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Application Of Factorial Design To The Stress Phenomenon Of *Bacillus Cereus* (Atcc 14579) Growth

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ABSTRACT

Background: A two level (2^3) factorial design of experiment (DOE) was employed to investigate the influence of nutrients concentrations and main operational parameters on the growth of *Bacillus cereus* (ATCC 14579) in a shake flask. The factorial models have been established from experimental design to study the individual and interactions effects toward the response within the selected variables nutrient concentration (4-16gl^{-1}), temperature (30^0C – 42^0C), agitation (140rpm-200rpm) and acclimatization time (24hours-72hours). These were statistically validated using analysis of variance (ANOVA). **Objective:** The present study aimed to use fractional factorial design of experiment to investigate the influence of growth limiting factors to the bacterial growth in a fermenting medium of orbital shaker. **Results:** The results revealed that the model terms were all significant with F-value of 251.07 at (p <0.004). The model term having the most significant effect on the response was nutrient concentration. And the magnitude of the main influence is in the ascending order nutrient concentration > temperature > acclimatization time. The analysis of the experimental response indicated that the interaction of nutrient concentration and temperature had the highest influence on the response. Whereas the interaction effects of nutrient and acclimatization time was found to be statistically insignificant. Based on the R^2 and adjusted R^2 the estimated model terms spell high degree of relationship between observed and predicted values, thus the prediction ability of the models is maintained. **Conclusion:** Although the interaction models terms have significant effects, their levels were only less likely comparable to linear effects. It could therefore concluded that nutrient concentration, temperature and to some extend acclimatization time were four to greatly limit growth at a specific ranges. In general, the predicted value was in reasonable agreement with the experimental data, further confirming the very good prediction ability of the model.

INTRODUCTION

Metabolism of microbial cells refers to as the concept of biochemical activities that enable the organisms to live, function, and replicate in an appropriate chemical milieu (such as a bacterial culture medium) as well as the chemical changes that result during this transformation(Imlay, 2014; Oliveira, Bonatto, Antonio, & Henriques, 2010; Zinkernagel, 2005) These biochemical activities involve the brake down of substrate through oxidation/dissimilation (catabolism) to release energy, as well uptake and utilization(assimilation/anabolism) of organic and inorganic substrates for growth and maintenance (biosynthesis) of bacterial cells(Swain *et al*. 2006; Abada 2014). These respective exergonic (energy-yielding) and endergonic (energy-demanding) reactions are

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catalyzed within the living bacterial cell by integrated enzyme systems. The chemical energy generated by substrate oxidations is conserved by formation of high-energy compounds such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP) or compounds containing the thioester bond (acetyl ~ SCoA) or succinyl ~ SCoA. ADP and ATP represent adenosine monophosphate (AMP) plus one and two high-energy phosphates (AMP ~ P and AMP ~ P~ P, respectively); the energy is stored in these compounds as high-energy phosphate bonds(Li *et al*. 2013; Zinkernagel 2005). *Bacillus cereus* cells are rod-shaped, Gram-positive bacteria that are naturally found in soil and vegetation, growing under mesophilic temperature range of 25^0C-35^0C. Like *P. putida; B. cereus* is able to withstand a Stress and starvation environmental conditions by evolving a set of strategies that allow survival under these harsh conditions. One of such strategy is the formation of stress-resistant endospores and as well uptake of external DNA, which allow the bacteria to adapt by recombination(Dos Santos et al., 2013; Zhong et al., 2014).

Bacterial adjustment to its immediate environment depends on a range of physical and chemical stimulants(Mosquera, González-Jaramillo, Orduz, & Villegas-Escobar, 2014; Munna et al., 2014; Siti, Nurhaslina, & Ku, 2013). Changes in any of the growth influencing factors such as nutrient availability, temperature, pH, aeration, redox potential, water activity, media concentration, and volume was reported to have an effect on bacterial growth rate, which is universally known as stress phenomenon(Munna et al., 2014). Various research studies were conducted to ascertain the relative important of individual effects of these growth factors. However, not much is reported concerning the interaction and complementary effects of these factors influencing the bacterial growth using experimental design of response surface methodology (RSM), in an orbital shaker.

Methodologies used for screening of the medium components fall into two major categories: classical and statistical. The former method which is a conventional approach, involves varying one independent variable at a time while fixing all other at certain level, and is known as "one-variable-at-a-time (OVAT), or one-factor-at-a-time, (OFAT)"(Singh *et al.* 2011; Navaneeth *et al*. 2009; Mosquera *et al.* 2014; Cook 1996). Although, this approach has been found to be useful to observe the individual effects of the media components and process conditions **(**Mandenius & Brundin, 2008; Tabbene, *et al.,* 2009), it is however, lacking in predicting the interaction and interrelationship between the various components influencing the realization of a particular response(s) (Cho, Kim, & Kim, 2009; Curtis, 2011; Navaneeth et al., 2009; Tabbene, *et al.*, 2009; Zhong et al., 2014). But found to be full of bias, as well as tiring and time consuming by having too much experimental runs This is further argued by the fact that variable cannot be studied by varying one factor at a time, as it often does not allow determination of actual optimum level of different components for a particular metabolic activity, as well as enable identification of vital factors affecting a process (Ridzuan, *et al.,* 2016). While the later method which include factorial experiments, partial factorial experiments, provide an alternative approach through screening of a particular process by considering individual/linear and mutual interactions among the variables and give an estimate of the combined effect of these variables on the final result (Onsekizoglu, *et al.,* 2010; Murthy *et al*. 2000; Mizumoto and Shoda, 2007; Pryor *et al.*, 2007(b)). Full factorial design is used to generate data for future response surface optimization studies, which facilitate determination of optimum conditions for any particular process(Hooshyar *et al.,* 2014; Ridzuan et al., 2016).

The relationship between the response and the input is given "Refer to Equ. 1"

$$\eta = f(x_1, x_2 \ldots .xn) + \varepsilon \quad (1)$$

Where η is the response, *f* is the unknown function of response, $x_1, x_2 \ldots .xn$ denote the independent variables, also called natural variables, n is the number of the independent variables and finally ε is the statistical error that represents other sources of variability not accounted for by *f*. Selected independent variables are assign levels based on different ranges each of the coded variables is assigned to a range from -1 to +1, so that they all affect the response more evenly, so the units of the parameters are insignificant. Generally, the polynomial model used a full quadratic equation, and is given "Refer to Equ. 2"

$$Y = \beta 0 + \mathbf{\Sigma}\, \beta iXi + \mathbf{\Sigma}\, \beta iiXi\ 2 + \mathbf{\Sigma}\, \mathbf{\Sigma}\, \beta ijXiXj \quad (2)$$

Where Y is the predicted response, β0, βi, βii and βij are regression coefficients for intercept, linear, quadratic and interaction coefficients respectively and Xi and Xj are coded independent variables. The system of equations given above is solved using the method of least squares (MLS) of multiple regression technique (Bas and Boyaci, 2007). Once the regression coefficients are obtained, the estimated response could be easily calculated using model equation.

The present study was envisaged with an aim to highlight a novel approach of applying factorial experimental statistical design to screen the individual and interactions effects of nutritional composition, acclimatization time and other physical parameters such as agitation speed, temperature, on the growth pattern in a shake flask. The outcomes of the screened factors influencing bacterial growth together with their optimum

values could further be utilized to optimize the process conditions of this isolates in modelling optimum growth conditions.

MATERIALS AND METHODS

Bacterial strain and Growth media:

Bacillus cereus (ATCC 14579) used in the research was obtained from the bacterial stock cultures of the faculty of chemical and natural resources engineering, University Malaysia Pahang. To ensure the authenticity of the bacteria identity, further molecular characterization protocols such as DNA extraction using Polymerase Chain Reaction (PCR), Sequencing and Phylogenetic identification is needed. The growth media (nutrient broth) used was of analytical grade (BD 234000, Merck (Malaysia) Sdn. Bhd) and is made up of peptone (5.0g/l) from meat and meat extract (3.0g/l).

Equipment (for experiment and analysis):

Equipment used for this research studies were auto-clave, H+P- Varioklav Steam Sterilizer ESCO, Shaker (B. Braun, German model), microbiological incubator (Mermmert-Germany/BE 600), UV-Visible Spectrophotometer (U-1800, Hitachi), pH Meter (Mettler Toledo), hot plate magnetic stirrer and analytical Balance (Mettler Toledo).

Preparation of enriched growth media:

Enriched culture media was prepared in accordance with the manufacturer's guidelines. Typically, 8g of nutrient broth was dissolved in 1000ml of deionized water in Schott bottles and shaken vigorously on a hot plate magnetic stirrer until it dissolved. The solution was then sterilized in an autoclave at 121^0C for 15 minutes; the sterilized media was then placed in a water bath to cool the media to 47^0C before pouring into various 20ml sampling bottles.

Inoculation and growth of B. cereus:

Inoculation of bacterial strain was done by suspending 1-3 loops (to ensure proper bacterial growth) from the stock culture (Shea *et al.* 2013) into a 20ml freshly prepared nutrient broth 10% (w/v%). The seeded culture was incubated at 37^0C for 24 hours. After 24 hours, the inoculum was transferred into a 500ml Erlenmeyer flask containing 150ml nutrient broth 30% (v/v) of the original volume of the shake flask (Standbury, *et al.*,1984). The sample was then placed inside a shaker. The experiments were run under the selected different ranges of nutrient concentration, temperature, agitation and acclimatization time. And pH was kept constant at a near neutral of 7.0±2 throughout the experiment, hence is not mention as a factor. The effect of these factors on the growth of *B. cereus* was monitored and analyzed. Aliquots samples were drawn at interval to measure growth of organism by estimating optical density at 600 nm. The process of inoculum transfer was aseptically performed inside a laminar flow, to avoid any contamination.

Factorial design for screening main parameters affecting bacterial cell growth:

In this study, four factors e.g. nutrient concentration, temperature, acclimatization time and agitation speed were selected and screened for their effect on cell growth of *B. cereus* (measured at OD 600nm) using a fractional two (2^3) level factorial design. These factors were selected based on the information from scientific articles. The levels of independent variables; nutrient concentration, temperature, acclimatization time and agitation speed, were based on the results obtained in a previous studies of OFAT (Azoddein, *et al.*, 2015). Each variable or factor was studied at two coded level; -1 (low-level) and +1 (high-level). Table 1 & 2 show a designed factors and levels employed for the experiment and a total of eight runs (2^3) were conducted in duplicate. The statistical software package Design Expert® (Version 7.0.3. State Ease, Minneapolis, MN) was used to design and analyze the experimental data. The effect of each variable and their interactions on the cell growth was statistically determined. True response surface was approximated over a small experimental region by a low-order polynomial. A first-order polynomial model is only able to estimate the main effects of the experimental factors and does not account for either interactions or curvilinear effects. If there is little curvature in the limited region, a first-order model with interaction is appropriate for modeling. Adding interaction terms introduces curvature into the response function (Onsekizoglu, *et al.*,2010). The first-order model with interaction terms proposed for each response variable (Yi) was based on the multiple linear regression method. A probability (P) value for a given factor less than 0.05 (95% confidence interval) was considered as significant. For three factors system a polynomial equation model in terms of coded factors was used to predict the response of bacterial cells to the selected variables:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (3)$$

Where β_i are the values of the regression coefficients, β_0 being the constant term, β_1, β_2 and β_3 the linear effects, β_{12}, β_{13} and β_{23} the interaction effects while the A(x_1), B(x_2), C(x_3) are the independent coded variables (nutrient concentration, temperature difference, acclimation time and agitation respectively). Stepwise deletion of terms was applied to eliminate the statistically non-significant terms. The goodness of fit of the model and significance of each regression coefficient was evaluated by regression analysis of the residual values, analysis of variance (ANOVA) and by the correlation coefficient R2. The statistical significance was checked by the F-test (Tabbene, *et al.,* 2009; Onsekizoglu *et al.* 2010).

Table 1: Actual Values of Experimental variables use in the 2^3 fractional factorial design

		Levels	
Variables	Units	Low (-1)	High (+1)
Nutrient Concentration(A/x_1)	g/l	4	16
Temperature (B/x_2)	^oC	30	42
Acclimatization time (C/x_3)	hours	24	72
Agitation speed (D/x_4)	rpm	140	200

Table 2: 2^3 Fractional Factorial Design Coded Levels Matrix

	Factors				Response
Run	Nutrient Concentration (A)	Temperature (B)	Acclimatization time (C)	Agitation speed (D)	Growth (OD 600nm)
1	1	-1	1	-1	1.958±0.022
2	1	-1	-1	1	3.000±0.019
3	-1	-1	-1	-1	1.644±0.032
4	-1	-1	1	1	0.901±0.017
5	-1	1	1	1	2.000±0.023
6	1	1	1	1	1.376±0.016
7	1	1	-1	-1	3.000±0.021
8	-1	1	-1	1	1.325±0.040

RESULTS AND DISCUSSION

The independent and dependent variables were found to have fitted to the first-order polynomial model equation with interaction terms (Eq. (3)) and for each response variable were examined for goodness of fit. Table 3 and 4 present the regression relationships for each response monitored. And these tables show that response quadratic model for growth efficiency has *F*-value of 251.07 indicating that the model is significant. And model terms of *P* value less than 0.05 implies that model term is significant (Ridzuan *et al*. 2016; Tabbene, *et al.,* 2009; Mohammad *et al.* 2014; Dutta *et al*. 2012; Ramakrishna & Susmita 2012; Yahaya *et al.* 2010; Siti Maryam Rusly *et al*. 2010; Hooshyar, *et al.,* 2014). The *P* values were used as a tool to check the significance of each of the coefficients, which in turn may indicate the pattern of the interactions between the variables. The smaller the value of *P*, the more significant was the corresponding coefficient (Heo,*et al.,* 2009). The significant models terms were A, B, and AB, while C and AC, were the insignificant values/models terms. The model term having the most significant effect on the growth response is A with *F*-value of *850.69* and *p<0.014 (i.e. p<0.05)* and the effect is in the ascending order A > B > *AB* > AC > *C*. The interaction of nutrient concentration and temperature difference (AB) was significant with F-value of *286.39*. Whereas the interaction effects of nutrient and acclimatization time (AC) was found to be statistically insignificant at 95% confidence level. Previous studies also revealed the main and interaction effects at 95% confidence interval; Tabbene, *et al.,* (2009), study the effect variables in bacterial cell growth, Ridzuan, *et al.,* (2016), effects of wax deposition in Malaysian crude oil, while Hooshayr, *et al.,* (2014), studied the effects of some selected variables on chromium (IV) biosorption.. The regression Eq. (4) represents the best description after the elimination of non-significant parameters at intercept/model (p>0.004) from the results summarized in Table 2.

$$Y= 1.90 + 0.59A + 0.17B + 0.34AB \quad (4)$$

Overall main and interactions effects of the variables were depicted in Figures 1, 2 and 3. It has been shown that the bar lengths of Pareto chart are proportional to the absolute value of the estimated effects at 95% confidence level, which indicate order of significance of each linear and interactions effects of the variables, with nutrient concentration demonstrated the most significant effect on both the growth of *B. cereus*. The interaction of nutrient concentration and temperature difference effects were very small in comparison with linear effects but it was also significant at 95% confidence level. This pattern agrees well with what was reported by Onsekizoglu, *et al.,* (2010). The final empirical models in terms of coded and actual parameters were determined as follows:

Coded: Y= 1.90 + 0.59A+ 0.17B + 0.094C + 0.34AB – 0.10AC (5)

Actual: Y= 2.78750 – 0.20875A – 0.066806B + 0.011174C+9.4930AB – 7.25694AC (6)

The goodness of fit of the model was evaluated by the coefficient of determination (R^2), adjusted-R^2, predicted-R^2, coefficient of variance (CV), prediction residual error sum of squares (PRESS), adequate precision and the lack of fit test for the model from the ANOVA table ((Onsekizoglu, *et al.,*2010). Tables 3 and 4 summarize the statistics used to test the adequacy of the model. The p value for the model was less than 0.05, hence indicating that the terms in the model have significant effects on the response. The coefficient of determination (R^2) is the proportion of variation in the response (s) attributed to the model. It is suggested that R^2 should be close to 1 for a good fit model (i. e. not less than 0.8 for biological processes). The estimated model for growth had satisfactory R^2 values of more than 90% variability in bacterial growth; however, it was argued that a large value of R^2 does not always imply that the regression model is good one. Thus, it is preferred to adopt the adjusted-R^2 for evaluation of model fitness, since it is adjusted for the number of terms in the model. The adjusted-R^2 should be over 90% which spell a high degree of relationship between the observed and predicted values. Table 4 shows that R^2 and adjusted-R^2 values for the models did not differ dramatically indicating non-significant terms have not been included in the model. Indeed, Table 4 indicates that all the fit indices indicated goodness of fit to the estimated model. The main and interaction effects of factors upon the responses are depicted in the three-dimensional surface plots (Figs. 5a and 5b). Figure 5a shows the combined effect of varying nutrient concentration and temperature at a defined acclimation time and agitation speed. Growth was observed to follow the normal curve between the temperature of 30^0C to 36.6^0C and up to 40^0C reaching the peak growth of almost OD 3.0 from there it was noticed to start an abnormal trend. Utilization of nutrient for growth (biosynthesis), is an endergonic process, hence require an optimum temperature to function well, although higher temperature affect the enzymatic activity of this process which resulted in a declined growth pattern at extreme range. The results were in agreement with the previous findings (Caroline, *et al.,* 2000; Heo, *et al.,* 2009). However, combined effect of nutrient concentration and acclimation time at a defined temperature and agitation speed was insignificant as indicated in Figure 5b.

The comparison plots of predicted versus the actual response values in Figures 4a and 4b, respectively, show very minimal variance of points from the diagonal point out that the model equations can be used to adequately represent the interaction of the three factors. The value of predicted (3.01) and actual (3.0), which were depicted graphically by the distribution of the predicted values near to the straight reasonable agrees with the experimental data (R^2 0.9984). Indeed, this further confirmed the very good prediction ability of the models.

Table 3: ANOVA table for the growth response

Source	Sum Squares	df	Mean Square	F Value	*p*-Value Prob > F	
Model	4.10	5	0.82	251.07	0.0040**	significant
A-Nutrient Conc	*2.78*	*1*	*2.78*	*850.69*	*0.0012**	significant
B-Temperature	*0.23*	*1*	*0.23*	*69.83*	*0.0140**	significant
C-Acclimat. Time	*0.071*	*1*	*0.071*	*21.67*	*0.0432**	insignificant
AB	*0.93*	*1*	*0.93*	*286.39*	*0.0035**	significant
AC	*0.087*	*1*	*0.087*	*26.78*	*0.0354**	insignificant
Residual	6.525E-003	2	3.263E-003			

**Intercept/Model p-value ($p<0.05$) *variables p-values ($p<0.05$)

The model F-value term 251.07 indicates that it is significant, with 0.40% chance of F-value with such magnitude could be due to noise. The probabilty is greater than Fisher-value of 0.5000 indicates that all model terms (A, B, C, AB and AC) were significant. Whereas value greater than 0.1000 indicates that a model is not significant

Table 4: Statistics used to test goodnessof fit of the models

Std. Dev.	0.057	R-Squared	0.9984
Mean	1.90	Adj R-Squared	0.9944
C.V. %	3.01	Pred R-Squared	0.9745
PRESS	0.10	Adeq Precision	41.857

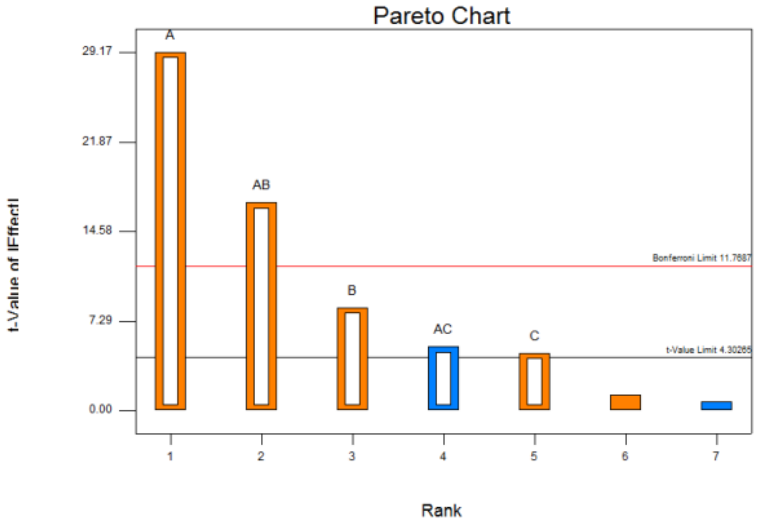


Fig. 1: Pareto's chart of the standardized effects for variables using the *B. cereus* growth response

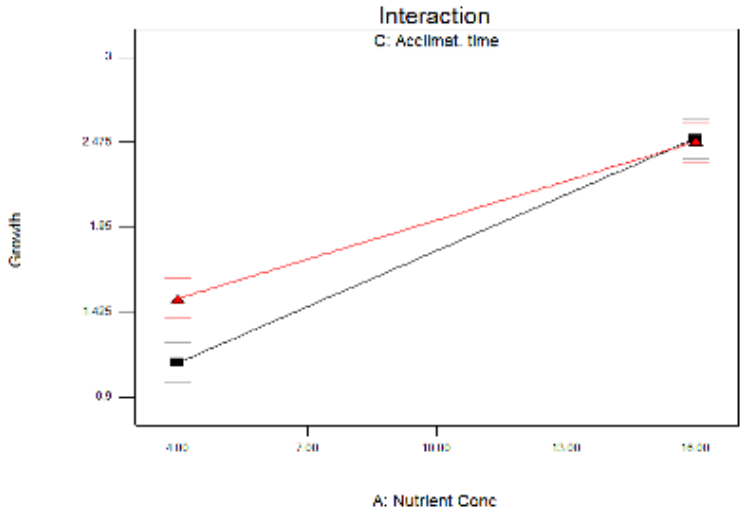


Fig. 2: Interaction effects of nutrient concentration and acclimation time on the *B. cereus* growth

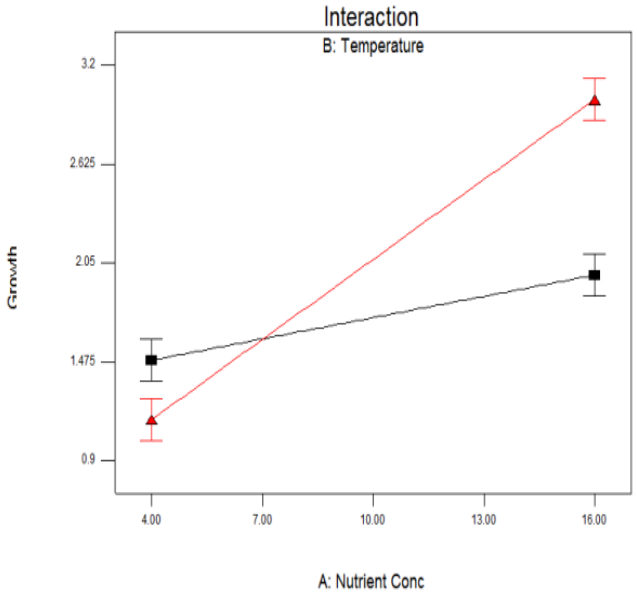


Fig. 3: Interaction effects of nutrient concentration and temperature on the *B. cereus* growth

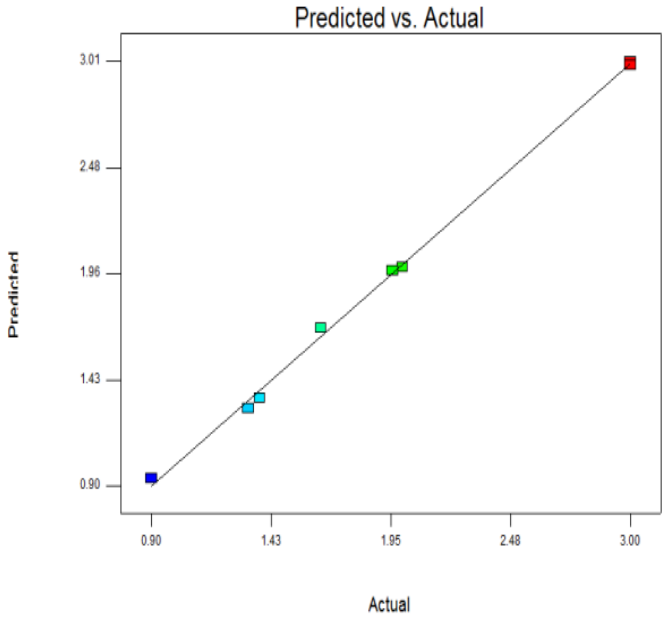


Fig. 4a: Experimental versus predicted ratio of the variables for *B. cereus* growth

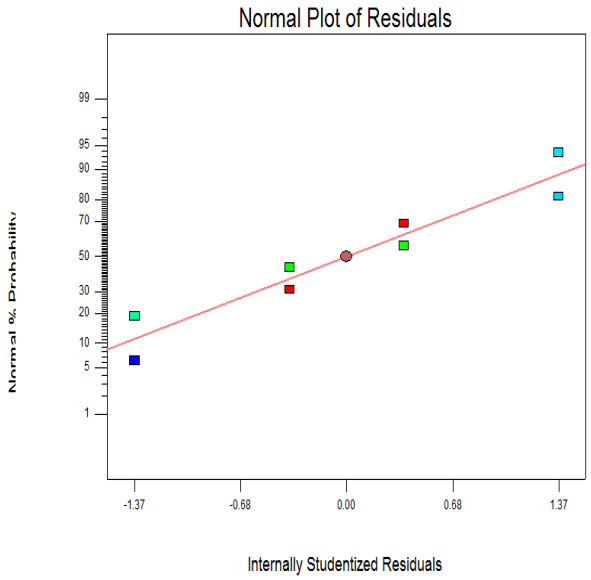
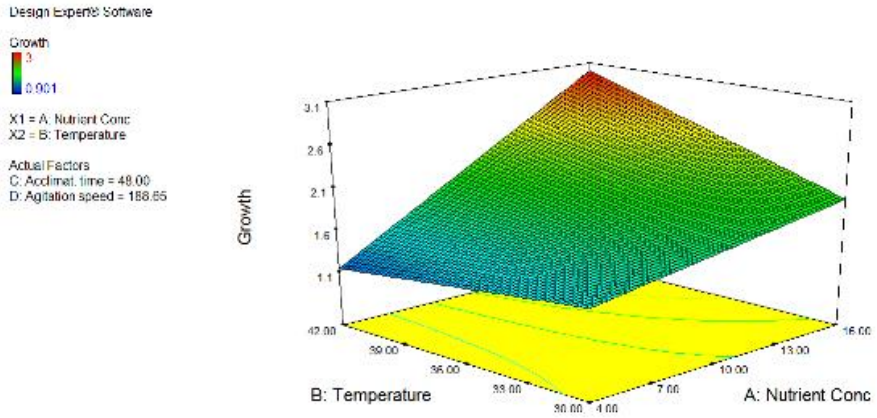


Fig. 4b: Studentized residuals versus predicted value of the variables for *B. cereus* growth

(a)



(b)

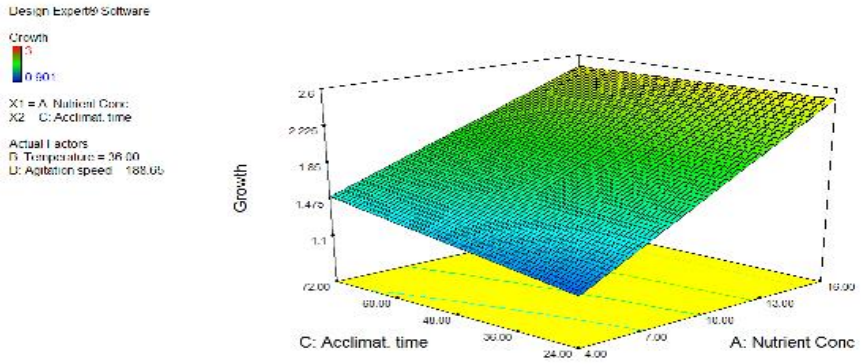


Fig. 5: Response surface plot of growth as a function of: (a) nutrient concentration (A) and temperature (B) at fixed acclimation time C=48hr and Agitation speed D=188.65 (b) nutrient concentration (A) and Acclimation time (C) at a fixed temperature (B) =36 and Agitation speed (C) =188.65

Conclusion:

A two level (2^3) factorial design with two center points was used for investigating the effects of varying nutrient concentration under different operational parameters of a shake flask. All the variables with the exception of agitation speed displayed a significant effect on isolate growth. The results indicated that nutrient concentration was more significant both in terms of linear and interaction effects on the response. Based on the adequacy testing tables; the estimated model terms spell high degree of relationship between observed and predicted values, thus further confirming the prediction ability of the models. In conclusion, the estimated models terms could further be utilized to optimize the process conditions of this isolates' growth.

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