40.00	2.00	16
	15	4	Block 1	15.00	8.50	8.00	25.00	5.00	120.00	40.00	2.00	14
	11	5	Block 1	15.00	8.50	3.00	25.00	2.00	150.00	60.00	2.00	12
	3	6	Block 1	15.00	8.50	3.00	10.00	5.00	120.00	60.00	4.00	13
	6	7	Block 1	25.00	5.50	8.00	10.00	5.00	120.00	40.00	4.00	16
	13	8	Block 1	15.00	5.50	8.00	25.00	2.00	120.00	60.00	4.00	15
	9	9	Block 1	15.00	5.50	3.00	25.00	5.00	150.00	40.00	4.00	12
	7	10	Block 1	15.00	8.50	8.00	10.00	2.00	150.00	40.00	4.00	12
	8	11	Block 1	25.00	8.50	8.00	10.00	2.00	120.00	60.00	2.00	16
	5	12	Block 1	15.00	5.50	8.00	10.00	5.00	150.00	60.00	2.00	12
	14	13	Block 1	25.00	5.50	8.00	25.00	2.00	150.00	40.00	2.00	21
	12	14	Block 1	25.00	8.50	3.00	25.00	2.00	120.00	40.00	4.00	16
	2	15	Block 1	25.00	5.50	3.00	10.00	2.00	150.00	60.00	4.00	19
	16	16	Block 1	25.00	8.50	8.00	25.00	5.00	150.00	60.00	4.00	14

Table 4: ANOVA parameters of the factorial design used for optimization of GP-2B production

Factors	Standardized Effects	Sum of Squares	% Contribution
A	3.5	49	48.04
B	-1.5	9	8.82
C	0	0	0
D	-0.25	0.25	0.25
E	-1.75	12.25	12.01
F	-0.25	0.25	0.25
G	-1	4	3.92
H	-0.75	2.25	2.21
AB	-0.5	1	0.98
AC	0	0	0
AD	-0.25	0.25	0.25
AE	-0.75	2.25	2.21
AF	2.25	20.25	19.85
AG	-0.5	1	0.98
AH	-0.25	0.25	0.25

Table 5: Statistical parameters showing significant and non-significant values in the factorial model used for GP-2B optimization

Source/ Factors	Sum of Squares	Mean Square	F Value	Prob> F	Prob> F (%)
Model	91	15.17	12.41	0.0007	0.07
A	49	49	40.09	0.0001	0.01
B	9	9	7.36	0.0239	0.239
D	0.25	0.25	0.2	0.6618	66.18
E	12.25	12.25	10.02	0.0114	1.14
F	0.25	0.25	0.2	0.6618	66.18
AF	20.25	20.25	16.57	0.0028	0.28

Figures

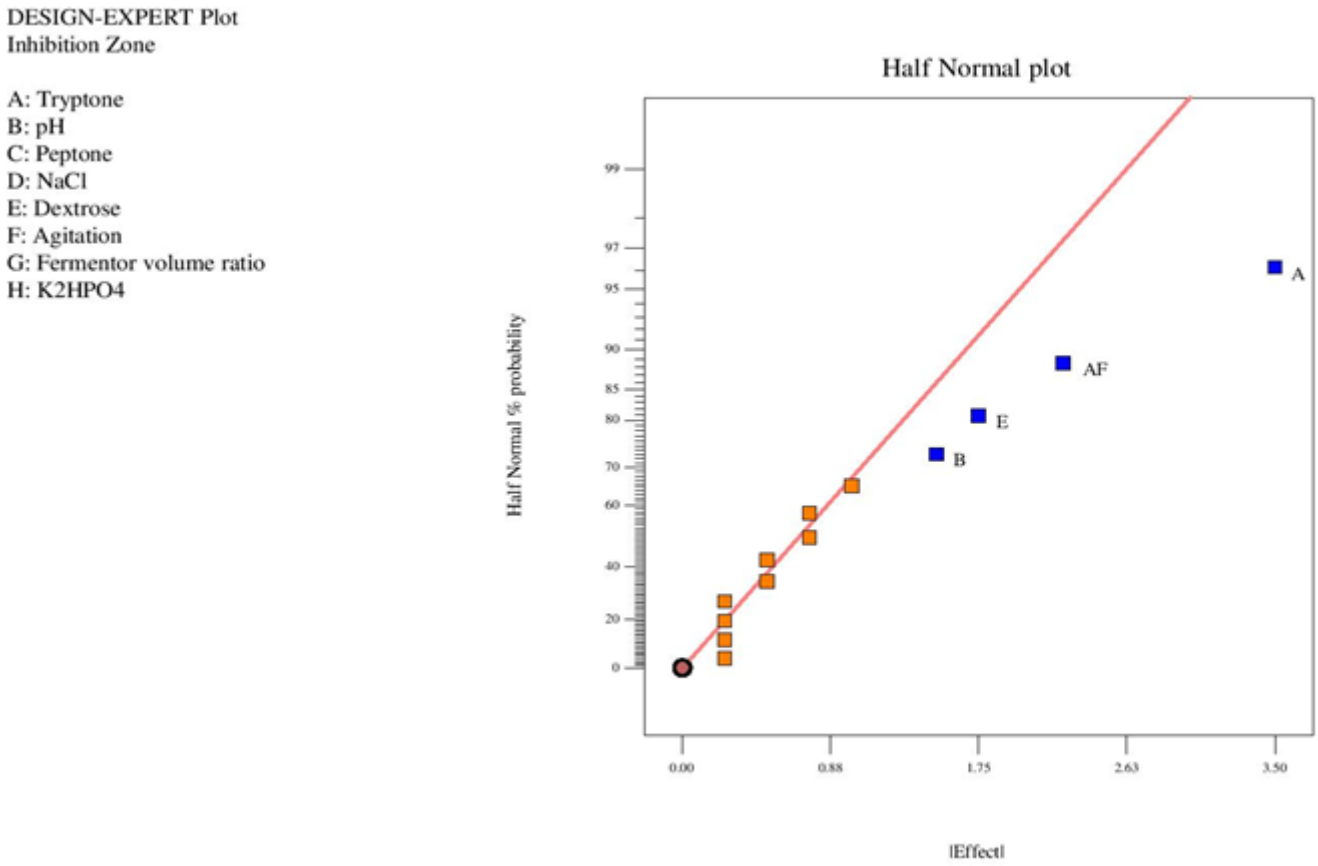


Figure 1

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

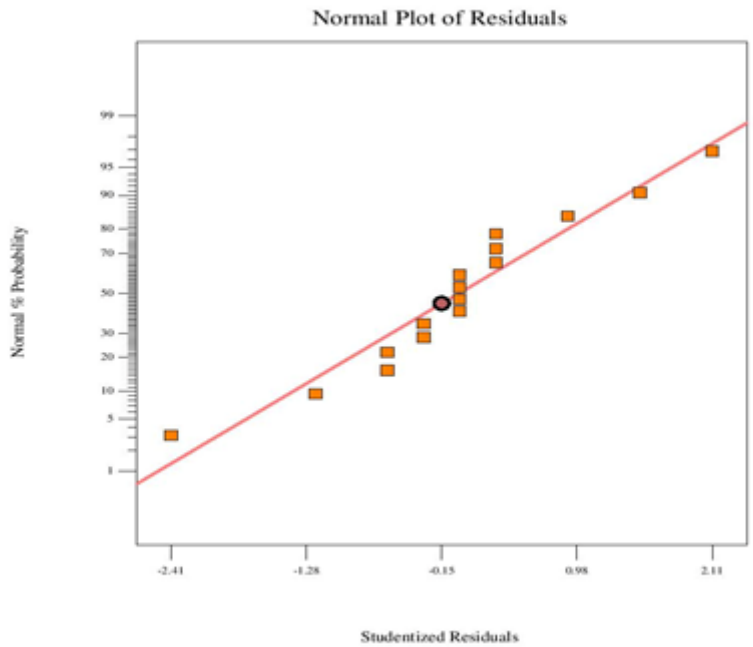


Figure 2

Percentage probability plot diagnostic of the factorial design describing correlation between Normalized residuals and Studentized residuals of the optimization model

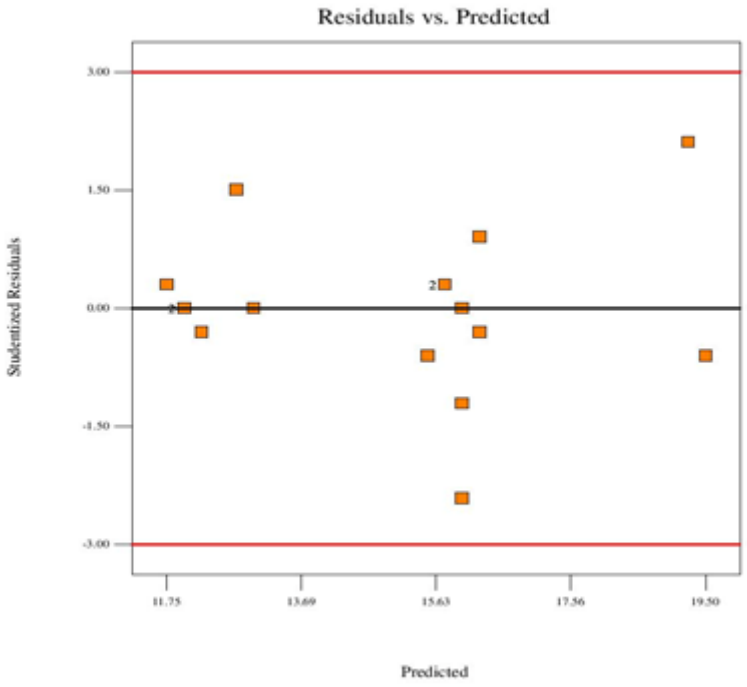


Figure 3

Diagnostic plot of the optimization model based on correlation between actual and predicted Response

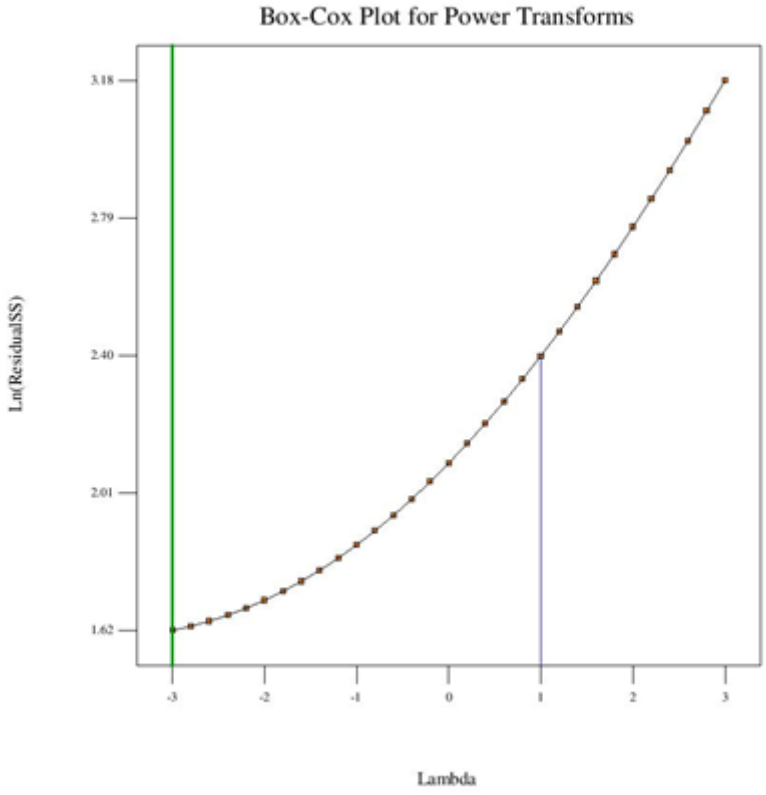
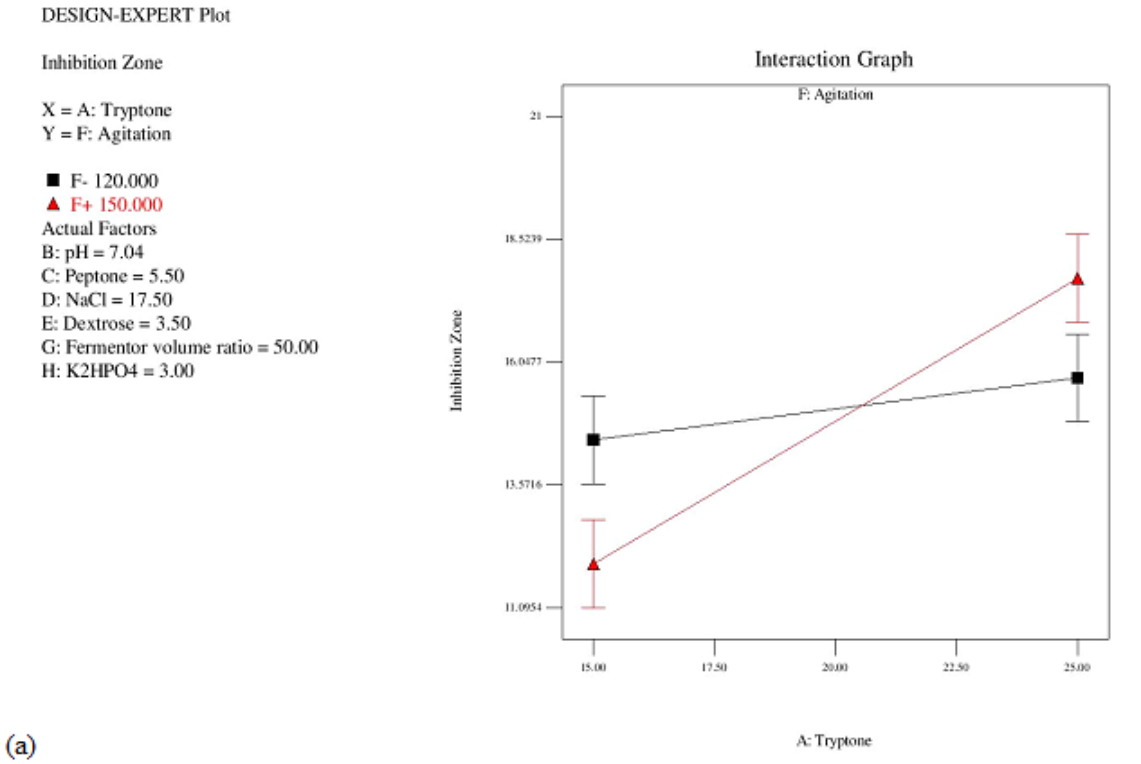
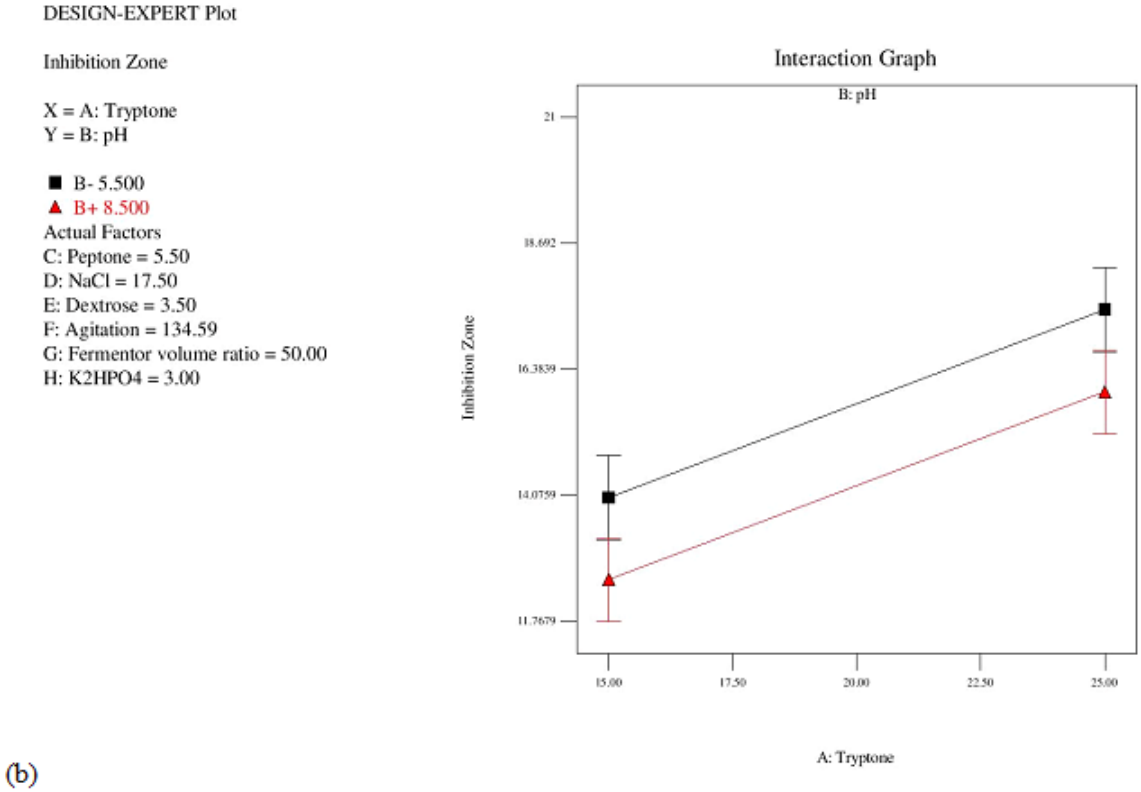


Figure 4

The Box-Cox plot diagnosis of the two-factorial model to determine if transformation of Response data is necessary (Blue line λ = 1 means Response data (ZI) requires no further transformation)



(a)



(b)

Figure 5

2D plot describing the presence or absence of interaction between two factors (a) tryptone and agitation and (b) tryptone and pH. Intersecting lines in (a) describes interaction between the two factors

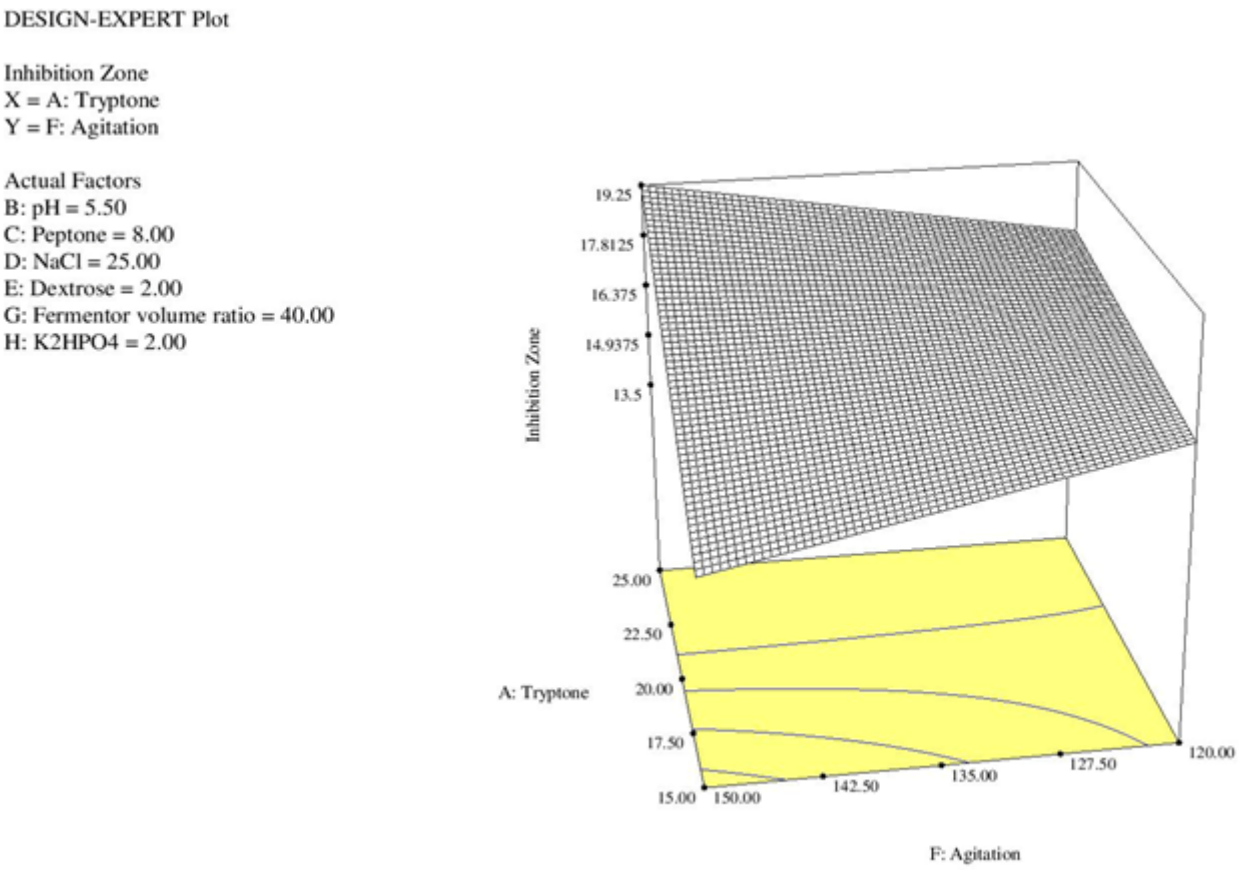


Figure 6

A curved Response surface plot of optimized fermentation conditions

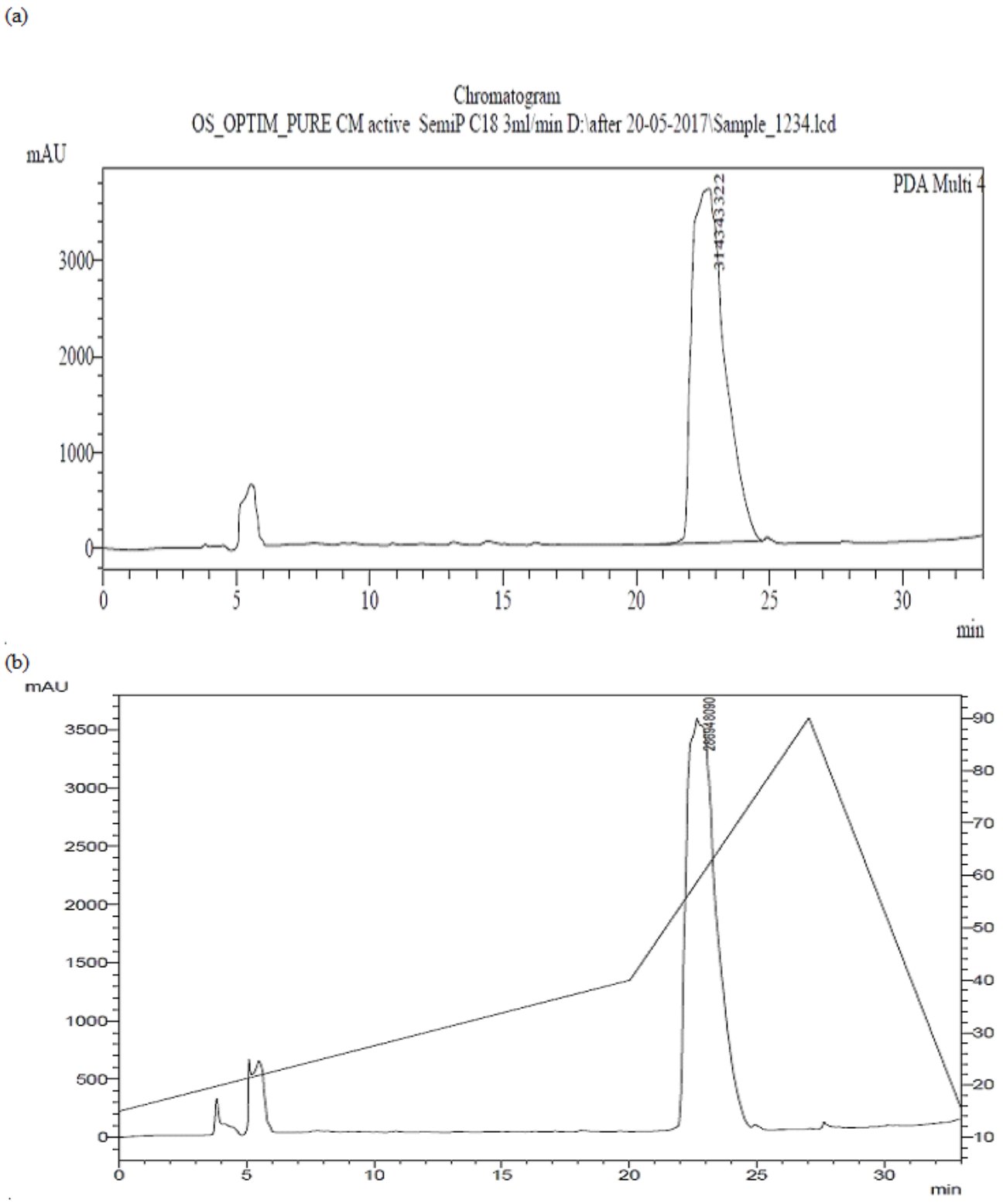


Figure 7

HPLC chromatogram of purified GP-2B produced under (a) optimized, and (b) normal fermentation conditions measured to determine antibiotic activity.