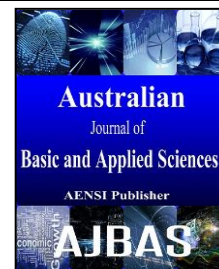




AUSTRALIAN JOURNAL OF BASIC AND APPLIED SCIENCES

ISSN:1991-8178 EISSN: 2309-8414
Journal home page: www.ajbasweb.com



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

Mani Malam Ahmad, Abd. Aziz Mohd Azoddien, Mior Ahmad Khusairi bin Mohd Zahari and Mazrul Nizam bin Abu Seman

Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang (UMP), Lebuhraya Tun Razak, 26300 Gambang, Kuantan, Pahang, Malaysia.

Address For Correspondence:

Mani Malam Ahmad, Faculty of Chemical and Natural Resources Engineering, University Malaysia Pahang (UMP), 26300, Gambang, Pahang, Malaysia.
E-mail: mmahamadu@gmail.com

ARTICLE INFO

Article history:

Received 19 August 2016

Accepted 10 December 2016

Published 31 December 2016

Keywords:

Factorial design, operational parameters, effect, *Bacillus cereus*, growth

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 ($4-16\text{gl}^{-1}$), temperature ($30^{\circ}\text{C} - 42^{\circ}\text{C}$), 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

Open Access Journal

Published BY AENSI Publication

© 2016 AENSI Publisher All rights reserved

This work is licensed under the Creative Commons Attribution International License (CC BY).

<http://creativecommons.org/licenses/by/4.0/>



Open Access

To Cite This Article: Mani Malam Ahmad, Abd. Aziz Mohd Azoddien, Mior Ahmad Khusairi bin Mohd Zahari and Mazrul Nizam bin Abu Seman., Application Of Factorial Design To The Stress Phenomenon Of *Bacillus Cereus* (Atcc 14579) Growth. *Aust. J. Basic & Appl. Sci.*, 10(17): 148-156, 2016

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°C-35°C. 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”

$$\eta = f(x_1, x_2, \dots, x_n) + \epsilon \quad (1)$$

Where η is the response, f is the unknown function of response, x_1, x_2, \dots, x_n 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”

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \sum \beta_{ij} X_i X_j \quad (2)$$

Where Y is the predicted response, β_0 , β_i , β_{ii} and β_{ij} are regression coefficients for intercept, linear, quadratic and interaction coefficients respectively and X_i and X_j 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 (Mermert-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°C for 15 minutes; the sterilized media was then placed in a water bath to cool the media to 47°C 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°C 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 (2³) 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 (2³) 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 (Y_i) 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:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 \quad (3)$$

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 R^2 . 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

Variables	Units	Levels	
		Low (-1)	High (+1)
Nutrient Concentration(A/x_1)	g/l	4	16
Temperature (B/x_2)	°C	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

Run	Factors				Response	
	Nutrient Concentration (A)	Temperature (B)	Acclimatization time (C)	Agitation speed (D)	Growth 600nm)	(OD
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; Hooshayr, *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:

$$\text{Coded: } Y = 1.90 + 0.59A + 0.17B + 0.094C + 0.34AB - 0.10AC \quad (5)$$

$$\text{Actual: } Y = 2.78750 - 0.20875A - 0.066806B + 0.011174C + 9.4930AB - 7.25694AC \quad (6)$$

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 30⁰C to 36.6⁰C and up to 40⁰C 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 probability 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 goodness of 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

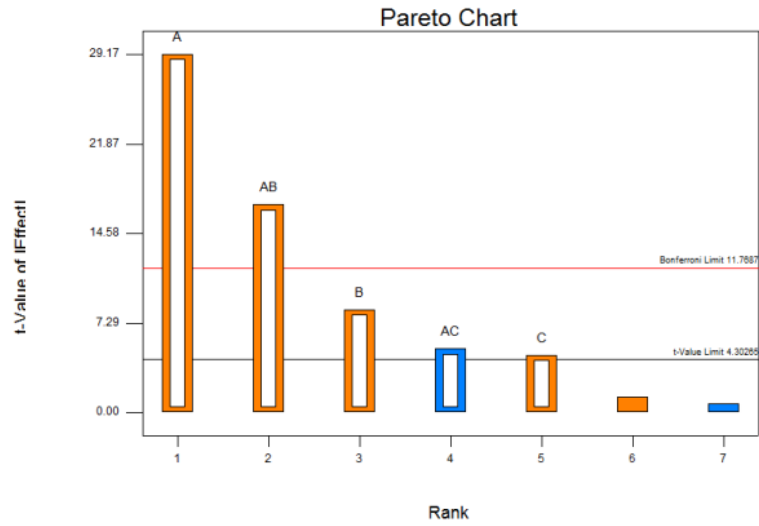


Fig. 1: Pareto’s chart of the standardized effects for variables using the *B. cereus* growth response

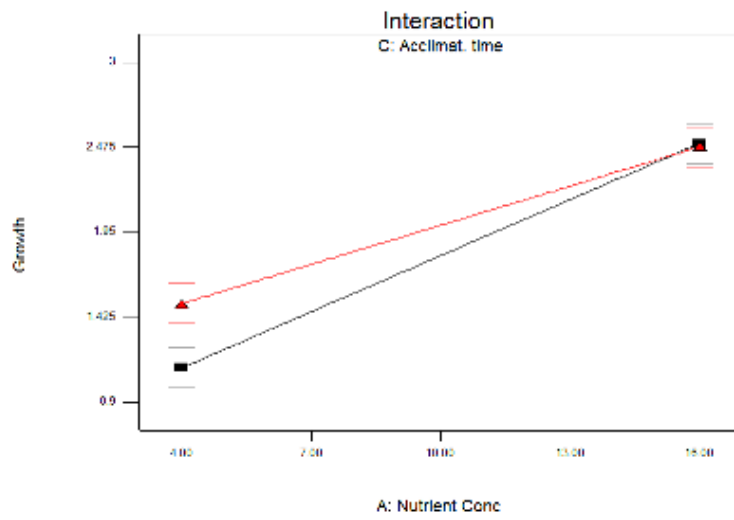


Fig. 2: Interaction effects of nutrient concentration and acclimation time on the *B. cereus* growth

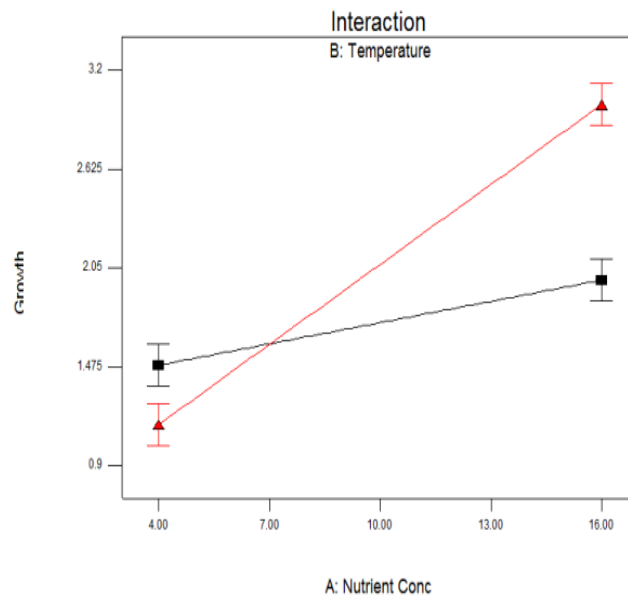


Fig. 3: Interaction effects of nutrient concentration and temperature on the *B. cereus* growth

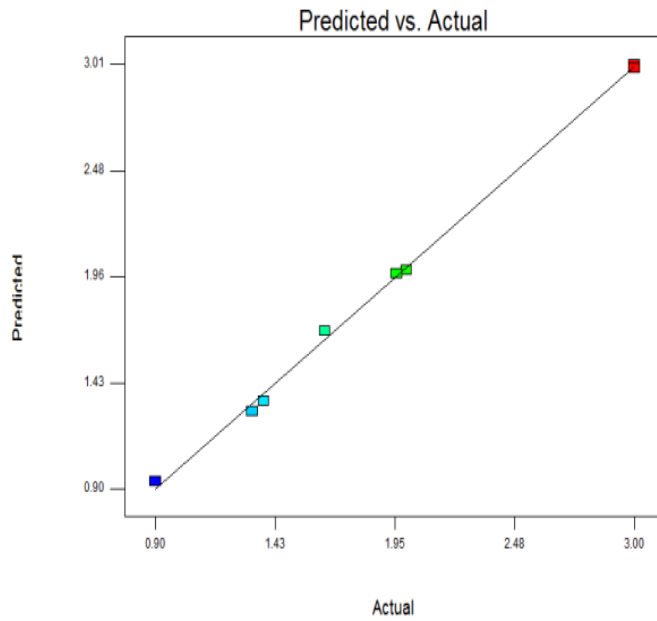


Fig. 4a: Experimental versus predicted ratio of the variables for *B. cereus* growth

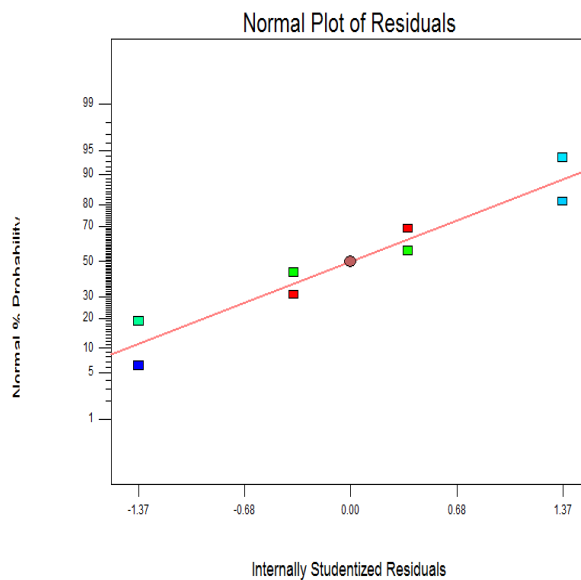
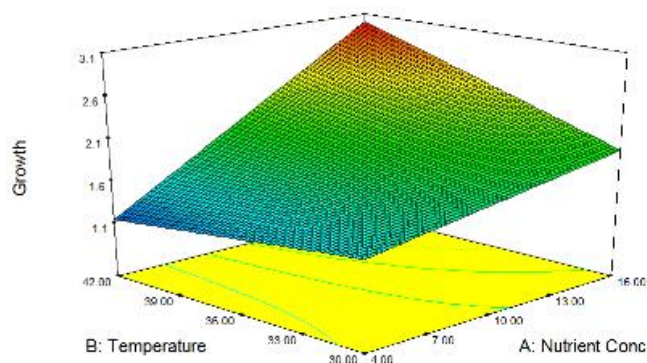


Fig. 4b: Studentized residuals versus predicted value of the variables for *B. cereus* growth

(a)

Design Expert® Software
 Growth
 3
 0.001
 X1 = A: Nutrient Conc
 X2 = B: Temperature
 Actual Factors
 C: Acclimat. time = 48.00
 D: Agitation speed = 188.55



(b)

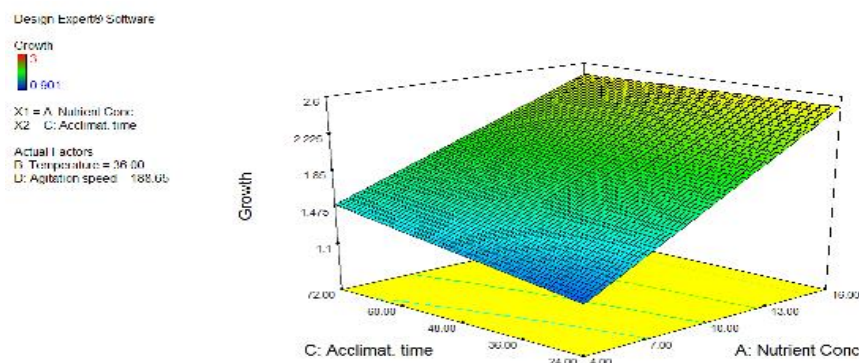


Fig. 5: Response surface plot of growth as a function of: (a) nutrient concentration (A) and temperature (B) at fixed acclimation time C=48hr and Agitation speed D=188.65 (b) nutrient concentration (A) and Acclimation time (C) at a fixed temperature (B) =36 and Agitation speed (C) =188.65

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.

REFERENCES

- Abada, E.A., 2014. Production optimization of extracellular amidase enzyme by newly isolated *Pseudomonas putida* AP-2 from agricultural soil. *Rendiconti Lincei*, 25(4): 523-530. <http://doi.org/10.1007/s12210-014-0347-4>
- Azoddein, A.A.M., R.M. Yunus, N.M. Sulaiman, A.B. Bustary and K. Sabar, 2015. Mercury Removal Using *Pseudomonas putida* (ATTC 49128): Effect of Acclimatization Time, Speed and Temperature of Incubator Shaker. *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*, 9(2): 204-209.
- Caroline, C., S.P hilippe, H. Soumille, C. Nguyen-the and P. Schmitt, 2000. Effect of temperature on growth characteristics of *Bacillus cereus* TZ415, 1-6. [http://doi.org/10.1016/S0168-1605\(00\)00197-5](http://doi.org/10.1016/S0168-1605(00)00197-5)
- Cho, J.-H., Y.-B. Kim and E.-K. Kim, 2009. Optimization of culture media for *Bacillus* species by statistical experimental design methods. *Korean Journal of Chemical Engineering*, 26(3): 754-759. <http://doi.org/10.1007/s11814-009-0126-6>
- Components, M. (n.d.). Optimization of Physical Parameters and Media Components for Alkaline Protease Production : A Statistical Approach.
- Cook, L.K.N., 1996. Optimizing Culturing Conditions for *Bacillus Subtilis*. *South African Avocado Growers' Association Yearbook*, pp: 54-58.
- Curtis, W.C., 2011. *Development Research*, 54(2): 267-270.
- Dos Santos, L.F., F.C. Bazani Cabral De Melo, W.J. Martins Paiva, D. Borsato, M.D.L. Corradi Custódio Da Silva and M.A.P. Colabone Celligoi, 2013. Characterization and optimization of levan production by *Bacillus subtilis* NATTO. *Romanian Biotechnological Letters*, 18(4): 8413-8422.
- Dutta, M., P. Ghosh and J.K. Basu, 2012. Statistical optimization for the prediction of ibuprofen adsorption capacity by using microwave assisted activated carbon. *Applied Science Research*, 4(2): 1053-1060.
- Heo, S., H. Lee and S. Ha, 2009. A Predictive Model for the Growth Rate of *Bacillus cereus* in Broth by Response Surface Methodology. *Biotechnology and Bioprocess Engineering*, 202-206. <http://doi.org/10.1007/s12257-008-0187-0>
- Hooshyar Hossini, Abbas Rezaee, S.R., 2014. Statistical screening of hexavalent chromium biosorption by *Sargassum*. *Iranian Journal of Health, Safety and Environment*, 1(JANUARY), pp: 36-42.
- Inlay, J.A., 2014. Transcription Factors That Defend Bacteria Against Reactive Oxygen Species. *Annual Review of Microbiology*, 69(1): 150612172656007. <http://doi.org/10.1146/annurev-micro-091014-104322>
- Jurtshuk, P. (n.d.). Chapter 4 Bacterial Metabolism.
- Li, Q., M. Wang, J. Feng, W. Zhang, Y. Wang, Y. Gu, S. Wang, 2013. Treatment of high-salinity chemical wastewater by indigenous bacteria--bioaugmented contact oxidation. *Bioresource Technology*, 144: 380-6.

<http://doi.org/10.1016/j.biortech.2013.07.004>

Mandeni, C.-F. and A. Brundin, 2008. REVIEW : BIOCATALYSTS AND BIOREACTOR DESIGN Optimization, Bioprocess Methodology, Using Design-of-experiments. *Biotechnol Progr*, 24: 1191-1203. <http://doi.org/10.1021/bp.67>

Mohammad, Y.S., S.B. Igboro, A. Giwa and C.A. Okuofu, 2014. Modeling and Optimization for Production of Rice Husk Activated Carbon and Adsorption of Phenol. *Hindawi Publishing Corporation Journal of Engineering*, 201: 1–10. <http://doi.org/10.1155/2014/278075>

Mosquera, S., L.M. González-Jaramillo, S. Orduz and V. Villegas-Escobar, 2014. Multiple response optimization of *Bacillus subtilis* EA-CB0015 culture and identification of antifungal metabolites. *Biocatalysis and Agricultural Biotechnology*, 3(4): 378-385. <http://doi.org/10.1016/j.bcab.2014.09.004>

Munna, M.S., S. Tamanna, M.R. Afrin, G.A. Sharif, C. Mazumder, K.S. Kana, R. Noor, 2014. Influence of Aeration Speed on Bacterial Colony Forming Unit (CFU) Formation Capacity. *American Journal of Microbiological Research*, 2(1): 47-51. <http://doi.org/10.12691/ajmr-2-1-7>

Navaneeth, S., S. Bhuvanesh, V.P.V.K. Bhaskar and S.K.J. Kandaswamy, 2009. Optimization of medium for the production of subtilisin from *Bacillus subtilis* MTCC 441. *Journal of Microbiol. Biotechnology*, 8(22): 6327-6331.

Olfa Tabbene, Imen Ben Slimene, Kais Djebali, M.-C.U. Maria-Luisa Mangoni and F.L., 2009. Optimization of medium composition for the production of antimicrobial activity by *Bacillus subtilis* B38. *Biotechnology Progress*, 25(5): 1267-1274. <http://doi.org/10.1002/btpr.202>

Oliveira, I.M., De, D. Bonatto, J. Antonio and P. Henriques, 2010. Nitroreductases : Enzymes with Environmental , Biotechnological and Clinical Importance. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, pp: 1008-1019.

Onsekizoglu, P., K.S. Bahceci and J. Acar, 2010. The use of factorial design for modeling membrane distillation. *Journal of Membrane Science*, 349: 225-230. <http://doi.org/10.1016/j.memsci.2009.11.049>

Ramakrishna, G. and M. Susmita, 2012. Application of response surface methodology for optimization of Cr (III) and Cr (VI) adsorption on commercial activated carbons. *Research Journal of Chemical Sciences*, 2(2): 40-48.

Ridzuan, N., F. Adam and Z. Yaacob, 2016. Screening of factor influencing wax deposition using full factorial experimental design. *Petroleum Science and Technology*, 34(1): 84-90. <http://doi.org/10.1080/10916466.2015.1122625>

Singh, S.K., S.K. Singh, V.R. Tripathi, S.K. Khare and S.K. Garg, 2011. Comparative one-factor-at-a-time, response surface (statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a psychrotrophic *Pseudomonas putida* SKG-1 isolate. *Microbial Cell Factories*, 10(1): 114. <http://doi.org/10.1186/1475-2859-10-114>

Siti, M.Z., R.C. Nurhaslina and H.K. Ku, 2013. Influence of agitation, pH and temperature on growth and decolorization of batik wastewater by bacteria *Lactobacillus delbrueckii*. *International Journal of Research and Reviews in Applied Sciences*, 14(2): 269-275.

Siti Maryam Rusly, Shaliza Ibrahim, S.M. Rusly and S. Ibrahim, 2010. Adsorption of Textile Reactive Dye by Palm Shell Activated Carbon: Response Surface Methodology. *World Academy of Science, Engineering and Technology*, 43(2): 892-895.

Standbury, P.F., A. Whitaker and S.J. Hall, 1984. P. of F. T. O. B. H. *Principle of Fermentation*. Standbury, P.F., Whitaker, A. and Hall, S. J. (1984). *Principles of Fermentation Technology*. Oxford: Butterworth Heinemann (2nd ed., Vol. 53). OXFORD: BUTTERWORTH HEINEMANN. <http://doi.org/10.1017/CBO9781107415324.004>

Swain, M.R., S. Kar, G. Padmaja and R.C. Ray, 2006. Partial characterization and optimization of production of extracellular alpha-amylase from *Bacillus subtilis* isolated from culturable cow dung microflora. *Polish Journal of Microbiology / Polskie Towarzystwo Mikrobiologów = The Polish Society of Microbiologists*, 55(4): 289-96. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/17416065>

Wikipedia, F., 2011. *Bacillus subtilis*. *Reproduction*.

Yahaya, N.K.E.M., M.F.P.M. Latiff, I. Abustan and M.A. Ahmad, 2010. Effect of preparation conditions of activated carbon prepared from rice husk by ZnCl₂ activation for removal of Cu (II) from aqueous solution. *International Journal of Engineering and Technology*, 10(6): 28-32.

Zhong, J., X. Zhang, Y. Ren, J. Yang, H. Tan and J. Zhou, 2014. Optimization of *Bacillus subtilis* cell growth effecting jjean-peptide production in fed batch fermentation using central composite design. *Electronic Journal of Biotechnology*, 17(3): 132-136. <http://doi.org/10.1016/j.ejbt.2014.04.010>

Zinkernagel, K.F.H.K.A.B.J.E. and R.M., 2005. *Basic Principles General Aspects of Medical Microbiology II Bacteriology General Bacteriology Bacteria as Human Pathogens III Mycology General Mycology Fungi as Human Pathogens IV Virology General Virology Viruses as Human Pathogens V Parasitology Protozo*. New York (Vol. 71). Retrieved from <http://pgim.srilanka.healthrepository.org/handle/123456789/9873>