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Response Surface Method for the Optimization of *Bacillus* sp. strain ZEID-14 Growth on Acrylamide as a Nitrogen Source

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ABSTRACT

Acrylamide contamination in food is mainly from raw material of plant-based origin. Acrylamide biodegradation by soil bacteria is an important remediation process. *Bacillus* sp. strain ZEID-14, which had previously been identified and exhibited the ability to break down amides, was examined further to determine the crucial parameters that contribute to the optimum growth of acrylamide. The Box-Behnken design was used to optimize the previously identified three significant components (pH, incubation time and acrylamide concentration). The model was supported by the diagnostic plots including the half-normal, Cook's distance, leverage vs runs, residual vs runs, Box-Cox, DFFITS, and DFBETAS. Predicted optimal conditions were determined using "Numerical Optimisation" toolbox of the Design Expert software. Two optimal conditions were tested. The model predicted a maximum growth of 10.686 (95% C.I., 10.458 to 10.913) which was verified through experimental results with a growth of 11.257 (95% C.I., 11.051 to 11.462) with the actual results being near to the predicted values but was significantly higher than the predicted values. The second numerical optimization gave a solution with a predicted maximum growth of 9.305 Log CFU/mL (95% C.I. from 9.011 to 9.614) which was verified through experimental results with a growth of 9.978 Log CFU/mL (95% C.I. from 9.830 to 10.126) with the actual results were also significantly higher than the predicted values. The RSM exercise gave far better growth on acrylamide than OFAT with a higher response of about 2 log CFU/mL unit indicating the utility of RSM over OFAT in the optimization of growth of this bacterium on acrylamide.

INTRODUCTION

The Maillard reaction is a cooking process that can result in the formation of acrylamide, a substance that is both carcinogenic and neurotoxic. Acrylamide can be created when meals that are heavy in carbohydrates are cooked at a high temperature. As a byproduct of the Maillard reaction, acrylamide may be found in foods that are rich in carbohydrates. The Maillard process is triggered whenever carbohydrates and amino acids are brought together. This is the primary pathway by which acrylamide can be produced [1]. On the other hand, acrylamide may be made from other carbonyl compounds [2]. On the other hand, acrylamide may be produced from a variety of other carbonyl compounds [2]. Cattle and fish both perished in Sweden and Norway as a direct result of acrylamide contamination in streams in the surrounding area. In the manufacturing of adhesives, plastics, and printed materials, as well as for the treatment of drinking water the most common application for acrylamide is in the formation of polyacrylamide, abbreviated as PAM. As of the year 2005, commercial polyacrylamides are frequently tainted by

the poisonous monomer of acrylamide, a situation that has had a substantial impact on our food supply chain as a direct result of the widespread use of these substances. The Roundup herbicide, which pollutes agricultural land with acrylamides, includes polyacrylamide in a concentration of thirty percent. Acylamide must be remediated by a biological process in order to address this problem, which must be addressed in order to be resolved [3].

In spite of the fact that Spencer and Schaumburg [4] discovered that acrylamide exposure in laboratory animals led to the development of cancer, it is still unknown whether or not this is also the case in humans who are subjected to the chemical. Acrylamide has been demonstrated to bind to DNA and mouse protamine at all phases of the spermiogenic process in mice, leading researchers to conclude that it is responsible for genetic damage [5]. Acrylamide exposure in rats has been linked to an increased risk of perinatal mortality, mutagenicity, clastogenicity, endocrine-related cancers, and male reproductive toxicity, according to research conducted on the subject [6]. According to Yang et al. [7], acrylamide may be mutagenic to the Salmonella strains TA100 and TA98 when exposed to it. Following administration of the medication, an increased number of chromosomal aberrations were seen in the bone marrow of mice that had received an intraperitoneal injection of acrylamide at a concentration of 50 mg/kg. The cases of chromosomal aberrations in mice lymphocytes that received intraperitoneal dosages of acrylamide up to 125 mg/kg did not substantially enhance when the acrylamide was provided. This finding was seen when the acrylamide was administered intraperitoneally [8]. The reproductive systems of male rats are also affected as a result of histological abnormalities in the seminiferous tubules that are induced by acrylamide. These histological abnormalities are caused by the chemical. It is possible that acrylamide will cause a burning feeling or a rash to occur if it is breathed in or absorbed through the skin. An overactive sweating gland, a sluggish physique, and trembling in the tongue are all signs that something is wrong with the neurological system [4].

Acrylamide, which has a high water solubility, has the ability to be absorbed via the skin, the lungs, the digestive system, and even the placental barrier. It is possible to assess the amount of acrylamide that the general public is exposed to as a result of their profession by measuring the amount of acrylamide adducts that are present in haemoglobin. As per the data, a total of 41 workers at an acrylamide production factory displayed neurotoxicity issues associated with the biomarker haemoglobin adducts. The level of haemoglobin adducts rose in workers from a Chinese plant that manufactures acrylamide, indicating that the workers had been subjected to extremely high levels of acrylamide [9]. As a result of acrylamide pollution in the water supply of the country, many cases of acute acrylamide poisoning have been documented in Japan. These occurrences have occurred in multiple people. Igisu et al. [10] made the discovery that an acrylamide concentration that was as high as 400 mg acrylamide/L was found in a well that had been polluted by a grouting operation that was 2.5 meters deep. This finding was published in Igisu et al., 1975. According to the findings, five people who drank poisoned drinking water experienced symptoms such as truncal ataxia and disorientation. These symptoms are assumed to be the result of acrylamide poisoning, which was produced by drinking the water. Recent studies have shown that plant-derived antioxidants and phytochemicals can be used to reduce the toxicity of acrylamide in humans and animals [11,12].

In order to get acrylamide poisoning, it has to either be breathed in contaminated air or consumed. This compound may be absorbed by the mucous membranes in the lungs, the digestive system, or the skin, depending on how it comes into contact with the body. On the other hand, it will be eliminated from the body via the urinary system. The facilitation of the acrylamide impact is contributed to by the presence of acrylamide in biological fluids as well as the distribution of acrylamide throughout the body. Despite the fact that it is rapidly metabolized and eliminated from the body after exposure, acrylamide nevertheless poses a risk to persons and employees due to the high degree of reactivity it exhibits against proteins [13–15]. The use of microorganisms for acrylamide remediation is gaining attention since in certain cases such as in soil, the matrx is complicated and will be more costly to remove acrylamide using physicochemical methods. Microorganisms that have been reported as capable of utilizing acrylamide include the yeast *Rhodotorula* sp. [16], the fungi *Aspergillus oryzae* [17] and bacteria [18–27], which present a far larger in numbers than yeast or fungi.

In fundamental research, the planning of experiments frequently takes a "intuitive" approach. Experiments in biology have always been conducted on a "one factor at a time" basis (OFAT). In this method, all of the factors and variables are kept the same, with the exception of the thing that is being investigated, and that thing's output is analyzed. This strategy has the potential to disclose significant "major effects" in biological research, however the interactions between components will result in incorrect words. Due to the intricacy of the process, regulating a large number of input factors is required in order to get optimal results. Even though numerous research on process optimization have employed OFAT to increase responsiveness, it will be important to understand the connections between components in order to optimize increasingly complicated procedures. Using an OFAT strategy, one axis would be optimized first, followed by the other. If, by some stroke of good fortune, the beginning of the investigation was reasonable in the first place, then the global maximum that maximizes the output variable may be identified. One thing to keep in mind, though, is that there is a possibility that the search was terminated at a local maximum or pseudo-optimum.

The results of an experiment could be noisy, and there might be a lot of intriguing data coming in. In situations like this, the selection of data points may be tweaked to optimize the amount of relevant information obtained through the use of statistically based experimental design, which can result in significantly more interesting data. The basic issue structure utilized by the DOE takes into account a number of aspects that are thought to impact process output. The design of the experiment that is ultimately selected is determined by which of several feasible designs yields the most amount of expected information. This criterion is frequently determined according to the precision or accuracy of the fitted model's estimates of the variable input or its forecasts of the output variable. In most cases, the dynamics of this partnership are a mystery. In its place, a model of the system is offered to characterize the system's output based on the elements that are influential. This so-called "response surface" model takes continuous inputs and, more often than not, takes the form of a first-order (linear) or second-order (quadratic) polynomial. Plackett-Burman experimental design is a common screening approach that is used to uncover key aspects early, when comprehensive system knowledge is typically lacking. This method was named after its creators, Plackett and Burman. It was developed in 1946 by statisticians Robin L. Plackett and J.P. Burman with the goal of identifying active variables with the fewest feasible experiments. Two-factor interactions can be

confusing to major effects when using a Plackett-Burman design. When there is little to no potential for two-way interaction, these are the kinds of designs that should be employed. In two-level multi-factor studies including more than four components, the Plackett-Burman design is useful for facilitating the detection of big main effects. PB does not check to see if the effect of one component is dependent on the influence of another factor, and because it is the smallest design, not enough data has been gathered to determine the importance of these effects are. For a more effective screening solution, think about doing an experiment with two factors or using a factorial design. Using this method results in a more accurate estimation of the optimal condition and calculates the interconnections between significant cultural factors. The response surface methodology is a more stringent approach to experimental point placement and response analysis (RSM). It is better to use the Taguchi or complete factorial design when there are not many elements that impact the design. When there are several aspects that impact a reaction or design, the response surface technique is beneficial.

It is essential that RSM be able to design and analyze trials in a sequential fashion. The person conducting the experiment will make educated guesses as to which factors will impact the response. An experiment performed during the preliminary screening phase can assess the significance of each element. This brings the total number of experimental components down, which in turn brings the total number of needed runs down as well. It is up to the fitted model to assess whether or not the data that have been collected come even near to a perfect answer. This enables an exploration into the issue space as well as the determination of the next area to experiment in. The collection of data points from a wide range of locations helps construct a process space perspective. During the last iteration of the experimentation process, the goal is to produce a model that more correctly mimics the actual function while operating within a constrained issue space. Each trial enhances our process model. Following a preliminary experiment, we now have the foundational components of the model. The mathematical modeling of biological systems can assist in answering difficult biological problems and understanding behavior that is counterintuitive. As was stated, it is essential to meticulously gather data from experiments. In order to generate a prediction model utilizing RSM's statistical analysis, experiments need to be carried out.

The response surface method, also known as RSM, is a statistical method that consists of several phases to accomplish the following: selecting an appropriate experimental design; determining the efficient levels/optimum points of numerous independent parameters; forecasting and validating model equations; and creating contour plots and response surfaces [28]. RSM has been used effectively to enhance biodegradation, biotransformation, and bioremediation processes such as the degradation of cyanide [29], phenol degradation [30], caffeine degradation [31], hexavalent chromium and molybdenum reduction to a less toxic form [32]. RSM optimizes optimum yield within a defined range of process, where the range is calculated by using mathematical and statistical softwares such as Design Expert® or MATLAB®. RSM's goal is to get the best possible results with the available resources. The ideal response, which can be seen visually, is depicted by 2-D and 3-D contour plots, which also indicate the influence of the levels of two factors and the potential of interactions by setting optimal concentrations for other parameters. Optimal responses may be viewed visually [33].

Two types of optimization methods are popular, which are Box Behnken (BB) and Central Composite Design (CCD) [34,35]. In this study, the Box-Behnken approach will be selected for the optimization of *Bacillus* sp. strain ZEID-14 growth on acrylamide due to a more compact experimental runs needed compared to the CCD.

MATERIALS AND METHODS

All chemical reagents were generated in large quantities and utilised in the analysis in their unpurified forms, and all of the materials used in this study were of analytical grade. In all cases, unless otherwise noted, experiments were carried out in triplicate.

Growth and maintenance of acrylamide-degrading bacterium

The bacterium was previously isolated from Sudan's agricultural soil as a molybdenum-reducing bacterium [36]. Characterization of this bacterium on acrylamide was conducted on minimal salts medium (MSM) supplemented with only acrylamide as the source of nitrogen and glucose as the sole carbon source. Revival of the bacterium from a 16% glycerol stock was carried out by growing overnight the pure culture in 10 mL of nutrient broth. From this, 0.1 mL was added into 45 mL of acrylamide enrichment medium in a 100 mL volumetric flask and the culture was incubated at 150 rpm for 48 h at 25 °C on an incubator shaker (Certomat R, USA). Minimal salt medium (MSM) for growth was supplemented with 0.5 g acrylamide g/L as the sole nitrogen source, glucose 10 g/L as the carbon source, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KH_2PO_4 6.8 g/L (buffering species and source of phosphorous), $FeSO_4 \cdot H_2O$ 0.005 g/L and 0.1 mL of trace elements [3].

The presence of the phosphate in the medium acts as a buffer system, maintaining a pH range that spans from 5.8 to 7.8. Acrylamide was the only source of nitrogen that was employed for the sterilisation process, and PTFE syringe filters with a pore size of 0.45 micron were used. In order to determine the number of bacteria present, samples of one milliliter each were successively diluted in sterile tap water and plated on nutrient agar. The presence of phosphate in the medium acts as a buffer system, maintaining a pH range that spans from 5.8 to 7.8. Acrylamide was filter-sterilized using PTFE syringe filters with a pore size of 0.45 micron. In order to determine the number of bacteria present, samples of one milliliter each were successively diluted.

Optimization study using RSM

RSM is a statistical technique used to develop and improve optimization process to achieve optimal response.[17] In this study, CCD was used as RSM, which is based on three steps such as: first, designing and experimental setup; second, response surface modelling through regression; and third, optimization (Du et al., 2010). The relationship and interrelationship among input variables and the experimental response variable were determined by fitting second order polynomial equation. The equation is given as:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_{ii}^2 + \sum_{i=1}^{k-1} \sum_{j>1}^{k} \beta_{ij} x_i x_j + \text{error}$$

where, y is the estimated response variable, β_0 is the regression constant, β_i is the linear regression coefficient, β_{ii} is the quadratic regression coefficient, β_{ij} is the bi-linear regression coefficient. A three-level, three-factor BBD was employed in this study (**Table 1**). The significant factors from a two-level factorial experiment (published elsewhere) were utilized in this study.

The response was bacterial growth measured as log CFU/mL. The BBD generated 17 experimental runs (**Table 2**) that were randomized to minimize the unpredictable variations in the observed responses due to uncontrolled extraneous factors. The experimental runs include 12 factorial points, and five center points that provide information on the interior of the experimental regions to evaluate the curvature effect.

Table 1. Coded and uncoded levels of the independent variables.

Factor	Name	Units	Mini-mum	Maxi-mum	Coded Low	Coded High	Mean	Std. Dev.
A	pH		6.00	7.50	-1 ↔ 6.00	+1 ↔ 7.50	6.75	0.5303
B	Acrylamide	g/L	0.3000	0.7000	-1 ↔ 0.30	+1 ↔ 0.70	0.5000	0.1414
C	Incubation	days	2.00	4.00	-1 ↔ 2.00	+1 ↔ 4.00	3.00	0.7071

Table 2. Experimental design and results of Box-Behnken for the growth of the bacterium on acrylamide.

Run	Factor 1 A:pH	Factor 2 B:Acrylamide g/L	Factor 3 C:Incubation days	Response 1 Growth Log CFU/mL
1	6.75	0.5	3	10.755
2	6.75	0.3	2	9.279
3	6	0.3	3	7.424
4	6.75	0.5	3	10.546
5	6	0.7	3	7.169
6	6.75	0.7	2	8.925
7	6.75	0.5	3	10.662
8	7.5	0.5	4	8.786
9	7.5	0.5	2	8.954
10	6	0.5	2	7.538
11	6	0.5	4	7.493
12	6.75	0.5	3	10.571
13	6.75	0.7	4	9.013
14	6.75	0.5	3	10.557
15	6.75	0.3	4	9.123
16	7.5	0.3	3	8.587
17	7.5	0.7	3	8.769

All experiments were performed in duplicate and their mean values are reported here. Data were analyzed using Design Expert 11.0, Stat-Ease, Inc (trial version) program including ANOVA to find out the significant factors among these variables.

Statistical Analysis

Values are means ± SD, in triplicate. One-way analysis of variance (with post hoc analysis by Tukey's test) or Student's t-test was used to compare between groups. P-value of < 0.05 was considered was significant.

RESULTS

Box-Behnken experimental design with 3 factors, namely; incubation period (days), acrylamide concentration (g/L) and pH, at 3 different levels (low, medium and high) was employed to investigate the effects on bacterial growth in log CFU/mL as the main response. The produced experimental runs served as the foundation for a series of tests that were carried out. Using the Design-Expert program, mathematical models, including linear, two-factor interaction, and quadratic, were tested for their ability to match the data in order to determine whether or not there was a correlation between the various components and the replies. On the other hand, it is suggested that BB be represented by a quadratic relation, which includes terms that are squared, products of two components, linear terms, and an intercept [37], and this will be used in this study. The design scheme of variables with actual value is illustrated in **Table 3**, along with experimental, predicted values of response and the residuals.

Table 3. Design scheme of variables with experimental, predicted values of response and the residuals.

Run	A: pH	B: Acrylamide (g/L)	C: Incub-ation (days)	Response. Bacterial growth (log CFU/mL)	Predicted response. Log CFU/mL	Residuals
1	6.75	0.5	3	10.755	10.618	0.137
2	6.75	0.3	2	9.279	9.248	0.031
3	6	0.3	3	7.424	7.480	-0.056
4	6.75	0.5	3	10.546	10.618	-0.072
5	6	0.7	3	7.169	7.127	0.042
6	6.75	0.7	2	8.925	8.992	-0.067
7	6.75	0.5	3	10.662	10.618	0.044
8	7.5	0.5	4	8.786	8.811	-0.025
9	7.5	0.5	2	8.954	8.943	0.011
10	6	0.5	2	7.538	7.513	0.025
11	6	0.5	4	7.493	7.504	-0.011
12	6.75	0.5	3	10.571	10.618	-0.047
13	6.75	0.7	4	9.013	9.044	-0.031
14	6.75	0.5	3	10.557	10.618	-0.061
15	6.75	0.3	4	9.123	9.056	0.067
16	7.5	0.3	3	8.587	8.629	-0.042
17	7.5	0.7	3	8.769	8.713	0.056

F-test evaluates the statistical significance of the model, analysis of variance (ANOVA) and P-value of a selected factor is shown in **Table 4**. The results demonstrated that the model is highly significant, which is evident from the F value of 340.53 with a low P-value of <0.0001. The lack of fit p value was not significant which means the model fits well. All factors are significant model terms Computing the correlation coefficient ($R2$: 0.9977, which is closer to unity) and the adjusted correlation coefficient (Adj $R2$: 0.9948), as shown in **Table 4**, verifies the model's reliability. Together, these two coefficients suggest that the model accounts for 99.5 percent of the total variation in response data.

With a difference of less than 0.2 between them, the Predicted R^2 and the Adjusted R^2 were in reasonable agreement with one another. Adeq Precision, of which in scientific terms, refers to the ratio of the amount of signal to the amount of noise in an experiment. It is preferable to have a ratio that is bigger than 4. A sufficient signal was obtained with a value of 51.87. Using this paradigm, one may move more easily across the design space.

The fact that the Lack of Fit p-value was >0.05 suggests that it is not statistically significant in comparison to the pure error. A lack of fit that is not large is considered to be positive because we want the model to be accurate.The predicted growth as the response can be obtained in terms of following coded factors (**Table 5**) and equation in terms of actual factors.

Table 4. ANOVA analysis of the fitted Box-Behnken design.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	23.61	9	2.62	340.53	< 0.0001	significant
A-pH	3.74	1	3.74	485.89	< 0.0001	
B-Acrylamide	0.0360	1	0.0360	4.68	0.0673	
C-Incubation	0.0099	1	0.0099	1.28	0.2949	
AB	0.0477	1	0.0477	6.20	0.0416	
AC	0.0038	1	0.0038	0.4910	0.5061	
BC	0.0149	1	0.0149	1.93	0.2071	
A^2	13.07	1	13.07	1696.24	< 0.0001	
B^2	3.18	1	3.18	413.11	< 0.0001	
C^2	1.86	1	1.86	240.89	< 0.0001	
Residual	0.0539	7	0.0077			
Lack of Fit	0.0221	3	0.0074	0.9262	0.5053	not significant
Pure Error	0.0318	4	0.0080			
Cor Total	23.66	16				

Std. Dev.	0.0878	R^2	0.9977
Mean	9.07	Adjusted R^2	0.9948
C.V. %	0.9679	Predicted R^2	0.9830
		Adeq Precision	51.8660

Table 5. Final equation in terms of coded and actual factors.

Coded growth equation =		Actual Growth equation =	
+10.62		-147.27692	
+0.6840	A	+42.94923	pH
-0.0671	B	+15.56688	Acrylamide
-0.0351	C	+4.07222	Incubation
+0.1092	AB	+0.728333	pH * Acrylamide
-0.0308	AC	-0.041000	pH * Incubation
+0.0610	BC	+0.305000	Acrylamide * Incubation
-1.76	A^2	-3.13173	pH^2
-0.8693	B^2	-21.73375	$Acrylamide^2$
-0.6639	C^2	-0.663850	$Incubation^2$

Table 6 shows the estimated coefficients of the components that were investigated, together with their respective standard errors, confidence limits, and variance inflation factors (VIF). The variance inflation factor, or VIF, is a statistic that determines how much a lack of orthogonality in the design increases the variance of a certain model coefficient. When specifically comparing the standard error for a model coefficient in an orthogonal design to the standard error for the same model coefficient in a VIF design, the standard error for the VIF design is greater by a factor equal to the square root of the VIF. As a rule, a VIF of one is desirable since it ensures that the coefficient is orthogonal to the other model components; in other words, the correlation coefficient is zero.

On the other hand, VIFs that are greater than ten are cause for worry while VIFs that are greater than one hundred are reason for concern since they indicate that coefficients were calculated incorrectly owing to multicollinearity, and VIFs that are greater than one thousand are the result of severe collinearity. The value of the VIF for all variables was found to be 1, which suggests that the regression analysis had a significant amount of multicollinearity. The construction of each component's confidence limit is what determines whether or not the regression coefficient of that factor is significant. Positive coefficient of estimates were found for all of the components that were investigated, with pH having the greatest value, followed by incubation length, and then acrylamide concentration.

Table 6. Coefficients in terms of coded factors.

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	10.62	1	0.0393	10.53	10.71	
A-pH	0.6840	1	0.0310	0.6106	0.7574	1.00
B-Acrylamide	-0.0671	1	0.0310	-0.1405	0.0063	1.00
C-Incubation	-0.0351	1	0.0310	-0.1085	0.0383	1.00
AB	0.1092	1	0.0439	0.0055	0.2130	1.00
AC	-0.0308	1	0.0439	-0.1345	0.0730	1.00
BC	0.0610	1	0.0439	-0.0428	0.1648	1.00
A^2	-1.76	1	0.0428	-1.86	-1.66	1.01
B^2	-0.8693	1	0.0428	-0.9705	-0.7682	1.01
C^2	-0.6639	1	0.0428	-0.7650	-0.5627	1.01

According to the OFAT methodology, these were also key contributing parameters in the development of this bacteria on acrylamide (the findings of which were reported elsewhere). This work was carried out using concentrations of acrylamide that were well within the range that has been reported to be tolerated by the majority of bacteria that degrade acrylamide. Acrylamide concentrations that are greater than 1000 mg/L are normally harmful to microorganisms. The propensity of acrylamide to produce alkylation products with the proteins found in microorganisms is the root of its toxicity. A longer incubation period allows for higher growth, and an incubation time ranging from two to five days for optimal development has been recorded in several acrylamide-degrading microorganisms. Therefore, the outcomes of incubation time are something that should be predicted. The majority of microorganisms that degrade acrylamide thrive in circumstances that are close to neutral, which is consistent with the findings of our study and the patterns that have been found in the published literature.

The perturbation plot (**Fig. 1**) of the model exhibits the comparative effect of all the operational parameters at a particular point in the design space. From the plot, it can be observed that factor A (pH) has the steepest curvature. The perturbation plot reveals the presence of two-factors interactions that implies synergistic effects. Moreover, all quadratic effects depicted a significant negative synergistic effect, (A^2), (B^2) and (C^2), at $p < 0.0001$, and the contributions were negative meaning an increase in these factors were detrimental to the response obtained, which is expected as the effect of pH is highly specific within a narrow range whilst higher concentrations of acrylamide are strongly growth inhibitory.

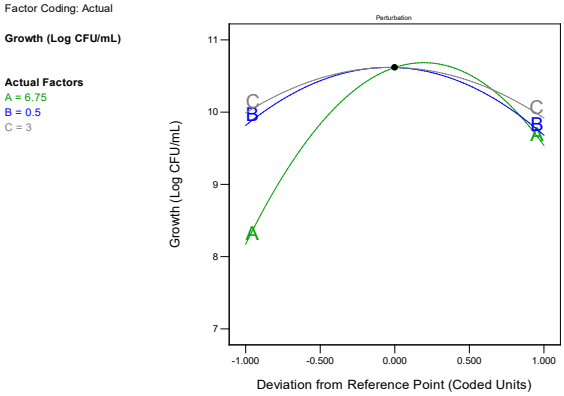


Fig. 1. Perturbation plot of operational parameters obtained for the Box-Behnken design.

In this regard, a half-normal probability plot of the residuals (**Fig. 2**) was constructed and analyzed to ensure the normality assumption. All of the internally studentized residuals values were found to be within 2 and along the straight line, which suggests that there is no requirement for a transformation of the response. This was discovered through research. A good fit may be seen in the graph that compares the actual experimental results to the model's projected values.

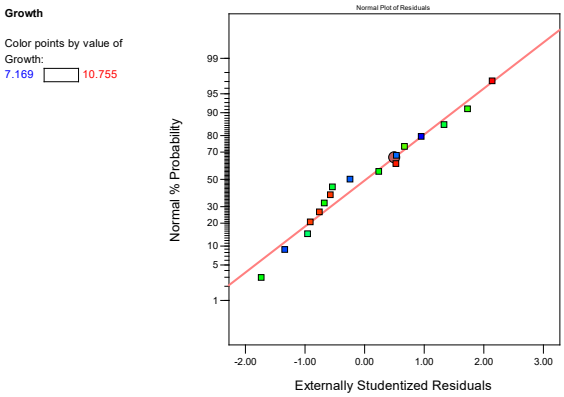


Fig. 2. Half-normal probability plot of the residuals.

The Box–Cox plot, which can be shown in **Fig. 3**, offers a helpful guidance for choosing the appropriate power law transformation based on the value of lambda. Due to the fact that the 95% confidence interval contains a value of 1 that corresponds to the value that was designed into the model, it is not recommended to alter the observed response further in order to suit the model. Recommended transformation [38] is as follows; $\lambda = 1$ no transformation, $\lambda = 0.5$ square root, $\lambda = 0$ natural log, $\lambda = -0.5$ inverse square root and $\lambda = -1$ inverse.

A good agreement can be seen between the anticipated predicted values and the experimental or observed values when looking at the plot of expected vs real data for the Box-Behnken design (**Fig. 4**). The leverages vs run plot shown in **Fig. 5** reveals that all of the acquired numerical values fall within the usual limits range of 0–1. This indicates the possibility that a design point will have an effect on how the model fits. If there is an issue with the data point, such as an unanticipated error, a high leverage point value more than one is considered "bad" since the error has a significant impact on the model. According to the plot of leverages vs runs, there are no data that are higher than the average leverage since data that are higher than this would impact at least one model parameter. A measurement of the response outlier that is equivalent to an experimental trial may be obtained from the plot of Cook's distances (**Fig. 6**). Cook's distances are values that cannot be negative, and the higher these values are, the more significant an observation is. For the majority of researchers, the threshold for determining whether or not an observation can be considered important is three times the dataset's mean value of Cook's D. The values of the Cook's distances are determined to be within a value of 1, and this analysis does not uncover any outliers. The comparison of residuals to run data, as shown in Figure, reveals no signs of serial correlation and hints that the data's features are random by nature.

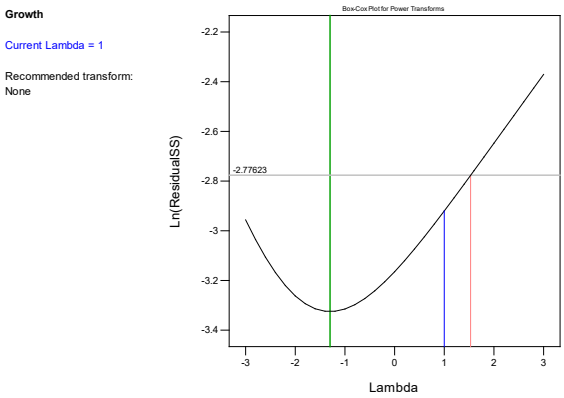


Fig. 3. Diagnostic plot in the form of Box-Cox plot for the Box-Behnken optimization studies.

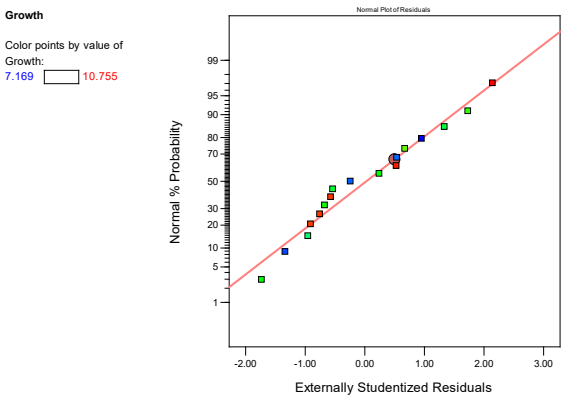


Fig. 4. Diagnostic plot in the form of the expected vs real data the Box-Behnken optimization studies.

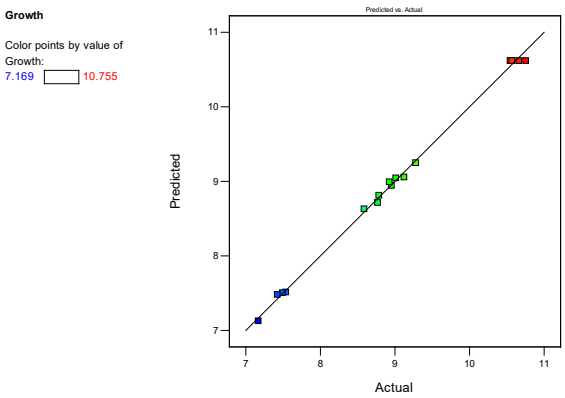


Fig. 5. Diagnostic plot in the form of the predicted versus actual plot for the Box-Behnken optimization studies.

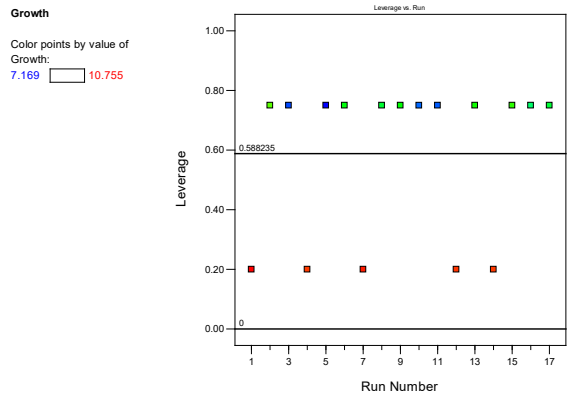


Fig. 6. Diagnostic plot in the form of leverage vs runs for the Box-Behnken optimization studies.

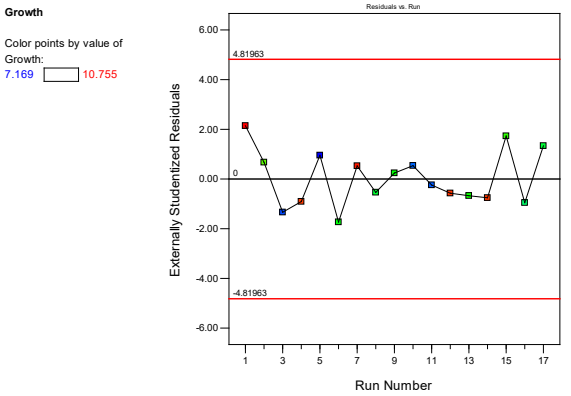


Fig. 7. Diagnostic plot in the form of residuals vs runs for the Box-Behnken optimization studies.

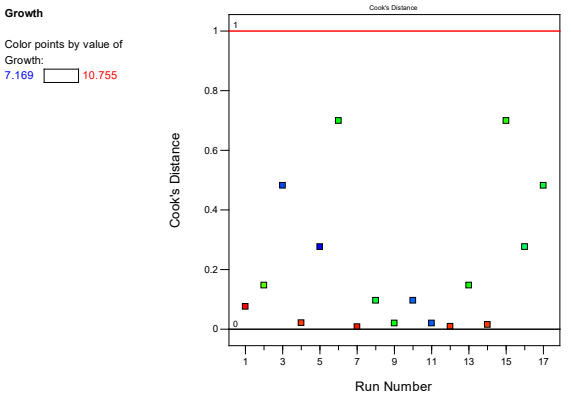


Fig. 8. Diagnostic plot in the form of Cook's distance vs runs for the Box-Behnken optimization studies.

It's not always a problem when influential points are brought up, but it is important to follow up on observations that are marked as extremely influential. A high result on an influence measure could indicate a number of different things, including a mistake in the data input process or an observation that is clearly not typical of the population of interest and so need to be excluded from the analysis. During the process of fitting a model, the inclusion of one or more data points that are sufficiently important might cause coefficient estimations to be thrown off and muddle the model's interpretation.

In the past, before conducting a linear regression, the potential of outliers in a dataset would be evaluated using histograms and scatterplots. This was done before running the linear regression. Both approaches of evaluating data points were subjective, and there was little way to determine how much influence each possible outlier had on the data representing the outcomes. This resulted in the development of a number of quantitative metrics, such as DFFIT and DFBETA. The DFFFITS algorithm assesses how much of an impact each particular example has on the value that was anticipated. It is possible to translate it to the distance according to Cook.

DFFITS, in contrast to Cook's distances, can take either a positive or a negative value. When the value is "0," the point in question is located precisely on the regression line. Leverage is what makes this possible. Mathematically speaking, it is the difference between the expected value with observation and the predicted value without observation. DFFITS is a representation of the externally studentized residual (ti) that has been exaggerated by high leverage points and decreased by low leverage points, as demonstrated by the alternative formula. The plots show the DFBETAS values (**Fig. 9**) and DFFITS values (**Fig. 10**) were within the size-adjusted threshold acceptable range with the exception of an extreme value, which was run 1 (DFBETAS), and runs 6 and 15 (DFFITS) (**Table 2**), which can also be seen in a half-normal probability plot above. However, these values barely were above the acceptable range and in overall do not affect the reliability of the model as a whole.

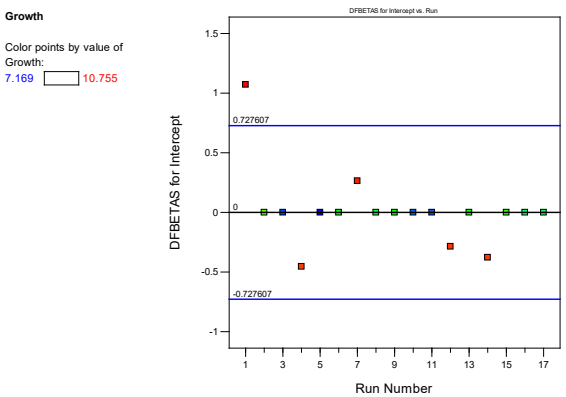


Fig. 9. Diagnostic plot in the form of DFBETAS for intercept vs runs for the Box-Behnken optimization studies.

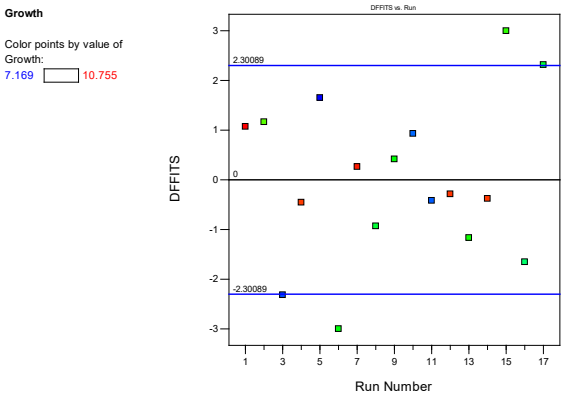
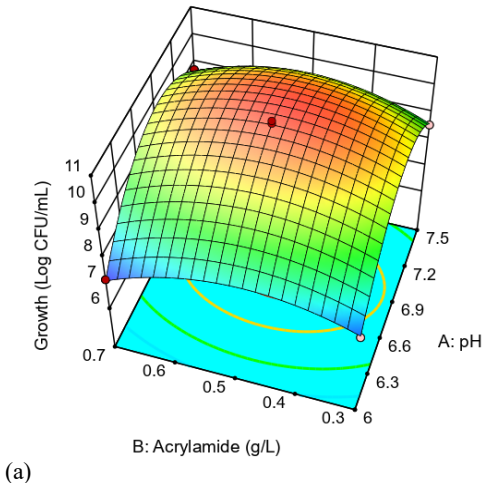


Fig. 10. Diagnostic plot in the form of DFFITS vs runs for the Box-Behnken optimization studies.

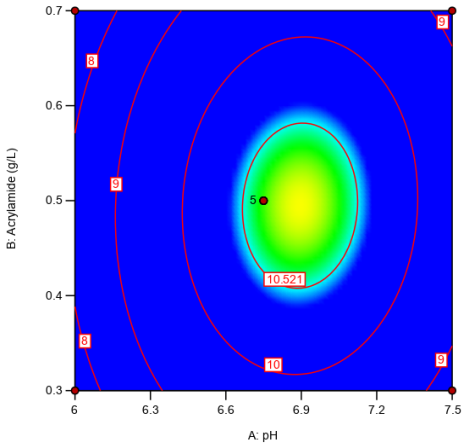
The model equation that was provided by the Design Expert program was used to construct the 3D plots, and they were created so that the interaction between the elements could be studied. Charting the answer against any two independent variables on the Z-axis allowed for the creation of the three-dimensional displays. In the middle of each of these graphs is a single variable that remains constant, while the other two variables are shown to be changing as the experimental range increases. Each figure illustrates the influence of the reciprocal interaction that occurs between two substantial independent elements, while simultaneously maintaining the status quo for the other two components that were investigated. The shape of the plot is determined by how they influence growth and how they communicate with one another, which are three factors that are independent of one another.

When the incubation period was held at midpoint (3 days), varying the pH and acrylamide concentration factors show an elliptical profile indicating a relationship of synergistic interaction (**Fig. 10a**) with a highest response of 10.685 log CFU/mL (95% confidence interval from 10.593 to 10.771) occurring at the studied region between the predicted acrylamide concentrations of 0.408 and 0.582 g/L and between the predicted pH regions of 6.67 and 7.12 (**Fig. 10b**). The elliptical shape of 3D wired frame and contour plot indicates the mutual interaction between independent factor was significant response surface model [38,39]. Within this bordering region (**Fig. 10c**), the 95% confidence interval of the maximum responses overlapped and was deemed not statistically different ($p>0.05$) [40].

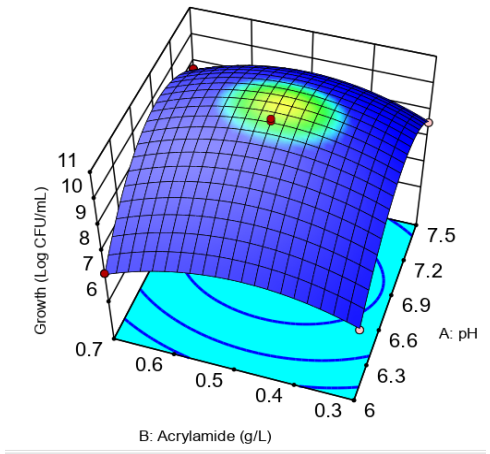
When the acrylamide concentration was held at midpoint (0.5 g/L), varying the incubation period and pH show an elliptical profile indicating a relationship of synergistic interaction (**Fig. 11a**) with a highest response of 10.685 log CFU/mL (95% confidence interval from 10.59 to 10.78) occurring at the predicted pH from 6.6 to 7.12 and incubation period from 2.47 to 3.47 (**Fig. 11b**). Within this bordering region (**Fig. 11c**), the 95% confidence interval of the maximum responses overlapped and was deemed not statistically different ($p>0.05$) [40].



(a)

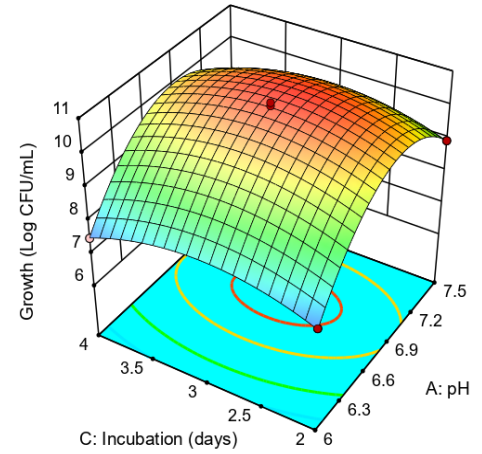


(b)

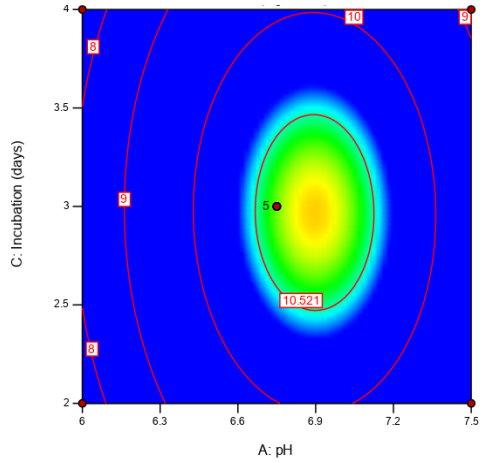


(c)

Fig. 10. The 3D response surface plots of between the factor incubation and acrylamide concentration (a), 95% confidence interval region of optimality visualized as 2D- (b) and 3D- (c) contour plots.



(a)



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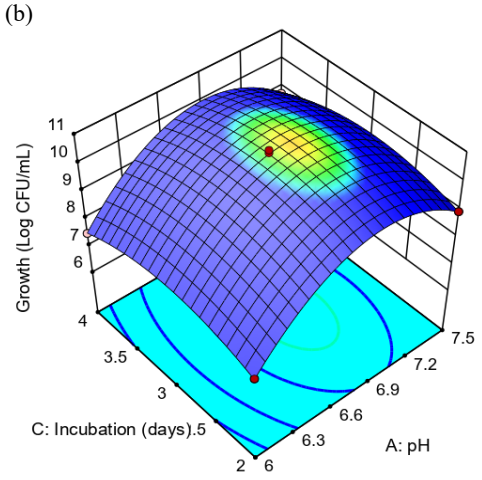
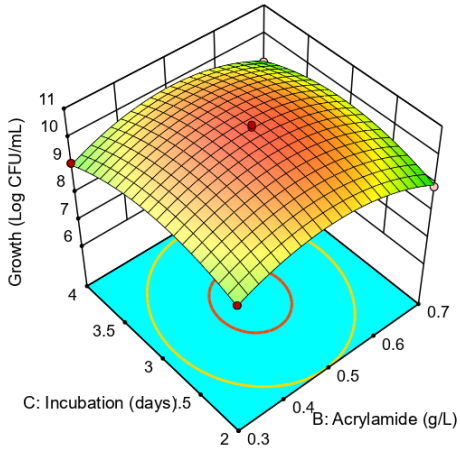


(c)

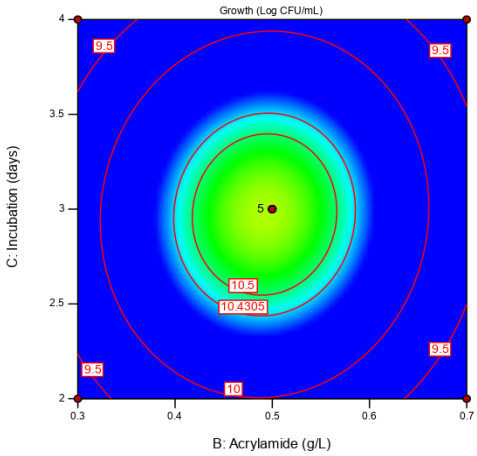
Fig. 11. The 3D response surface plots of between the factor pH and incubation period (a), 95% confidence interval region of optimality visualized as 2D- (b) and 3D- (c) contour plots.

When the pH was held at midpoint (6.75), varying the incubation period and acrylamide concentration factors show a spherical profile indicating weak interaction (**Fig. 12a**) with a highest response of 10.62 log CFU/mL (95% confidence interval from 10.52 to 10.71) occurring at the predicted incubation period of 2.98 and acrylamide concentration of 0.49 g/L.

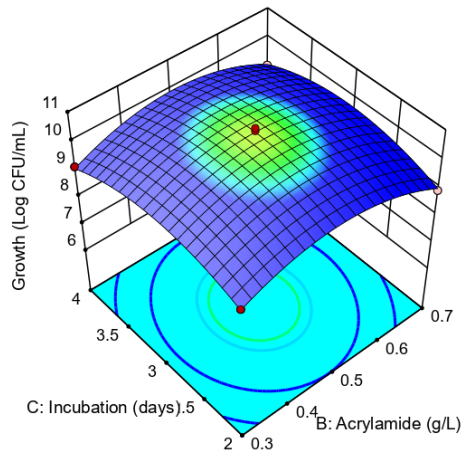
This optimum region also occurs between the predicted acrylamide concentrations of 0.4 and 0.58 g/L and incubation periods of 2.44 and 3.52 days (**Fig. 12b**). Within this bordering region (**Fig. 12c**), the 95% confidence interval of the maximum responses overlapped and was deemed not statistically different ($p>0.05$) [40].



(a)



(b)



(c)

Fig. 12. The 3D response surface plots of between the factor acrylamide and pH (a), 95% confidence interval region of optimality visualized as 2D- (b) and 3D- (c) contour plots.

Verification of BB experimental design of RSM for the growth of the bacterium on acrylamide

Predicted optimal conditions were determined using "Numerical Optimisation" toolbox of the Design Expert software. Two optimal conditions were tested. The first was for finding the optimum growth under the range of factors employed whilst the second was to predict the optimum growth at the highest acrylamide concentration tolerable, which was 0.7 g/L. The predicted value of the dependent variable for both sets of design experiment were suggested with different combinations of the parameter value. **Table 7** shows the solutions for the verification of the first predicted model.

The model predicted the maximum growth of 10.686 (95% C.I., 10.458 to 10.913) which was verified through experimental result with a growth of 11.257 (95% C.I., 11.051 to 11.462) with the actual results were near to the predicted values but was significantly higher than the predicted values. The predicted combination to give the desired maximum response based on requirement of **Table 8** was at pH 6.89, acrylamide concentration of 0.494 g/L and an incubation period of 2.97 days. On the other hand, the predicted combination to give the desired maximum response based on the requirement for the conditions where growth at the highest desirable acrylamide concentration as shown in **Table 9** was at pH 6.92, acrylamide concentration of 0.671 g/L and an incubation period of 3.0 days.

The first solution suggested was run according to the suggested data with the desirability value (**Table 8**). The second numerical optimization gave a solution with a predicted a maximum growth of 9.305 Log CFU/mL (95% C.I. from 9.011 to 9.614), which was verified through experimental result with a growth of 9.978 Log CFU/mL (95% C.I. from 9.830 to 10.126) with the actual results were also significantly higher than the predicted values.

Table 7. Suggested parameter for each variable for maximum growth of the bacterium on acrylamide based on the Box-Behnken design.

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:pH	is in range	6	7.5	1	1	3
B:Acrylamide	is in range	0.3	0.7	1	1	3
C:Incubation	is in range	2	4	1	1	3
Growth	maximize	7.169	10.755	1	1	3

Table 8. Suggested parameter for each variable for maximum growth of the bacterium on maximum acrylamide concentration based on the Box-Behnken design.

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:pH	is in range	6	7.5	1	1	3
B:Acrylamide	maximize	0.3	0.7	1	1	3
C:Incubation	is in range	2	4	1	1	3
Growth	maximize	7.169	10.755	1	1	3

Comparison of optimisation parameters between OFAT and RSM

In comparison, results from OFAT (published elsewhere) and RSM were gathered and compared to each other (**Table 10**). A statistically better and higher response of about 2 log CFU/mL was achieved through RSM optimisation.

Table 9. Verification results between experiments and predicted response.

RSM target solution	Desira-bility	Predicted mean (95%, C.I.) log CFU/mL	Experimental verification (95%, C.I.)	Statistical significant Difference between predicted and experiment
All factors within range, Maximum growth	0.981	10.686 (10.458 to 10.913)	11.257 (11.051 to 11.462)	No significant Difference ($p>0.05$)
Acrylamide concentration maximum, Maximum growth	0.851	9.305 (9.011 to 9.614)	9.978 (9.830 to 10.126)	No significant Difference ($p>0.05$)

Table 10. Comparison of optimum conditions and results obtained between OFAT and RSM for growth of the bacterium on acrylamide.

	OFAT		RSM	
Factors	Optimum value	Max growth (Log CFU/mL)	Optimum value	Max growth (Log CFU/mL)
pH	6.5 to 7.5	9.21	6.89	11.26
Incubation period (d)	3		2.97	
Acrylamide (g/L)	0.5		0.494	

When compared to CCD designs, BB designs often feature fewer design points, and as a result, they are easier on the wallet to maintain and operate when resources are few (Kumar et al., 2019). The Box-Behnken design will never have more than three levels per factor, in contrast to the CCD, which can have as many as five levels per factor [42].In a Box-Behnken design, the design points are located at combinations of the variables that represent the low, high, and midpoints. For example, if the experiment's operating temperature ranges from 10 to 60 degrees Celsius, the lowest temperature point will be 10 degrees Celsius and the highest temperature point will be 60 degrees Celsius, with 30 degrees Celsius serving as the midway. Box-Behnken does not include a limit breaker, also known as an extreme setting, therefore in contrast to CCD, the minimum temperature will not dip below 10 degrees Celsius, and the maximum temperature will not rise over 60 degrees Celsius. When we want our goal scale to stay inside the safe range because of physical or conceptual limits, this feature is quite crucial (e.g., when the temperature starts at zero with no negative range).

Central composite designs are a type of complete fractional factorial design that include center points and are complemented by a collection of axial points [42]. As a result, both its upper and lower limits always fall outside of the limit range of the target scale. Box and Behnken (BB) came up with the idea of an incomplete factorial design with three levels as a time-saving replacement for the labor-intensive full factorial design [37]. Polynomials of the second order are required to be utilized in the modeling process in order to effectively capture linear, quadratic, and interaction effects. Box and Behnken came up with this workable concept in order to cut down on the number of tests that were necessary, particularly in the process of fitting quadratic models [37]. +1, 0 and -1 are the three levels of factorial designs that are used for constructing experiment matrices. In order to get the desired level of accuracy in the end product, the core point has been replicated several times.

There is not an experimental point in this design at which all of the components have their most extreme values. This capability could come in handy during trials in which unfavorable occurrences might take place as a result of harsh conditions. In terms of labor efficiency, the Basic Block Design (BB) is only slightly superior to the Central Composite Design (CCD), but it is noticeably superior to the Full Factorial Design (FFD). The BB has just two key limitations: the number of experimental components must be equal to or more than three, and the BB should not be used to fit equations other than second order polynomials. Both of these requirements must be met for the BB to be valid [38].

CONCLUSION

The Box-Behnken design was adopted in optimization of three factors influencing the growth of the bacterium on acrylamide. These factors include pH, incubation time and acrylamide concentration. The important contributing factors or parameters were analysed using ANOVA, pertubation's plot and other diagnostic plots. The diagnostic plots such as half-normal, Cook's distance, residual vs runs, leverage vs runs, Box-Cox, DFFITS, DFBETAS all supported the model with the exception of two values, which were at Runs 1 and 15, with residual values of 0.14 and 0.07, respectively. Remedies for these outliers in future works include running the experiments in blocks or the outright removal of these outliers. Predicted optimal conditions were determined for finding the optimum growth under the range of factors employed and to predict the optimum , which was 1 g/L. Predicted optimal conditions were determined using "Numerical Optimisation" toolbox of the Design Expert software. Two optimal conditions were tested. The first was for finding the optimum growth under the range of factors employed whilst the second was to predict the optimum growth at the highest acrylamide concentration tolerable, which was 0.7 g/L. In both verification experiment, the actual results were near to the predicted values but was significantly higher than the predicted values. This means that other methods which employ more runs such as CCD or a different optimization approach such as Artificial Neural Network may be employed in the future to close the difference between the model predicted values and actual experimental values. Despite this, the RSM exercise gave far better growth on acrylamide than OFAT, indicating the utility of RSM over OFAT in optimization of growth on acrylamide.

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