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Growth Kinetics of a Native Toxigenic Isolate of Bacillus Cereus under the Influence of Incubation Temperature, pH and Sodium Chloride in Broth System

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Authors' contributions

This work was carried out in collaboration between both authors. Author MCV designed the work and finalized the manuscript. Author SVD conducted laboratory experimental trials, compiled data and documented literature relevant to the study. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The growth behavior of vegetative cells and spores of a native toxigenic food isolate of Bacillus cereus CFR 1534 was studied under the influence of incubation temperature, pH and sodium chloride.
Place and Duration of Study: The study was undertaken in the Department of Human Resource Development, CSIR-Central Food Technological Research Institute, Mysore 570020, India. The duration of study was during the period October 2009 to February 2010.
Methodology: The experimental design was a central composite design (CCD) based on 3 factors and 5 levels. The factors for vegetative cells were incubation temperature (12-48ºC), pH level (5.5-7.5) and sodium chloride (2-6%). With spores, the ranges of pH and NaCl levels were the same, while incubation temperature range was 22 to 42ºC.
Results: Multiple regression analysis of experimental data relating to lag phase duration (LPD) and growth rate (GR) of *B. cereus* across the influencing factors in broth system

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revealed that LPD of vegetative cells and spores was primarily influenced by incubation temperature. In vegetative cells of B. cereus, the LPD was in the range of 3.1 to 31.5 h with the highest being observed at 48ºC and pH 6.5. The GR had the lowest of 0.2/h to highest of 2.2/h at 30ºC and pH 7.5. With spores, the lowest LPD of 5.8 h was at 42ºC and highest of 20.5 h at 22ºC and that of GR was in the range of 0.2/h at 22ºC to 0.7/h at 32ºC. Experimental tubes of vegetative cells with GR estimates of 1.3/h and above revealed positive reactions for toxigenic traits of haemolytic and lecithinase activities associated with B. cereus.

Conclusion: In the case of vegetative cells, incubation temperature in the range of 12-20ºC resulted in higher LPD, while in spores, higher LPD was observed with incubation temperatures of 22-26ºC. This could provide a basis to design protocols for a safe food in the food chain.

Keywords: Bacillus cereus; vegetative cells; spores; lag phase duration; growth rate; response surface plots.

1. INTRODUCTION

In the global scenario, microbial food safety is of high significance as it relates to the health of human population. Food safety legislation on a global platform and also at the national level is focused towards meeting the safety of foods [1]. In the Indian food legislation, the Food Safety and Standards Authority of India (FSSAI) are empowered to lay down microbiological standards for different category of foods that are designated under Indian Food Code [2]. Spore forming bacteria are known to cause serious quality and safety problems in the food industry, as heat processing as well as freezing does not necessarily ensure killing of spores in food matrix [3]. In the present scenario of microbial food safety, occurrence of *Bacillus cereus* in foods is viewed with greater significance, because of the existence of this bacterial species in dual phase i.e. vegetative cells and spores. Among several of the factors, storage temperature, pH, nutrients and water activity do affect the survival/growth of this microorganism [4,5,6]. The focus of many research studies has been to understand the origin of heterogeneity in resistance of spores and also the diversity and role of germinant receptors [7,8].

The apparent heat resistance pattern of *B. cereus* spores in relation to temperature, pH, chlorides/phosphates of metals, macro-nutrients, acidulants and heating menstruum have been studied to assess thermal inactivation profile, recovery efficiency and heat resistance characteristics [9,10,11,12]. Further, predictive models including validation studies have been in use to define growth kinetics of microorganisms in broth systems and food matrices under the influence of selected intrinsic and extrinsic factors [13,14,15]. The models for growth kinetics have been generally centered on logarithmic phase of microbial growth. However, it is the lag phase duration (LPD) in microbial growth pattern, which is to be considered more relevant, as it gets related to the time required for the cells to adjust physiologically to a new environment. Growth rate (GR) is another important kinetic parameter in growth behavioral studies [16-21].

In different phases of food chain, the prevailing practices of hygiene and sanitation provide ample scope for contamination with *B. cereus*. The ability of strains of *B. cereus* to prevail and dominate in a diverse range of foods is due to their being opportunistic and ubiquitous in nature [22]. Irrespective of food processing parameters, the survival and growth of *B. cereus* in food systems are greatly influenced by extrinsic factors [23-27]. Among these factors,

storage temperature appears to have greater influence as strains of *B. cereus* are mesophiles. At the same time, they also exist as psychrophiles, thermodurics and thermophiles in a given food environment [28].

The majority of Indian foods are in the optimum levels of acidic and neutral pH with reasonably lower amount of salt. Besides, the prevailing practices of storage and marketing in Indian scenario envisage to select temperatures that exist across the country taking into consideration different geo-climatic seasons. Several research studies on the growth of *B. cereus* have selected extrinsic factors that usually simulate market conditions [27]. Further, the focus has been on the behavioral pattern of spores of *B. cereus* and less emphasis on vegetative cells. Considering the prevailing environmental conditions and product profile of Indian foods along with the equal importance of vegetative and spore phases of *B. cereus* in food safety, the objective of present study was to study the growth kinetics in terms of LPD and GR of vegetative cells and spores of a native toxigenic isolate of *B. cereus* CFR 1534 as a function of temperature, pH and sodium chloride concentration in a selected broth system.

2. MATERIALS AND METHODS

2.1 Materials

All glasswares, media and other materials used in the present study were either wet sterilized or dry sterilized. Wet sterilization was carried out at 121ºC for 20 min in an autoclave and dry sterilization at 180ºC for 4 h in a hot air oven. All bacteriological media used were those of dehydrated media procured from Hi-Media Lab., Mumbai, India. The media were prepared as per manufacturer's instructions [29]. The water used in the experimental trials was Milli-Q water (A10 Elix 3, Millipore Corporation, Billerica, USA).

2.2 Bacterial Culture and Preparation of Vegetative Cells and Spore Suspension

This included a native toxigenic food isolate of *B. cereus* CFR 1534 that was positive for toxigenic traits of phosphatidylinositol phosphholipase C, haemolysin BL and sphingomyelinase [22]. The culture was maintained at 6ºC on brain heart infusion (BHI) agar slant in the Culture Collection Stock of Human Resource Development of the parent Institution and propagated twice successively in BHI broth for 18 h at 37ºC prior to use in experimental trials. The suspension of vegetative cells and spores of *B. cereus* CFR 1534 was prepared as described earlier [30].

2.3 Experimental Design

The experimental design was a central composite design (CCD) based on 3 factors and 5 levels. The factors for vegetative cells were incubation temperature (12-48ºC), pH level (5.5-7.5) and sodium chloride (2-6%). Similarly, in the case of spores, the ranges of pH and NaCl levels were the same, while incubation temperature range was 22 to 42ºC. Multiple tubes of pH adjusted nutrient broth (10 ml aliquots) with requisite levels of NaCl as per the experimental design were prepared and sterilized. As 10 ml aliquot of nutrient broth contains 0.5% NaCl, the requisite levels were added from the prepared stock solution. The individual tubes were inoculated with aliquots of 0.1 ml inoculum of test culture (vegetative cells and spores in separate sets) to give a final concentration of 3.3 $\log_{10}$ CFU/ml and incubated at

desired temperatures in a BOD Incubator (Sub-Zero, Industrial and Laboratory Tools Corporation, Chennai, India). Experimental samples of culture broths were enumerated for the viable counts of inoculated *B. cereus* for the respective set of parameters by removing the tubes periodically from incubation and surface plating of 0.1 ml aliquots of appropriate serial dilutions on pre-poured nutrient agar plates in duplicates. The inoculated plates were incubated for 24-48 h at 37ºC and characteristic colonies of *B. cereus* appearing in the incubated plates were counted and expressed as average $\log_{10}$ CFU/ml.

2.4 Determination of Lag Phase Duration and Growth Rate

The derived average viable populations of *B. cereus* in CFU/ml obtained from two experimental trials were transformed to $\log_{10}$ values. At each combination of treatment variables, the log values were plotted against time (h) to obtain growth curves using DMFit curve fitting software programme version 2.0 (Institute of Food Research, Norwich, UK) as a function of Baranyi model to determine lag phase duration (LPD) and growth rate (GR) values [31].

2.5 Model Development and Response Surface Plots

The generated experimental data in the individual sets of vegetative cells and spores were used to perform multiple regression analysis (Microsoft Excel Software Programme 2010, Microsoft Corporation, Redmond, WA, USA) to derive equations for individual parameters of LPD and GR, respectively, which were further expressed as a quadratic function of incubation temperature, pH level and NaCl concentration using the following equation:

$$PrdR = \mathbf{x}_1 + \mathbf{x}_2(T) + \mathbf{x}_3(P) + \mathbf{x}_4(N) + \mathbf{x}_5(T)^2 + \mathbf{x}_6(TxP) + \mathbf{x}_7(P)^2 + \mathbf{x}_8(PxN) + \mathbf{x}_9(N)^2 + \mathbf{x}_{10}(TxN)$$

Where R is any one of the growth parameters (LPD or GR); $\mathbf{x}_n$ (n=1, 2, 3……..10) are the coefficients; T is the incubation temperature (°C); P is the pH; and N is the concentration of sodium chloride (%).

Statistical testing of the model was performed using analysis of variance (ANOVA), which was used to test the significance and adequacy of the model. Statistical significance was determined based on Fischer (*F)* test. The value of significance of *F* (P<0.05) show that model terms are significant, whereas values greater than 0.1 indicate no significance. The feasibility to predict growth responses depends on these statistical indices [32]. Further, observed and predicted values were subjected to Chi-Square Test to assess the goodness of the fit, wherein value of 1.0 could establish a high degree of correlation between observed and predicted values.

Three dimensional response surface plots were generated to depict the interaction of the dependent and independent variables. The effects of independent variables on LPD and GR were evaluated using these three-dimensional plots obtained by imposing a constant value to one variable at a time.

2.6 Assessing for Toxigenic Traits

Individual culture broth tubes of the experimental design was assayed for (i) haemolytic and lipolytic activities in blood agar and tributyrin agar plates, respectively and (ii) toxigenic traits of phosphatidylinositol phospholipase C (*pi-plc*) and haemolysin BL (*hbl*) with respective primers of Pi-PLC and Ha-1 by PCR as described earlier [22]. The PCR conditions were as

follows: (i) Initial denaturation: 94ºC for 5 min; (ii) Denaturation: 94ºC for 1 min; (iii) Annealing: 50ºC for 1 min; (iv) Extension: 72ºC for 1 min; (v) Final extension: 72ºC for 8 min; Total No. of cycles: 35 of steps (ii) to (iv).

2.7 Statistical Analysis

The experimental trials were carried out independently, in triplicate and calculations were performed in Microsoft Excel Program, 2010 (Microsoft Corporation, Redmond, WA, USA). The mean values with statistical analysis were considered in presenting the data. Those with very small *P* values ($P<0.05$) were considered significant and others as non-significant.

3. RESULTS AND DISCUSSION

3.1 Lag Phase Duration and Growth Rate in Vegetative Cells of *B. cereus*

The experimental and predicted LPD and GR values are presented in Table 1. Among the set of factors/variables used, it was observed that 4% NaCl was associated with all those significant values of LPD and GR. The experimental LPD values ranged from 3.1 to 31.5 h, with the lowest LPD recorded under defined conditions of 30ºC and pH of 5.5 and the highest at 48ºC and pH of 6.5. Similarly, the experimental GR values ranged from the lowest of 0.2/h in a combination of 48ºC and pH 6.5 and the highest of 2.2/h at 30ºC and pH 7.5. The derived regression coefficients, their standard errors and *P* values for LPD and GR values are shown in Table 2. The R^2 value of 0.78 for LPD indicates an optimum degree of correlation between experimental and predicted values, while that of 0.49 for GR shows a poor correlation. In the case of LPD, the linear coefficient temperature alone was significant with a very small *P* value ($P<0.05$). In contrast, for GR values, the linear coefficients of pH and cross product coefficient (pH x pH) were significant with very small *P* values ($P<0.05$). It could be visualized that in vegetative cells of *B. cereus*, incubation temperature primarily influenced LPD and pH level affected GR. Further, results of Chi Square test (0.85 and 0.99) did establish goodness of the fit and a high degree of correlation between observed and predicted values for both LPD and GR values of vegetative cells (data not shown).

The assay for selected toxigenic traits in experimental broth tubes revealed positive reaction for lecithinase activity in agar plate assay and positive amplification in PCR with Pi-PLC primers in only vegetative cells with GR estimates in the range of 1.3–2.2/h (Fig. 1A). Besides, the other trait namely haemolysin BL was recorded only with GR estimates of 1.5, 1.6 and 2.2/h (Fig. 1B).

The coefficients derived by multivariate analysis were utilized to generate response surface plots for LPD and GR values of vegetative cells of *B. cereus* CFR 1534 as a function of varying incubation temperatures and NaCl concentrations at 3 defined pH levels of 5.5, 6.5 and 7.5. The individual response surface plots at the defined pH levels for LPD and GR values are shown in Fig. 2 and 3, respectively. Among the range of LPD values generated, at pH levels of 5.5 and 6.5, lower LPD values were observed with 30-36ºC (Fig. 2A and 2B). At the lowest and highest incubation temperatures, LPD values were in higher range. In contrast, at pH 7.5 with incubation temperatures of 30-36ºC, the LPD values were very low with negative values being observed initially at NaCl concentrations of 2 and 2.5%. However, with increasing NaCl concentrations, the LPD values revealed an increasing trend, with higher values at 12 and 48ºC, respectively and lower values at 24-36ºC (Fig. 2C).

Table 1. Experimental and predicted LPD and GR values of vegetative cells of *B. cereus* CFR 1534

Exptl. No.	Temp. (°C)	pH level	NaCl conc. (%)	LPD ± SD		GR ± SD	
				Exptl. LPD (h)	Prd LPD(h)	Exptl. GR (/h)	Prd GR (/h)
1	19.3	5.9	2.8	21.0±7.1	18.4	1.3±0.5	1.3
2	40.7	5.9	2.8	12.1±0.1	13.0	1.4±0.6	1.3
3	19.3	7.1	2.8	17.3±6.4	14.8	1.0±0.9	1.5
4	40.7	7.1	2.8	5.8±0.4	9.5	1.4±0.3	1.3
5	19.3	5.9	5.2	19.2±1.0	16.4	0.7±0.5	1.0
6	40.7	5.9	5.2	6.9±2.5	10.2	0.9±0.4	0.7
7	19.3	7.1	5.2	17.0±2.2	17.0	1.2±0.3	1.5
8	40.7	7.1	5.2	7.4±1.2	10.9	0.9±0.1	1.0
9	12.0	6.5	4.0	29.9±1.4	35.0	1.5±0.1	1.0
10	48.0	6.5	4.0	31.5±1.6	25.3	0.2±0.0	0.5
11	30.0	5.5	4.0	3.1±0.1	4.3	1.3±0.1	1.4
12	30.0	7.5	4.0	4.2±1.7	1.9	2.2±0.0	1.8
13	30.0	6.5	2.0	6.6±1.6	7.4	1.4±0.1	1.4
14	30.0	6.5	6.0	8.7±0.8	6.8	1.2±0.4	1.0
15	30.0	6.5	4.0	4.4±0.2	7.7	1.6±0.2	0.9
16	30.0	6.5	4.0	6.7±1.7	7.7	0.9±0.1	0.9
17	30.0	6.5	4.0	7.0±2.3	7.7	0.6±0.1	0.9
18	30.0	6.5	4.0	7.7±2.2	7.7	7.7±2.2	7.7
19	30.0	6.5	4.0	8.8±0.6	7.7	0.9±0.2	0.9
20	30.0	6.5	4.0	9.7±0.3	7.7	0.9±0.3	0.9
21	30.0	6.5	4.0	9.4±2.3	7.7	0.9±0.2	0.9

Table 2. Coefficients, associated standard errors and P values derived by multivariate analysis for LPD and GR values of vegetative cells of *B. cereus* CFR 1534

Source	LPD			GR		
	Coefficient	SE	P	Coefficient	SE	P
Intercept	-75.01	132.6	0.58	32.08	15.0	0.05
T	-4.39	1.5	0.01	0.07	0.2	0.67
P	53.12	36.6	0.17	-9.24	4.1	0.05
N	-7.88	13.5	0.57	-1.25	1.5	0.43
T^2	0.07	0.01	5.01E	0.00	0.0	0.62
T x P	0.00	0.2	0.98	-0.00	0.0	0.82
P^2	-4.63	2.7	0.11	-0.70	0.0	0.82
P x N	1.45	1.8	0.44	0.11	0.2	0.59
N^2	-0.15	0.7	0.82	0.07	0.1	0.38
N x T	-0.01	0.1	0.89	-0.00	0.0	0.65
R^2		0.78			0.49	

T, incubation temperature (°C); P, pH level; N, NaCl concentration (%); R^2, Coefficient of determination. Chi-Square Test between observed and predicted values revealed high degree of goodness to fit with values of 0.85 and 0.99, respectively, for LPD and GR

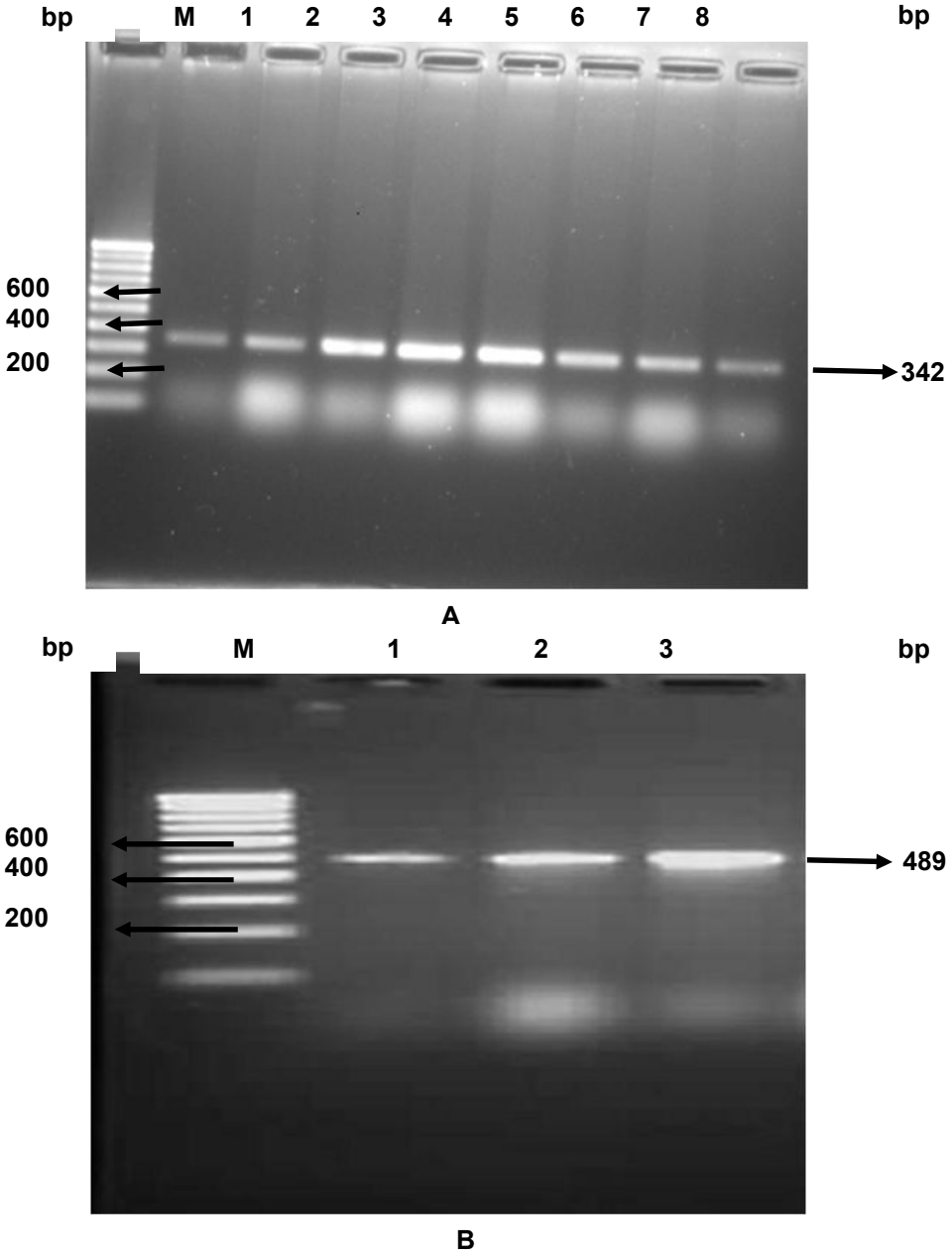

Fig. 1. Agarose gel electrophoretic pattern of positive amplification in PCR with Pi-PLC (A) and Ha-1 (B) primers in experimental broth tubes with GR estimates of vegetative cells of *B. cereus* CFR 1534. In (A): Lane M, 100 bp marker; Lanes 1-8, broth tubes with GR estimates of 1.3, 1.3, 1.4, 1.4, 1.4, 1.5, 1.6 and 2.2/h, respectively; In (B): Lane M, 100 bp marker; Lanes 1-3, broth tubes with GR estimates of 1.5, 1.6 and 2.2/h, respectively

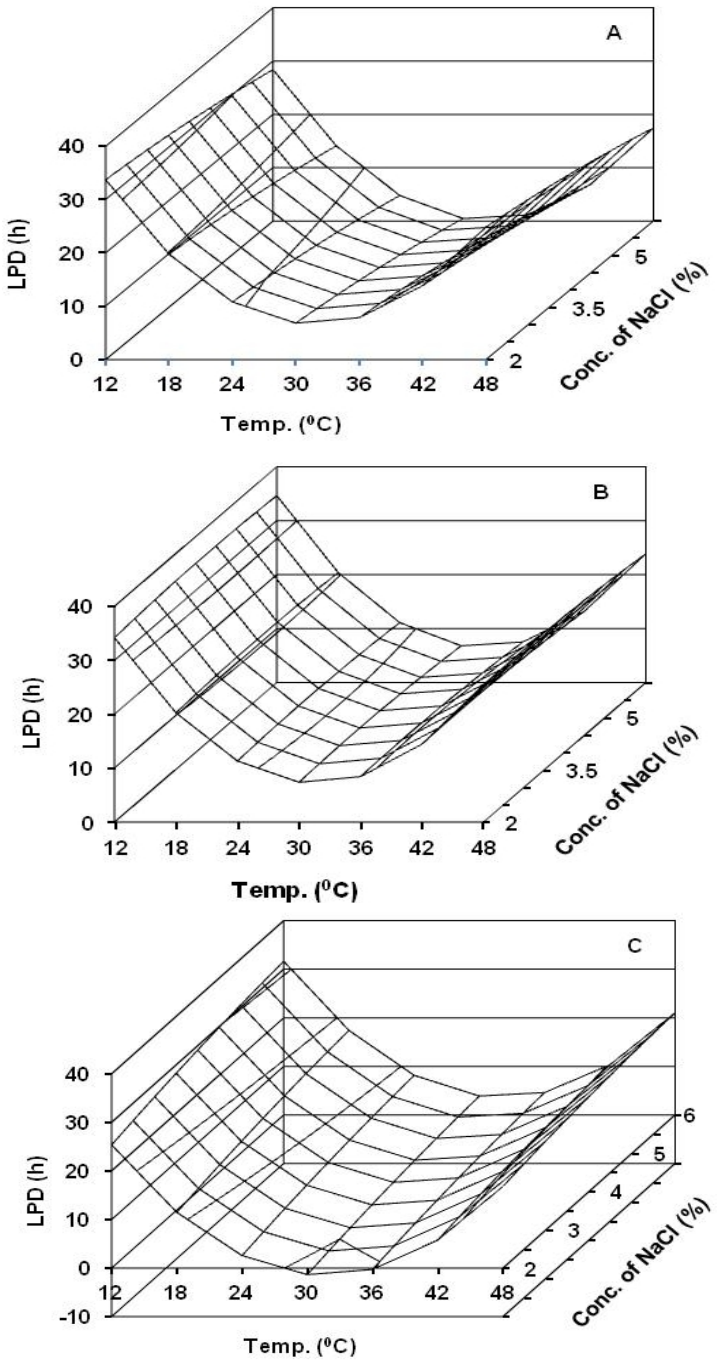

Fig. 2. Response surface plots for LPD in vegetative cells of *B. cereus* CFR 1534 grown in nutrient broth under the influence of incubation temperature and NaCl level at pH 5.5 (A), 6.5 (B) and 7.5 (C), respectively

The response surface plots derived for GR values appeared to have a varied pattern at pH levels of 5.5, 6.5 and 7.5 included in the present study, as GR was influenced by pH level. At pH 5.5, GR values of less than 1.0/h was observed at (i) 42ºC and NaCl concentration of 5.5-6% and (ii) 48ºC with NaCl levels of 4.5-6% (Fig. 3A). The GR values obtained over the range of influencing factors at pH 6.5 were lower than those obtained at pH 5.5 (Fig. 3B). At pH 7.5 (Fig. 3C), the generated GR values were higher, ranging from a minimum of 1.3/h at 48ºC and NaCl levels of 4 to 5.5% to a maximum of 2.0 to 2.5/h at the combinations of (i) 12-36ºC with NaCl levels of 2 and 6% and (ii) 18-24ºC with NaCl levels of 2.5, 4.5, 5, 5.5 and 6%.

Earlier research investigations on similar lines with determination of growth kinetic parameters as a function of temperature, pH, sodium chloride and sodium nitrite observed LPD of 16.86 h under defined set conditions of 19ºC, pH of 2.5 and NaCl concentration of 2.5% [33]. Most of the values reported in their study correlate with those obtained in the present experimental trials under similar conditions for growth of vegetative cells. The effect of salt concentration on growth of *B. cereus* with continuous gradients of pH and temperature revealed that at NaCl concentration of 0.5%, growth occurred over the entire temperature range examined (14 to 41ºC) with a minimum pH of 4.7. When the salt concentration was increased to 3 and 5%, growth was observed in the temperature range with a decrease by 1ºC on either side of the range with minimum pH levels of 4.9 and 5.5, respectively. No growth was observed at 7% NaCl level [34]. It could be seen that a similar pattern of growth accompanied by varying LPD was observed in this study.

The LPD values for *B. cereus* and other microflora in pasteurized milk was determined in an earlier study, wherein these values were found high enough to allow the background flora to grow to a population level of 4 $\log_{10}$ CFU/ml in pasteurized milk stored at low temperatures (5 to 13ºC), before *B. cereus* could reach the risk population level [35]. Although deviation did exist when the present experimental LPD values was compared with an established Pathogen Modeling Program (PMP), the value of 3.1 h obtained at a combination factors of 30ºC, pH 5.5 and 4% NaCl was quite near to the predicted value of 3.9 h by PMP. Similarly, a model developed to describe the growth kinetics (LPD and GR) of *B. cereus* at different temperatures, pH and concentrations of sodium lactate and sodium chloride indicated that the predicted data was comparable with that of PMP [20].

Studies have focused on the factors that tend to influence growth profile of *B. cereus*. A 4-factor growth model using vegetative cells of *B. cereus* with controlling factors of temperature (10-30ºC), pH (4.5-7.0), NaCl (0.5-1.5%) and CO_2 (10-80% v/v) gave accurate predictions for doubling time estimates [36]. Similarly, variables like temperature, pH and water activity significantly affected the location of growth/no growth boundaries in *B. cereus*, wherein the culture was unaffected by the combined variables, as long as they were above the minimum values for growth [16]. Studies [13] with an emetic strain of *B. cereus* F4810/72 revealed GR values in the range of 0.01 to 0.90/h in tryptic soy broth as a function of temperature (10 to 40ºC), pH (5.5 to 8.5) and NaCl concentration (0 to 8%). This study was extended to describe the effect in cooked rice, wherein the predictive model was significant (P<0.01) and there was a close relation between predicted and experimental values (R^2 of 0.98). This was well supported with bias and accuracy factor values [14]. The primary model used in their study showed a good fit to the Gompertz equation used to obtain growth rate. The GR values of 0.2 to 2.2/h observed in the broth medium in this study almost comes very near to those reported using Gompertz function.

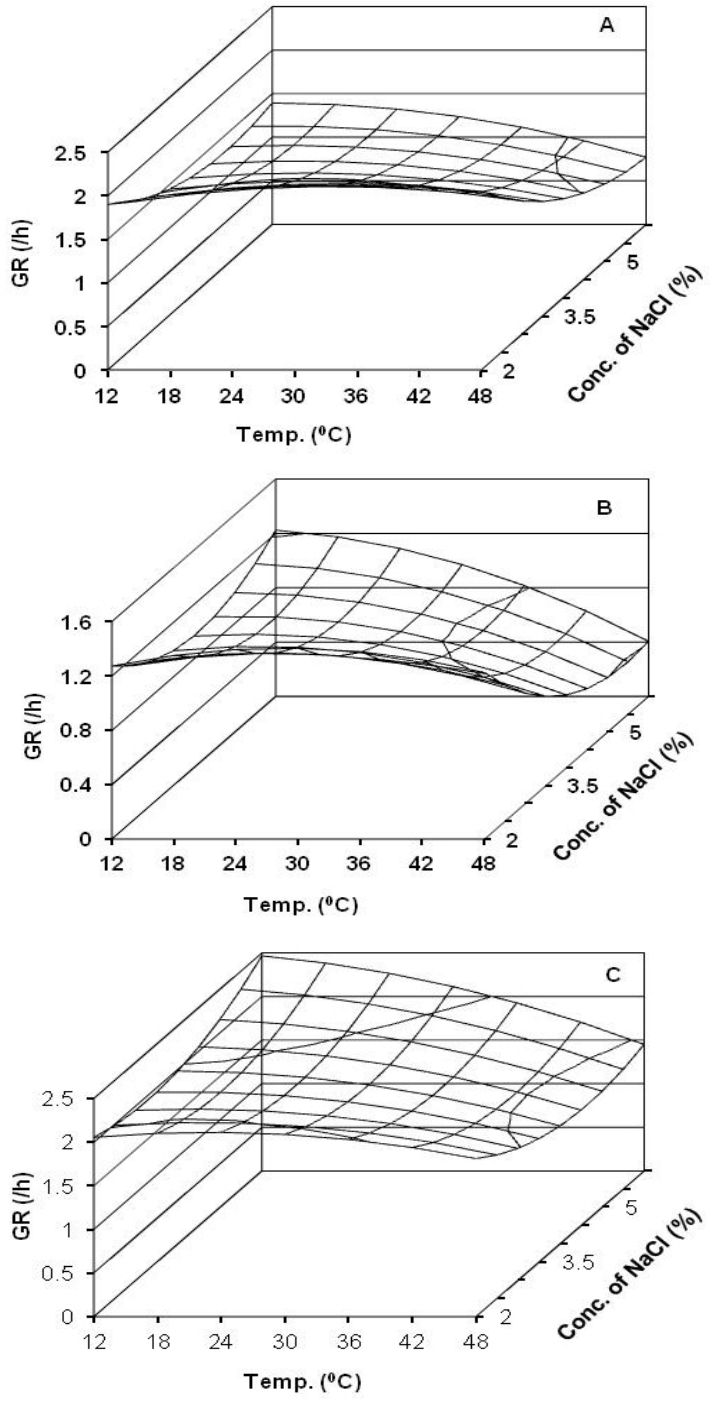

Fig. 3. Response surface plots for GR in vegetative cells of *B. cereus* CFR 1534 grown in nutrient broth under the influence of incubation temperature and NaCl level at pH 5.5 (A), 6.5 (B) and 7.5 (C), respectively

The toxigenic potential of *B. cereus* culture used in this study was evident with the growth of vegetative cells, wherein GR estimates of 1.3/h and above did reveal positive results with the primers of phospholipase C and haemolysin BL (Fig. 1A and B) as well as lecithinase activity in agar plate assay. The detection of *pi-plc* gene gives an indication about the possibility of the culture being virulent, as phospholipase C can cause degradation of cell and mucous membranes leading to necrosis [37]. Similarly, the detection of *hbl* enterotoxin complex is also related to major health hazard attributed to *B. cereus* [38,39].

3.2 Lag Phase Duration and Growth Rate in Spores of *B. cereus*

The experimental and predicted LPD and GR values in the spores of *B. cereus* CFR 1534 are presented in Table 3. Similar to the vegetative cells, even in spores of *B. cereus*, 4% NaCl and pH 6.5 were common defined factors and variables associated with significant LPD and GR values. The experimental LPD was in the range of 5.8 h at 42ºC to 20.5 h at 22ºC. In respect of GR values, the range was between 0.2/h at 22ºC and 0.7/h at 32ºC. The derived regression coefficients, their standard errors and *P* values of LPD and GR are shown in Table 4. The R^2 value of 0.81 for LPD indicates a fairly high degree of correlation between experimental and predicted values, while a value of 0.58 for GR does reflect a poor correlation. In the case of LPD, the linear coefficients of temperature and cross product coefficient (temperature x temperature) were significant with very small *P* values ($P<0.05$). Contrastingly, for GR values, the coefficient values and cross product coefficients were not significant. It could be visualized that incubation temperature appeared to be the primary influencing factor for the LPD of spores of *B. cereus* CFR 1534 and for GR, none of the factors used in the experimental design appeared to have any major influence. Further, results of Chi Square test (0.99 and 1.0) did establish goodness of the fit and a high degree of correlation between observed and predicted values for both LPD and GR values of spores (data not shown). In contrast to vegetative cells, with spores there was no visualization of any positive reactions for haemolysin and lecithinase activities in plate assay and so also no positive amplification with Pi-PLC and Ha-1 primers in PCR.

Similar to the vegetative cells, the derived coefficients were utilized to generate response surface plots for spores of vegetative cells of *B. cereus* CFR 1534 at defined pH levels of 5.5, 6.5 and 7.5. The individual response surface plots at the defined pH levels for LPD and GR values are shown in Fig. 4 and Fig. 5, respectively. The LPD values generated were in the range of 4.6 to 24.0 h. At pH 5.5, lower values (4.6 to 8.0 h) were recorded at temperatures of 34-42ºC and NaCl levels of 2, 2.5 and 3% (Fig. 4A**)**. At pH 6.5, lower LPD values of 4.6 to 8.0 h were observed at temperatures of 34-42ºC and NaCl levels of 2, 2.5, 3 and 3.5% (Fig. 4B**)**. Interestingly, at pH 7.5 (Fig. 4C**)**, the LPD values ranged from a minimum of 9.9 to a maximum of 21.4 h.

The surface plots generated for GR values in the spores of *B. cereus* CFR 1534 were quite interesting. At pH 5.5, the GRs ranged from the lowest of 0.05/h at 42ºC and 6% NaCl to the highest of 0.52/h at 30ºC and 3% NaCl. The trend in behavior was an increase from the initial GR values at 2-3% NaCl and temperatures of 22-42ºC, after which at levels of 3.5-6.0% NaCl, the GR values progressively decreased (Fig. 5A). In a similar manner, at pH 6.5, the lowest GR value of 0.07/h was at 22ºC and 6% NaCl and the highest of 0.56/h was at 34ºC and 4% NaCl as well as 38ºC and 4.5% NaCl (Fig. 5B). At pH 7.5, the GR values generated were negative at 22ºC and NaCl levels of 2-6% with the lowest of -0.04/h (at 4.5% NaCl). A similar trend to that of pH 5.5 and 6.5 with respect to initial increase and subsequent decrease does exist at the temperatures included in the experimental design,

except for 22ºC. However, the increase in GR values extend from 2-5% NaCl and shows marginal decrease at 5.5 and 6.0% NaCl levels (Fig. 5C).

Table 3. Experimental and predicted LPD and GR values of spores of *B. cereus* CFR 1534

Exptl. No.	Temp. (°C)	pH level	NaCl conc. (%)	LPD ± SD		GR ± SD	
				Exptl. LPD (h)	Prd LPD(h)	Exptl. GR (/h)	Prd GR (/h)
1	26.1	5.9	2.8	10.5±1.6	12.4	0.4±0.5	0.5
2	37.9	5.9	2.8	7.1±2.5	5.6	0.5±0.6	0.5
3	26.1	7.1	2.8	13.0±1.4	13.8	0.3±0.9	0.2
4	37.9	7.1	2.8	8.9±4.5	7.9	0.4±0.3	0.5
5	26.1	5.9	5.2	15.2±3.8	16.1	0.4±0.5	0.4
6	37.9	5.9	5.2	12.7±1.6	11.9	0.3±0.4	0.4
7	26.1	7.1	5.2	13.9±0.1	15.5	0.3±0.3	0.3
8	37.9	7.1	5.2	14.3±0.8	12.3	0.6±0.1	0.6
9	22.0	6.5	4.0	20.5±1.0	17.4	0.2±0.1	0.3
10	42.0	6.5	4.0	5.8±0.1	8.9	0.6±0.0	0.5
11	32.0	5.5	4.0	11.5±4.7	11.2	0.6±0.1	0.5
12	32.0	7.5	4.0	12.3±1.6	12.7	0.3±0.0	0.4
13	32.0	6.5	2.0	7.0±0.7	6.9	0.4±0.1	0.4
14	32.0	6.5	6.0	13.5±0.8	13.6	0.4± 0.4	0.4
15	32.0	6.5	4.0	7.8±1.6	9.2	0.4±0.2	0.5
16	32.0	6.5	4.0	9.5±2.6	9.2	0.6±0.1	0.5
17	32.0	6.5	4.0	7.8±1.6	9.2	0.7±0.1	0.5
18	32.0	6.5	4.0	9.8±1.3	9.2	0.5±0.2	0.5
19	32.0	6.5	4.0	10.7±2.8	9.2	0.5±0.2	0.5
20	32.0	6.5	4.0	10.5±0.3	9.2	0.5±0.3	0.5
21	32.0	6.5	4.0	8.3±1.6	9.2	0.4±0.2	0.5

Table 4. Coefficients, associated standard errors and P values derived by multivariate analysis for LPD and GR values of spores of *B. cereus* CFR 1534

Source	LPD			GR		
	Coefficient	SE	P	Coefficient	SE	P
Intercept	180.14	81.7	0.05	0.14	4.8	0.98
T	-3.81	1.6	0.04	-0.03	0.1	0.80
P	-34.13	20.3	0.12	0.35	1.2	0.78
N	0.98	7.7	0.90	-0.18	0.5	0.70
T^2	0.04	0.01	0.02	0.00	0.0	0.13
T x P	0.07	0.2	0.72	0.02	0.0	0.14
P^2	2.71	1.4	0.12	-0.09	0.1	0.30
P x N	-0.68	1.0	0.49	0.06	0.1	0.34
N^2	0.26	0.4	0.47	-0.03	0.1	0.14
N x T	0.09	0.1	0.36	0.00	0.0	0.74
R^2		0.81			0.58	

T, incubation temperature (ºC); P, pH level; N, NaCl concentration (%); R^2, Coefficient of determination. Chi-Square Test between observed and predicted values revealed high degree of goodness to fit with values of 0.99 and 1.0, respectively, for LPD and GR

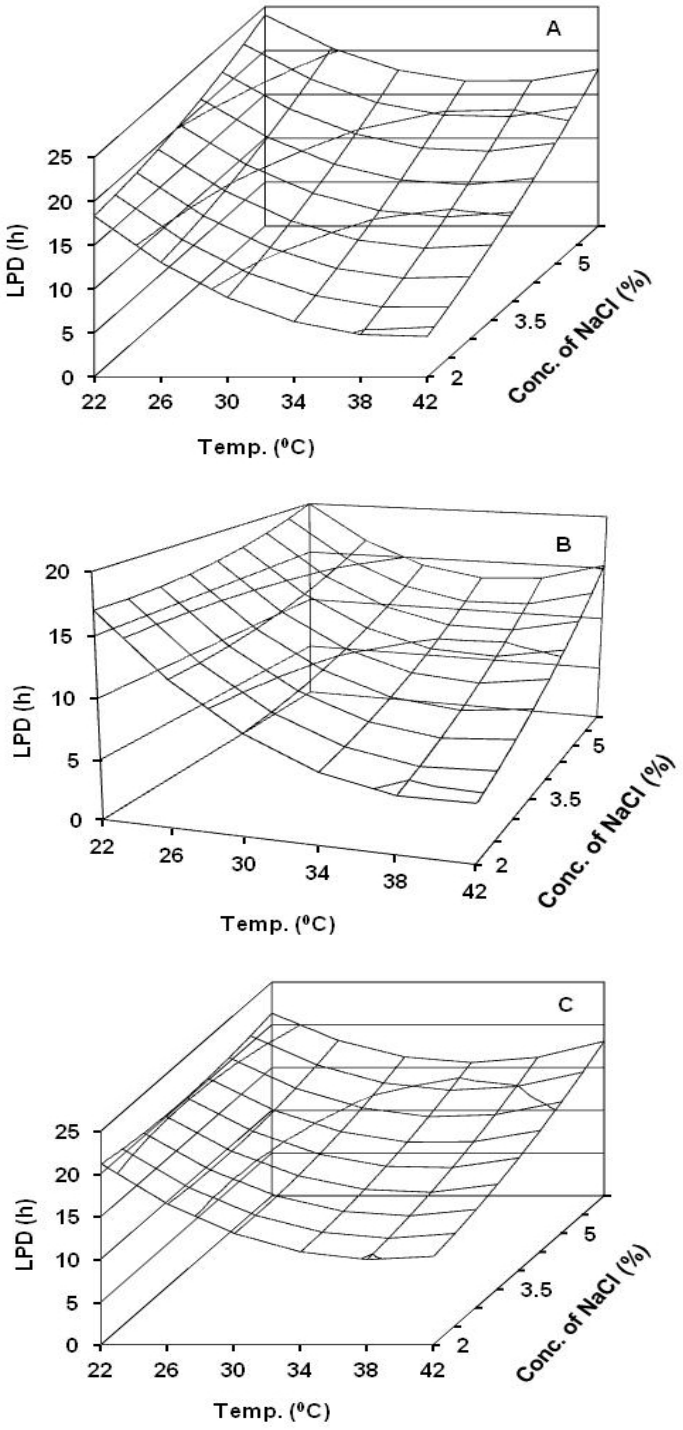

Fig. 4. Response surface plots for LPD in spores of *B. cereus* CFR 1534 grown in nutrient broth under the influence of incubation temperature and NaCl level at pH 5.5 (A), 6.5 (B) and 7.5 (C), respectively

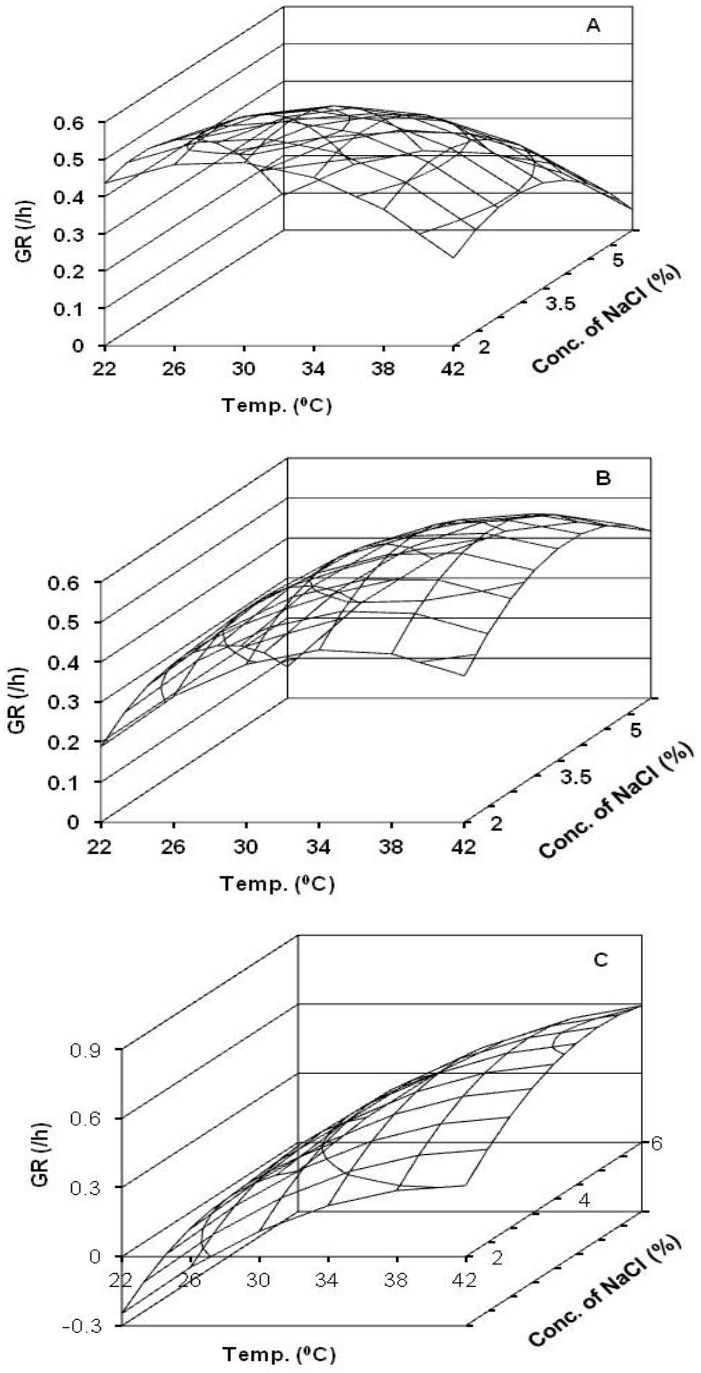

Fig. 5. Response surface plots for GR in spores of *B. cereus* CFR 1534 grown in nutrient broth under the influence of incubation temperature and NaCl level at pH 5.5 (A), 6.5 (B) and 7.5 (C), respectively

In contrast to the vegetative cells, the LPD of spores would include the time for germination and outgrowth of the spores to transform to vegetative cells prior to going in to proper growth phase. This could be the reason for non-visualization of elaboration of toxigenic traits with spores, as GR values were lower than that of vegetative cells. The relationship between the apparent heat resistance of *B. cereus* spores in combination with pH and NaCl concentration of the recovery medium using a simple 3-parameter model was studied [12]. Similar to the present study, higher LPD values of 12.9 h and 16.85 h were reported at a combination of 45ºC, pH of 6.5 and 4% NaCl level and 30ºC, pH 6.0 and 3% NaCl [26,40]. A response surface model developed to describe the effect of temperature (20-40ºC), pH (4.5-6.5) and a_w (0.94-0.99) on germination of *B. cereus* ATCC 11778 revealed that germination was affected (P <0.05) by interactions of a_w with temperature and pH and by temperature in its quadratic term [18], an observation almost similar to the present study. Study relating to validation of empirical models did indicate that re-parameterized function as applied to broth and food model systems can provide estimates of desired minimum processing parameters to determine potential changes in heat resistance of spores of *B. cereus* [15]. In this study, the derived models could be used to predict responses within the range of influencing factors studied and for which experimental data were collected. In this regard, a similar view point was proposed in one of the very earlier studies [41].

4. CONCLUSION

Among the influencing factors included in the present study, LPD and GR values of *B. cereus* was primarily influenced by incubation temperature. Irrespective of pH and sodium chloride levels, lower incubation temperatures result in higher LPDs and GRs with both vegetative cells and spores of *B. cereus*. Response surface plots generated from data obtained from multiple regression analysis over the experimental range of influencing factors revealed that based on LPD and GR values, it is possible to deduce combination of factors that could minimize the proliferation of *B. cereus* in food matrix. The generated data can find potential application to develop HACCP protocols, so as to derive safe foods in the food chain.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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