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Beneficial effects of bio-controlling agent *Bacillus cereus* IB311 on the agricultural crop production and its biomass optimization through response surface methodology

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ABSTRACT

Disease in agricultural field is a big problem that causes a massive loss in production. In this present investigation, we have reported a soil-borne bacterium *Bacillus cereus* IB311 which is antagonistic to plant pathogens (*Pseudomonas syringae* and *Agrobacterium tumefaciens*), and could make a substantial contribution to the prevention of plant diseases. To prove the practical application, the strain was directly applied in agricultural field. The results demonstrated that *B. cereus* IB311 has increased the production (20% and 26% in term of average pod number per plant, average seed number per pod, and seed yield per experimental plot) in ground nut (*Arachis hypogaea* var. Koushal, G201) and sesame (*Sesamum indicum* var. Kanak), respectively. To reduce the production cost, the biomass production was optimized through response surface methodology (RSM). Interactions of three variables (glucose, beef extract and inoculum) were studied using Central Composite Design. According to our analysis, optimum production of *Bacillus cereus* IB311 (5.383 µg/ mL) may be obtained at glucose 1.985%, beef extract 1.615% and inoculums size 0.757%. Therefore, we strongly believe that the application of this strain in agricultural field as bio-controlling agent will definitely enhance the production yield and will reduce the disease risk.

Key words: *Bacillus cereus*, bio-control, phytopathogens, RSM, field application, crop production.

INTRODUCTION

Plants harbor a wide range of bacteria, which may be beneficial or pathogenic. The types of interactions include mutualism, proto-cooperation, commensalism, neutralism, competition, amensalism, parasitism and predation (Bull and Koike 2015). Plant disease is an impairment of the

normal state of a plant that interrupts or modifies its vital functions. Till date, many plant bacterial phytopathogens have been identified such as *Agrobacterium tumefaciens*, *Dickeya* (*dadantii* and *solani*), *Erwinia amylovora*, *Pectobacterium carotovorum*, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Xanthomonas oryzae*, *Xanthomonas campestris*, *Xanthomonas axonopodis* and *Xylella fastidiosa*. Virtually, all species of plants are subject to bacterial disease. The occurrence and prevalence of bacterial plant diseases vary time to time, depending on the presence of the

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pathogen, environmental conditions, and the crops and varieties grown (Mansfield et al. 2012). The organism that suppresses the pest or pathogen is referred to as the biological control agents (BCA). According to the members of the U.S. National Research Council, 'Biological control is the use of natural or modified organisms, gene, or gene products, to reduce the effect of undesirable organisms and to favor desirable organisms such as crops, beneficial insects, and microorganisms' (O'Neil 1997). These bio-control candidates represent an eco-friendly alternative to the use of chemical pesticides in agriculture. Microbes that contribute to disease control are most likely those that could be classified competitive saprophytes, facultative plant symbionts, and facultative hyperparasites.

Among the 20 genera of bacteria, *Bacillus* spp., *Pseudomonas* spp., and *Streptomyces* spp. are widely used as bio-control agents (Islam et al. 2012). There is a huge demand of chemical residue free crops in both domestic and international market. Therefore, soil-borne bacteria that are antagonistic to plant pathogens could make a substantial contribution in preventing plant diseases. Rhizospheric soil has conventionally been used as a model environment for screening of putative agents for the biological control of soil-borne plant pathogens (Majeed et al. 2015). Therefore, the objectives of this present investigation were to (i) isolate bacterial strains from soils of anjeer orchard (ii) characterize and identify the most potential anti-phytopathogenic candidate (iii) practical application of this bio-controlling agent in agricultural field and (iv) optimize environmental and nutritional condition for maximum production of this bio-controlling agent through Response Surface Methodology (RSM).

MATERIALS AND METHODS

PLANT PATHOGENIC BACTERIA AND THEIR

CULTURE MEDIUM

Two plant pathogenic bacteria viz. *Agrobacterium tumefaciens* MTCC No. 609 and *Pseudomonas syringae* MTCC No. 1604 were grown in Tryptone Yeast Extract Broth (YEB, HiMedia, India) and Cetrimide Broth (CB, HiMedia, India) medium, respectively.

ISOLATION OF BACTERIA FROM SOIL

Soil samples were collected from agricultural field of the village Khalad, Purandar, Pune district of Maharashtra, India following the methods of Krieg (1987). The field had no history of application of pesticides and fertilizer, at least six months prior to the collection. One gram of soil was dissolved in 10 mL of sterile double distilled water and serial dilution was done accordingly. 100 μ L sample from each dilution was taken and plated in NA medium plates (pH 7.2), followed by 24 h incubation period at 30 °C. Colonies were selected on the basis of distinctive morphology. Pure cultures were obtained through repeated streaking method. The cultures were stored at 4°C for further use.

SCREENING OF SOIL ISOLATES FOR PLANT PATHOGEN ANTAGONISM

A total of 36 bacterial strains were tested for antimicrobial activity. A loop full pure culture was inoculated in 200 mL of LB media and the supernatant was collected by centrifugation (10,000 \times g, 4°C). Solvent extraction was done by adding ethyl acetate to the supernatants and the mixture was agitated for 45 min. Solvent phase was then separated, collected and dried by solvent evaporation. The crude powder was weighed and then resuspended in DMSO, sterilized using syringe filters and stored at 4°C. The crude powders after dissolving in DMSO were tested for antibacterial activity against test organisms (phytopathogens) following the standard disc diffusion method (Serrano et al. 2004). The Minimum Inhibitory

Concentration (MIC) and the Minimum Bacterial Concentration (MBC) were determined using broth-dilution method (Tyler et al. 1988). The number of bacteria killed in the MIC was determined by serial dilution technique.

CHARACTERIZATION OF THE SCREENED ISOLATES

The most promising strain was selected and subjected to morphological characterization. Single colony of the isolate was subjected to Gram staining, endospore staining and motility analysis. The results were observed by light microscopy under suitable magnification. Carbohydrate fermentation tests (CFT) were studied using HiCarbo KB009-KT kit (HiMedia, India) containing a set of 35 carbohydrates (Table I).

16S RDNA SEQUENCE AND PHYLOGENY ANALYSIS

Bacterial genomic DNA was isolated using gene O-spin microbial DNA isolation kit. Bacterial 16S gene region was amplified using standard PCR reaction and the products were checked on 1% agarose by agarose gel electrophoresis, and the amplicon size was compared using reference ladder. The PCR products were then purified using Gene O-spin PCR product purification kit and were directly sequenced using an ABI PRISM Big Dye Terminator V 3.1 kit (Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. BLAST analysis was performed at BlastN site at NCBI server (<http://www.ncbi.nlm.nih.gov/blast>). Multiple sequence alignments were done using CLUSTAL W and the phylogenetic tree was constructed using neighbor-joining algorithm. For statistical validation of the tree, boot-strap (10,000 replicates) method was used. All the phylogenetic analysis was carried out

TABLE I
Morphological and biochemical characterization of the isolate IB131.

Isolate IB131	Results
Morphological parameter	
Size	4mm
Shape	Filamentous
Margin	Entire
Colour	Creamy
Opacity	Translucent
Elevation	Flat
Consistency	Butyrous
Sporulation	Present (subterminal)
Motility	-
Gram stain	+
Biochemical parameter (HiCarbo KB009-KT Kit)	
Lactose, Xylose, Fructose, Galactose, Raffinose, Trehalose, Sucrose, L-arabinose, Mannose, Inulin, Sodium gluconate, Salicin, Dulcitol, ONPG, Inositol, Sorbitol, Mannitol, Mannitol, Adonitol, Arabitol, Erythritol, α -methyl-D-glucoside, Rhamnose, Cellobiose, Melezitose, α -methyl-D-mannoside, Xylitol, Esculin hydrolysis, D-arabinose, Sorbose	-
Maltose, Dextrose, Melibiose, Glycerol, Citrate utilization, Malonate utilization	+

+ : positive test result; - : negative test result.

using MEGA6 (Tamura et al. 2013).

APPLICATION OF BIO-CONTROLLING AGENT IN AGRICULTURAL FIELD

The selected bacterial strain was inoculated in 200 mL of LB media (pH 7.0) and incubated for 5 days at 37°C, 110 rpm. The broth was then centrifuged at $10,000 \times g$, 20 min, 4°C. The cell pellet was collected and the cfu was adjusted to 1.5×10^{10} cfu/ mL. Field trials were conducted in Ravi season in experimental field. Soil type was red laterite. The field was kept idle for 6 months prior to seed sowing for avoiding effects of any pesticide. Plots of 3.5 m \times 4.0 m were laid out and brought to a fine tilt by ploughing. Soils of the plots were mixed well ensuring leveling and rows were made in 30 cm apart. Randomized complete block design (RBCD) model was followed for the experiments. Untreated (TS1) experimental plots were taken as control, whereas other plots supplied with 100% recommended dose of NPK (60:60:50) (TS2) were also maintained as positive control. Plots treated with experimental strain (TS3), 90 ml 1.5×10^{10} cfu/ mL mixed with 5 Kg of double autoclaved powdered soil to broadcast over one plot area (3.5 m \times 4.0 m). All the experimental plots were irrigated, as required to maintain the moisture level at 15 %. In each case, treatment was carried out one hour before sunset (Chattopadhyay et al. 2014). After preparation of the field, surface sterilized seeds of ground nut (*Arachis hypogaea* var. Koushal, G201) and sesame (*Sesamum indicum* var. Kanak) were showed. Row to row distance was maintained at 30 cm, whereas, plant to plant distance was maintained 20 cm.

In order to analyze the productivity, yield parameters (average pod number per plant, PN; average seed number per pod, SN; seed yield per experimental plot, SY) were measured. The experimental results were statistically analyzed using ANOVA. Duncan's multiple range test (DMRT) was used to determine group mean value,

when ANOVA was found significant at $P < 0.05$ (Chattopadhyay et al. 2014).

OPTIMIZATION OF BIOMASS PRODUCTION BY RESPONSE SURFACE METHODOLOGY

Three variables (glucose, beef extract and inoculum size) central composite design (CCD) for response surface methodology was carried out to optimize the production of the selected isolate. Statistical analysis was done using 'DESIGN-EXPERT® 10.0.3' software package. Level of different factors was taken as -1, 0, and +1 (Table II). Twenty experiments were conducted to maximize the effect of unexplained variability using biomass dry weight as response. Relationship between coded value and actual value in this experiment was given in Equation 1.

$$X_i = x_i - x_0 / \Delta x; i=1,2,3 \quad (1)$$

Where, x_i denotes coded value, x_0 denotes actual value, and Δx denotes step change of x_i . Optimum conditions for production were predicted from a second order polynomial model as described below in Equation 2.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (2)$$

where Y denotes response, b_0 denotes model constant X_1 , X_2 , and X_3 were the variable b_1 , b_2 , and b_3 are linear interaction b_{12} , b_{13} , and b_{23} are cross product interaction, b_{11} , b_{22} , and b_{33} are quadratic interaction.

RESULTS AND DISCUSSION

ISOLATION, SCREENING AND SELECTION

The strain IB131 has been identified as the most promising bio-controlling candidate against the bacterial phytopathogens on the basis of the inhibition zone (Table III). The highest zone of inhibition was recorded against *P. syringae* (21

TABLE II
Central Composite Design showing production of *Bacillus cereus* strain IB311 at different variable combinations.

Run	Coded value			Actual value			
	Glucose (A)	Beef extract (B)	Inoculum size (C)	Glucose (%) (A)	Beef extract (%) (B)	Inoculum size (%) (C)	Biomass mg/ mL
1	-1	-1	-1	2	0.5	1	3.8
2	+1	-1	-1	6	0.5	1	4
3	-1	+1	-1	2	2	1	5.6
4	+1	+1	-1	6	2	1	5.7
5	-1	-1	+1	2	0.5	2	3
6	+1	-1	+1	6	0.5	2	4.5
7	-1	+1	+1	2	2	2	5.3
8	+1	+1	+1	0.5	2	2	5.3
9	- α	0	0	7.5	1.25	1.5	4.3
10	+ α	0	0	4	1.25	1.5	4.9
11	0	- α	0	4	0.1	1.5	2.4
12	0	+ α	0	4	2.4	1.5	5.4
13	0	0	- α	4	1.25	0.25	4.5
14	0	0	+ α	4	1.25	2.75	4.9
15	0	0	0	4	1.25	1.5	5
16	0	0	0	4	1.25	1.5	4.8
17	0	0	0	4	1.25	1.5	4.8
18	0	0	0	4	1.25	1.5	5
19	0	0	0	4	1.25	1.5	5.3
20	0	0	0	4	1.25	1.5	5.1

TABLE III
Antiphytopathogenic activity of the isolate IB131.

Name of the phytopathogenic bacteria	Zone of inhibition (mm @ 30 μ g/ mL)	Approximate count in 60 μ L at 12 h fresh culture (cfu)	MIC (μ g/ mL)	MBC (μ g/ mL)
<i>A. tumefaciens</i> MTCC 609	9	0.11x10 ⁶	8	16
<i>P. syringae</i> MTCC 1604	21	0.42 x10 ⁶	2	8

mm), followed by *A. tumefaciens* (9 mm). The MIC value of the crude powder was determined to be 8 and 2 μ g/ mL, respectively (Table III). The number of phytopathogenic bacteria inhibited by the MIC was also determined. The number of pathogenic bacteria in 60 μ L of broth were measured to be 0.11x10⁶ (*A. tumefaciens*) and 0.42x10⁶ (*P. syringae*) cfu (Table III). Furthermore, the MBC

value of the crude powder against *A. tumefaciens* and *P. syringae* were obtained to be 16 and 2 μ g/ mL, respectively (Table III).

The bacterial species; *A. tumefaciens* and *P. syringae* are considered to be potential phytopathogen based on their scientific and economic importance in plant diseases (Mansfield et al. 2012). Members of the species *A. tumefaciens*

are plant pathogens that cause tumors mostly at the crown of dicotyledonous plant species (Subramoni et al. 2015). The diversification of lineages within *P. syringae* has involved a number of adaptive shifts from herbaceous host onto various species of tree, resulting in the emergence of highly destructive diseases such as bacterial cancer of kiwi and bleeding cancer of horse chestnut (Nowell et al. 2016). Our results clearly indicated the inhibition potentiality of the isolate IB131 against phytopathogen, as a minimum concentration of crude powder (2 µg/ mL) inhibited about 0.42×10^6 cfu of *P. syringae*. Furthermore, the higher value of MBC compared to MIC inferred that the nature

of the crude powder was bacteriostatic rather than bacteriocidal.

CHARACTERIZATION AND IDENTIFICATION OF THE STRAIN IB131

The strain IB131 was found to be Gram-positive, rod-shaped, aerobic bacteria (Table I). The candidate strain IB131 (Table I) actively utilized glucose, reduced nitrogen, and synthesized catalase enzyme, but unable to utilize mannitol, and was therefore predicted to be a strain of *Bacillus* (Wong et al. 1988). 16S ribosomal RNA gene sequence spanning 1183 bp was submitted to NCBI GenBank (accession no. KX685929). The phylogenetic

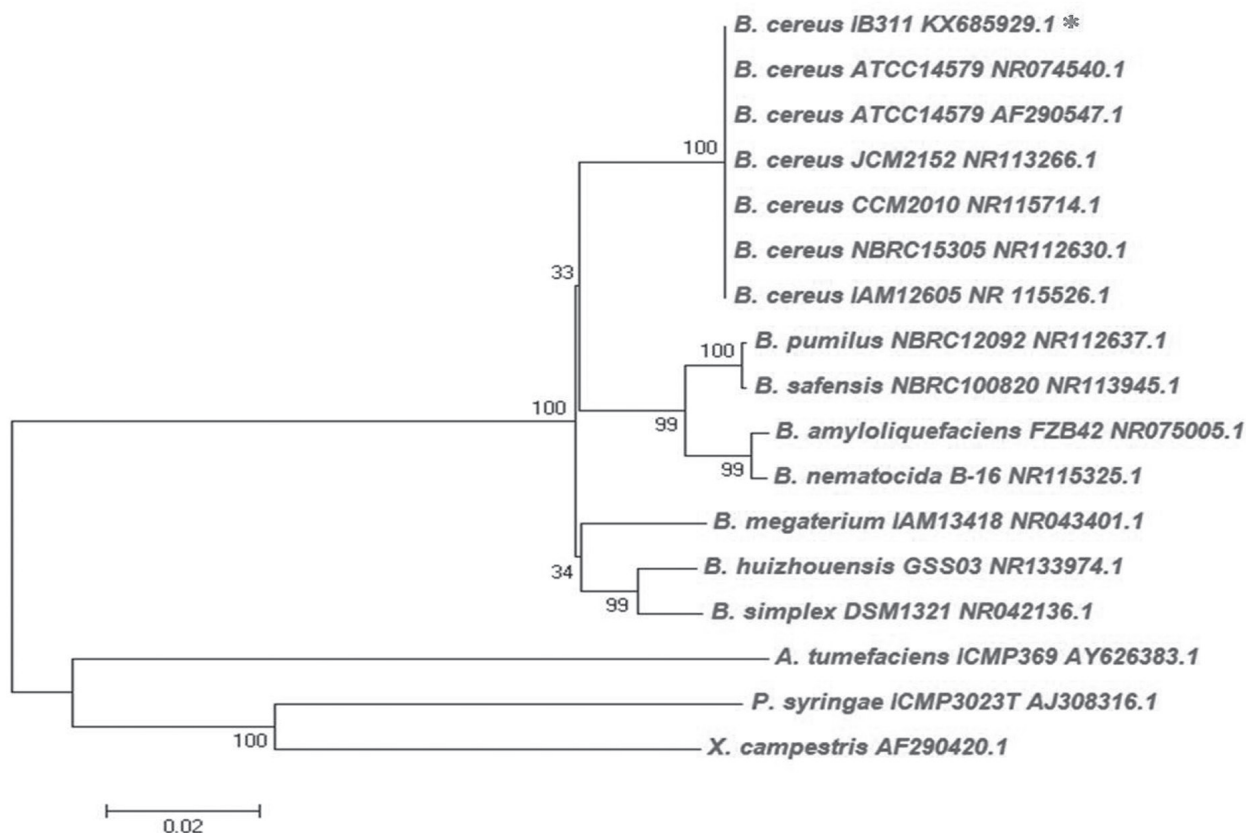


Figure 1 - Neighbour-joining tree based on partial 16S rRNA gene sequences showing relationships of *Bacillus cereus* strain IB311 (denoted with asterisks) with other close homologous strains. Three phytopathogenic bacteria (viz., *Agrobacterium tumefaciens*, *Pseudomonas syringae* and *Xanthomonas campestris*) were used as outgroups. Bootstrap values greater than 50% are highlighted at the nodes (100 replications). The scale bar represents 2 substitutions per 100 bases. Evolutionary analyses were conducted in MEGA 6.06.

relationship of the isolate IB131 exhibited 100% homology to *B. cereus* ATCC 14579, *B. cereus* JCM 2152 and *B. cereus* NBRC 15305 (Fig. 1). Therefore, based on morphology, biochemical and 16S rDNA sequence based phylogeny the strain was identified as *B. cereus* IB131.

B. cereus is a gram-positive, facultative anaerobic, rod shaped, endospore-forming bacterium, which occurs ubiquitously in soil (Sarrías et al. 2002, Guinebreiere et al. 2003, Kuta et al. 2009, Tallent et al. 2012). Furthermore, *Bacillus* produce spores resistant to UV light and heat, which allows them to survive in adverse environmental conditions, and permits easy formulation for commercial purposes (Raaijmakers et al. 2002). In general, *Bacillus* spp. produces several kinds of antibiotics, including bacillomycin, fengycin, mycosubtilin, and zwittermicin, which are effective in controlling the growth of target pathogens (Pal and McSpadden Gardener 2006). *B. cereus* UW85 was isolated from a root of a field-grown alfalfa plant from Arlington, WI, and identified for its ability to suppress damping off, a disease caused by *Phytophthora megasperma* f. sp. medicaginis

on alfalfa. This strain was reported to produce two antibiotics (zwittermicin A and kanosamine) that contribute to its ability to suppress certain plant diseases (Emmert and Handelsman 1999, Emmert et al. 2004, Lozano et al. 2016).

APPLICATION OF BIO-CONTROLLING AGENT IN SESAME AND GROUND NUT AGRICULTURAL FIELD

The results showed that the yield criteria viz., PN, SN and SY were influenced by different treatments $TS3 \geq TS2 > TS1$ (Fig. 2). In details, the SY was found maximum when treated with the experimental strain (TS3), and about 20% increment in ground nut and 26% increment in sesame yield was achieved in comparison to positive control (TS2). Similarly, PN was significantly increased in TS2 and TS3 treated field, compared to TS1 (Fig. 2a, b). The difference in seed number (SN) was not significantly distinct in case of ground nut at $p > 0.05$ level (Fig. 2a), however, this difference was significant in case of sesame (Fig. 2b).

The yield parameters directly prove the beneficiary effect of the selected bacterium

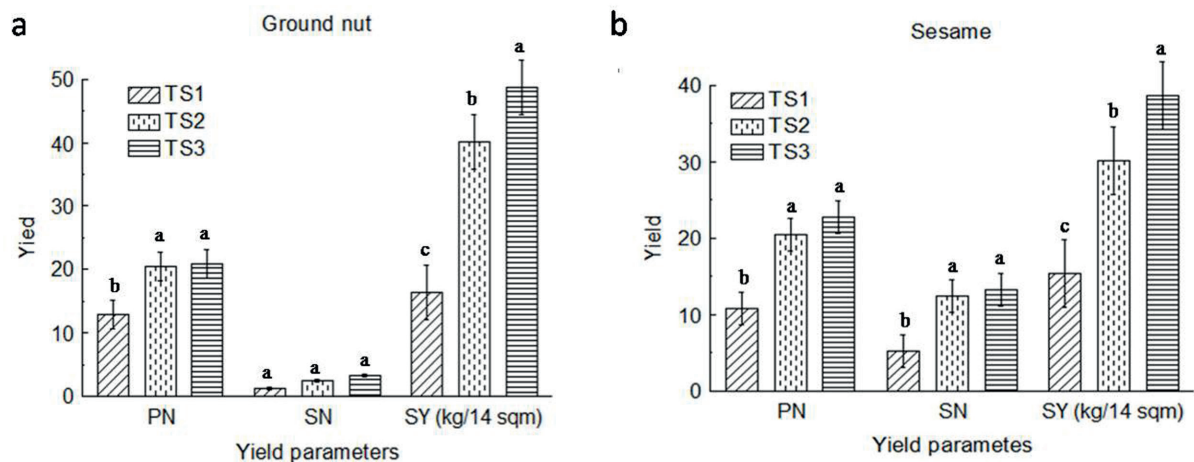


Figure 2 - Use of bio-controlling agent in agricultural field. **a:** Effect of *B. cereus* IB311 on ground nut (*Arachis hypogaea*) production in field trial experiments. **b:** Effect of *B. cereus* IB311 on sesame (*Sesamum indicum*) production in field trial experiments. PN- average pod number per plant, SN- average seed number per pod, SY- seed yield per experimental plot. TS1- untreated experimental plots (negative control), TS2- experimental plots treated with 100% recommended dose of NPK (positive control), TS3- experimental plots treated with *B. cereus* IB311.

B. cereus IB311. In the present investigation, *B. cereus* IB311 was found to possess anti-phytopathogen activity in laboratory condition and found to enhance yield in the field. Therefore, we may conclude that anti-phytopathogen activity of the strain might be one of the reasons of yield enhancement in the field. Previously, several reports have been published regarding the inhibition of plant pathogens (Makovitzki et al. 2007, Ramzan et al. 2014, Ahemad and Kibret 2014); however, the information regarding the direct application of such bio-controlling agent in agricultural field is scanty.

OPTIMIZATION OF BIOMASS PRODUCTION BY RSM

In this present investigation, CCD was used and experimental data of 20 runs were summarized in Table II, which indicated the interaction between different variables (glucose, beef extract and inoculum size). Among the three parameters studied in this investigation, the inoculum size was found to be an insignificant variable. According to the results obtained from the analysis of variance, a second degree polynomial model was fitted to this present experiment (Table IV). The P value for lack of fit ($P>F$) is 0.0074, suggesting that this model adequately fits the data and there is only 0.74% chance that it is due to noise. The “lack of fit” F value is 6.51, which indicated an insignificant

lack of fit. The model F value of 10.32 and low probability value imply the significant model fit (Table IV). The coefficient of determinant (R^2) was 0.8902, which indicated the variability of the model, as well as real relationship between variables. This R^2 value explained the variability of the model by 99.9% and only 0.1% by chance. The coefficient of variance (C.V.) of the model was 8.24%, which indicated the degree of precision. The adequate precision value was 10.535, which simply indicated the signal to noise ratio (Table V). According to Cao and Jin (2004), the desired adequate precision value must be greater than 4 for reliability of model. Multiple regression analysis of the experimental data, followed by second degree polynomial equation (3) was found to describe the interaction between glucose, beef extract and inoculum size for *B. cereus* IB311 production.

$$\begin{aligned} \text{Biomass (dry wt.)} = & 1.65423 + 0.23049\text{Glucose} \\ & + 3.35353\text{Beefextract} - 0.11821\text{Inoculumsize} \\ & - 0.142111\text{Glucose} \times \text{Beefextract} + 0.13684\text{Glucose} \\ & \times \text{Inoculumsize} - 0.043750\text{Beefextract} \times \text{Inoculum} \\ & \text{size} - 0.026320\text{Glucose}^2 - 0.60625\text{Beefextract}^2 - \\ & 0.11607\text{Inoculumsize}^2 \end{aligned} \quad (3)$$

Where, glucose, beef extract and inoculum size are the three variables already mentioned in Table II. The three dimensional surface plots were drawn from the inter-relationship of two variables

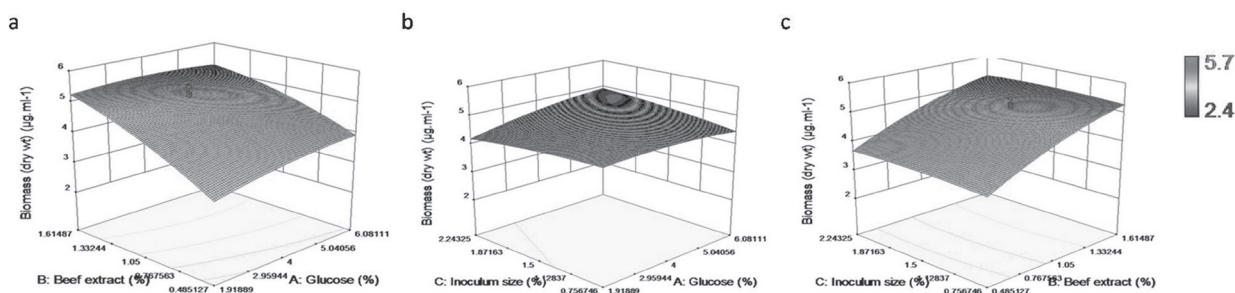


Figure 3 - Response surface graph of *B. cereus* IB311 production in terms of biomass (dry wt.). **a**: The effect of glucose and beef extract on *B. cereus* IB311 production. **b**: The effect of glucose and inoculum sizes on *B. cereus* IB311 production. **c**: The effect of beef extract and inoculum size on *B. cereus* IB311 production.

TABLE IV
ANOVA for quadratic model showing interaction between three variables.

Source	Sum of square	Degree of freedom	Mean square	F value	P value (Prob>F)
Model	12.05	9	1.34	9.00	0.0010
A-Glucose	0.22	1	0.22	1.47	0.2530
B-Beef extract	10.29	1	10.29	69.24	<0.0001
C-Inoculum size	5.240E-003	1	5.240E-003	0.035	0.8548
AB	0.32	1	0.32	2.14	0.1741
AC	0.13	1	0.13	0.88	0.3697
BC	1.589E-003	1	1.589E-003	0.011	0.9197
A2	0.15	1	0.15	0.98	0.3454
B2	1.14	1	1.14	7.69	0.0197
C2	0.052	1	0.052	0.35	0.5678
Residual	1.49	10	0.15		
Lack of fit	1.30	4	0.32	10.32	0.0074
Pure error	0.19	6	0.031		
Cor total	13.53	19			

TABLE V
Statistical information of the model.

Source	Response
R square	0.8902
Adjusted R square	0.7913
Coefficient of variation (%)	8.24
Standard deviation	0.39
Adequate precision	10.535

considering one variable constant. Surface response of the plots indicates the effect of glucose, beef extract and inoculum size on *B. cereus* IB311 production (Fig. 3). In order to determine the optimum conditions of *B. cereus* IB311 production, combined effect of these three variables was also checked. Theoretically, the optimum biomass of *B. cereus* IB311 (5.383 µg/ mL) can be obtained considering glucose 1.985%, beef extract 1.615% and inoculum size 0.757%. To validate the data, we have conducted an experiment with these possible values. Results obtained were 5.45 µg/ ml

dry weight produced at glucose 2%, beef extract 1.5% and inoculum size 0.75%.

Optimization of nutritional (carbon source and nitrogen source) is one of the most important steps to enhance the production in both laboratory experiments, as well as in fermentation industries. Changes in one of these parameters can have a dramatic effect on the yield of cells and the stability of protein product. Many bacterial bio-controlling agents are used in the field directly including *Bacillus thuringiensis* and *Serratia entomophila* (Chattopadhyay et al. 2017). Product formulation also requires mass production of BCA (Chattopadhyay et al. 2017). Therefore, in the present investigation, we opt for optimization of mass production of the selected strain. Results of this study are consistent with those of previous studies, where the culture media had a significant influence on production of *B. cereus* (De Sarrau et al. 2012, Singh et al. 2013). Singh et al. (2013) used response

surface methodology and artificial neural network to optimize cultural conditions of L-glutaminase production from *Bacillus cereus* MTCC 1305. The production of L-glutaminase was enhanced by 1.58-fold after optimization of cultural conditions.

CONCLUSIONS

This is the first report on use of response surface methodology to improve production of bio-controlling agent like *Bacillus cereus*. The application of response surface methodology not only resulted in an enhancement in biomass production but also minimized the production cost. Our investigation also has clearly demonstrated that the *B. cereus* IB311 has beneficiary effect in agricultural field. Due to safe, cost effective and positive impact on agricultural field, this bacterial strain will be a good bio-controlling candidate.

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