Biocatalysis and Agricultural Biotechnology 18 (2019) 101011

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Optimization of protease production in indigenous *Bacillus* species isolated from soil samples in Lagos, Nigeria using response surface methodology

Yewande Suberu[a,b,*], Idowu Akande[a], Titilola Samuel[a], Adekunle Lawal[b], Ademola Olaniran[c]

[a] Department of Biochemistry, College of Medicine, University of Lagos, PMB 12003, Lagos
[b] Department of Biotechnology, Federal Institute of Industrial Research, Oshodi, PMB 21023, Lagos, Nigeria
[c] Department of Microbiology, College of Agriculture, Engineering and Sciences, University of KwaZulu-Natal, Westville Campus, Private Bag X54001, Durban, South Africa

ARTICLE INFO

Keywords:
Bacillus isolates
Box-Behnken design
Protease activity
Protease optimization

ABSTRACT

Proteases catalyse the hydrolysis of peptide bonds in proteins and offer a huge potential for application in industries, including detergent, dairy, leather, baking, pharmaceutical and beverage industries. In this study, indigenous *Bacillus* species were isolated from soil samples collected from abattoir, refuse and non-refuse sites in Lagos, Nigeria and optimized for protease production. The isolates were purified on *Bacillus* agar and screened for protease production on casein agar. Three strains showing high potential for protease production were identified as *Bacillus cereus* ABBA1, *Bacillus subtilis* RD7 and *Bacillus subtilis* NRD9 via amplification and analysis of 16S rRNA genes. Protease optimization was done Insilco using Box-Behnken Design (BBD) by response surface methodology (RSM) with Design-Expert software and then validated experimentally. Factors optimized include temperature, pH, carbon and nitrogen source and inoculum density. Statistical analysis was done using ANOVA. The results obtained from the Insilco experimental model revealed high protease activity of 159.43 U/ml, 141.28 U/ml and 138.17 U/ml while experimental validation generated a high protease activity of 200.56 U/ml, 176.00 U/ml and 163.76 U/ml for strains ABBA1, RD7 and NRD9, respectively in optimized medium. This corresponds to 33.54-, 42.21- and 36.64- fold increase in protease production compared to the unoptimized protease production medium. The optimum conditions for extracellular protease production obtained from quadratic model of RSM were 40 °C, pH 8.5, 2.5% (v/v) inoculum density, 1.5 g/L maltose and 2.0 g/L beef extract powder. The model prediction agreed with the experimental data ($R^2 = 0.98$) and was statistically significant ($p \leq 0.05$). This results further confirms the need to optimize the production parameters to achieve maximum yield and economical use of available resources during production of industrially important enzymes.

1. Introduction

Proteases (E.C 3.4) are enzymes that hydrolyse peptide bonds between amino acid groups of proteins. Hydrolytic enzymes constitute about 75% of the worldwide sales of industrial enzymes used in various applications with proteolytic enzymes constituting about 60%. (Ninghoujam and Kshetri, 2010; Rai et al., 2010; Ningthoujam et al., 2009 and Chu, 2007). They are ubiquitously found in a wide diversity of sources such as plants, animals, and microorganisms but microbial sources are preferred to produce these proteases due to their technical and economic advantages including; rapid growth, limited space required for their cultivation, and ease of genetic manipulation (Rai et al., 2010).

Microbial proteases have wide potentials for application in different industries including detergent, dairy, leather, baking, pharmaceutical and beverage industries. These hydrolytic enzymes are involved in the food industry for enhancing nutritional value, digestibility, palatability, flavour and reducing allergenic compounds as well as in the management of domestic and industrial wastes, they are also involved in the synthesis and structural elucidation of proteins (Singh, 2016).

Currently, the worldwide sales of industrial enzymes are estimated at about $4.2 billion in value (Singh, 2016). Proteases which constitutes one of the three largest groups of industrial enzymes are projected to reach a global market of approximately $ 2.21 billion in terms of value by 2021 at a Compound Annual Growth Rate (CAGR) of 6% from 2016 to 2021. Proteases of microbial origin accounts for the largest share in the market in terms of value, followed by the animal and plant source respectively (Proteases Market by Source, 2016). Of all the bacterial genera used for production of various industrially important enzymes, the *Bacillus* genus is the most widely exploited for production of various

* Corresponding author. Department of Biochemistry, College of Medicine, University of Lagos, P.M.B 12003, Lagos, Nigeria.
E-mail address: talktoyewande@yahoo.com (Y. Suberu).

https://doi.org/10.1016/j.bcab.2019.01.049
Received 18 October 2018; Received in revised form 24 January 2019; Accepted 27 January 2019
Available online 06 February 2019
1878-8181/

enzymes.

Bacillus species are an important source of antibiotics, enzymes, insecticides and vitamins (Dave et al., 2015). *Bacillus* species have been the major workhorse of industrial microorganisms with roles in microbiology. These *Bacillus* species are attractive industrial organisms because of their high growth rates leading to short fermentation cycle times, capacity to secrete proteins into the extracellular medium and their GRAS (generally regarded as safe) status with the Food and Drug Administration for species, such as *Bacillus subtilis* and *Bacillus licheniformis* (Alcaraz et al., 2010). Presently there is ample information about the biochemistry, genetics and physiology of the bacteria *Bacillus*, which could facilitate further development and greater exploitation of these indigenous *Bacillus* for protease production by industries in Nigeria. However, this does not seem to be the case as commercial production of these proteases are underexploited in the country and may require optimization of the production process.

Response surface methodology (RSM) is applied to optimize and model certain number of variables leading to the optimization of various environmental parameters as well as the culture parameters which may enhance the production of value-added products of commercial importance to many folds. (Dave, et al., 2015). This leads to improved yield of enzymes and in turn reduction in production cost for their various commercial applications in the food, detergent and pharmaceutical industries (Dave, et al., 2015; Ferreira et al., 2009). Usually, researchers employ “one parameter at a time” strategy for the optimization; but this method is time consuming and hectic, therefore, the alternative utilization of statistical tools which makes the process easier is being explored. Response surface methodology (RSM) is an example of a statistical tool being applied widely for the optimization, modelling and analysis of problems related to the production of industrial enzymes (Benjamin et al., 2014a,b) and it was therefore applied in the present study to obtain the better yield of the protease enzyme. This study thus, focuses on the isolation of indigenous *Bacillus* species from soil samples and statistical optimization of the production of protease from these indigenous *Bacillus* species employing RSM technique.

2. Materials and methods

2.1. Sample collection

Soil samples for isolation of microbes were obtained from the 5–10-cm layers below soil surface at an Abattoir (ABBA) (GPS coordinates: 6°32'59.19''N, 3°20'25.1082E), Refuse dump site (RD) (GPS coordinates: 6°32'47.2956''N, 3°21'4.4028 E), and Non-refuse dump site (NRD) (GPS coordinates: 6°32'47.2956''N, 3°21'4.4028 E) in Lagos Nigeria in February 2016. The soil samples were collected using sterile McCartney bottles and taken to the laboratory for further processing.

2.2. Isolation of Bacillus species from soil samples

One (1) gram of each soil sample was accurately and aseptically weighed into 9 ml of sterile distilled water in a test tube. These tubes were placed in a water bath at 90 °C and maintained at this temperature for 30 min. Serial dilution was carried out and from an appropriate dilution of 10^{-4}, 10^{-5} and 10^{-6} of each soil sample (ABBA, RD and NRD) respectively, 0.1 ml of each sample was inoculated into *Bacillus* and nutrient agar (NA) using the pour plate method and swirled. The plates were allowed to solidify and incubated at 37 °C for 24 h. The *Bacillus* isolates were purified by successive streaking into freshly prepared *Bacillus* and nutrient agar. The pure isolates were then screened for protease production by inoculating them onto fresh Nutrient agar supplemented with 2% casein and incubation at 37 °C for 24 h. Zone of clearing which is indicative of protease production was observed and measured. Isolates with the highest zone of clearing were kept for further studies.

2.3. Identification of protease producing organism

The cellular morphology of the bacterial isolates was examined under high powered microscope. Morphological identification was done by gram staining following standard microbiological procedures. Biochemical tests of bacterial isolates were performed using Analytical Profile Index (API) kits (bioMerieux API, USA). Further, the identification of the selected bacteria isolates (ABBA1, RD7 and NRD9) was confirmed by the amplification of the 16S rRNA gene using the oligonucleotide primers: 63f (5′-CAGGCCTAACACATGCAAGTC-3′) and 1387r (5′GGGCGG(A/T) GTGTACAAGGC-3′) and conditions previously designed and described by Marchesi et al. (1998). DNA extraction was done using the Quick-DNA Fungal/Bacterial Miniprep kit following manufacturer's instructions (Zymo Research Corp. USA). PCR was performed in a 25 µl reaction consisting of 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM of dNTPs, 0.5 µM of each primer and 2U of Supertherm Taq polymerase (Southern Cross Biotech., Cape Town, South Africa). Thermal cycling was done in a T100 thermal cycler (Bio-Rad, Hercules, California, USA) at the following cycling conditions:95 °C for 10 min and 35 cycles of 94 °C for 30 s, 55 °C for 10 s and 72 °C for 45 s and a final elongation at 72 °C for 10 min. The amplified DNA (5 µl) was electrophoresed in a 1.5% agarose gel at 70 V for 60 min. Thereafter, the gel was stained in Ethidium bromide for 10 min and visualized under a UV light (Syngene, Cambridge. UK). The amplified products were sequenced at a biotechnology lab (Inqaba Biotech, Pretoria, South Africa) and the sequences edited using Chromas Ver. 2.2.4 (Technelysium Pty Ltd, Brisbane, Australia). The sequences obtained were then compared against the sequences in GenBank database using the basic local alignment search tool (BLAST) to identify the organism (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree and Evolutionary analyses was constructed with 1000 bootstrap replicates by using MEGA 7 version 5.2 software (Tamura et al. 2011; Kumar et al., 2016).

2.4. Production of protease

2.4.1. Inoculum preparation and inoculation

The bacterial culture *Bacillus cereus* strain ABBA1, *Bacillus subtilis* strain RD7 and *Bacillus subtillis* strain NRD9 obtained from this study was subjected to protease production by submerged fermentation. For the growth of inoculum, a loopful of bacteria culture was transferred from stock to 100 ml Luria bertani broth in 250 ml Erlenmeyer shake flask and the inoculated flasks were incubated overnight at 37 °C and 150 rpm. Cells were harvested from the broth and their absorbance (A) was measured at 600 nm. The cells were standardized to Optical Density of 1 (Dave et al., 2015). The prepared Inoculum was used for submerged fermentation.

2.4.2. Selection of carbon and nitrogen sources

In the preliminary study, several carbon and nitrogen sources were tested by using one factor optimization method. Different substrates such as glucose, fructose, maltose, sucrose, lactose and starch were used as carbon source for the better production of protease, similarly different nitrogen substrates like yeast extract, beef extract, malt extract, peptone, gelatin and ammonium chloride were added as organic nitrogen sources. 1% w/v of each substrate was added into 100 ml of basal medium used for protease production and thereafter 1 ml of inoculum was inoculated into the medium and then subjected to submerged fermentation.

2.4.3. Submerged fermentation

The production of protease was studied in 250 ml Erlenmeyer shake flasks containing various carbon and nitrogen sources as a substrate. The basal medium used for protease production was (g/L), potassium dihydrogen phosphate (0.6), dipotassium hydrogen phosphate (0.3), sodium chloride (0.5), magnesium sulphate (0.1) and distilled H_20 to make 1 L (Aygan et al., 2011). The flask was sterilized at 121 °C for

15 min at 15 lbs pressure, allowed to cool and inoculated with 1 ml (v/v) inoculum (Dave et al., 2015). The contents of the flasks were mixed thoroughly and incubated at 37 °C for 24 h.

2.5. Optimization of fermentation parameters by response surface methodology (RSM) using Box-Behnken Design (BBD)

2.5.1 Protease production by submerged fermentation: RSM using BBD was used to optimize protease production which involves full factorial search by observing simultaneous, systematic and efficient variation of important components on the fermentation process based on the preliminary findings. The production of protease was studied in flasks containing varying concentrations of the selected carbon and nitrogen source as a substrate. The basal medium used for protease production was (g/L), potassium dihydrogen phosphate (0.6), dipotassium hydrogen phosphate (0.3), sodium chloride (0.5), magnesium sulphate (0.1) and distilled H_20 to make 1 L (Aygan et al., 2011). Five important parameters namely initial pH of the medium (X1), temperature (X2), maltose concentration (X3), beef extract concentration (X4) and inoculum density (X5), were selected as independent variables and the protease enzyme activity (U/ml) was the dependent response variable. Each of these independent variables were studied at three different levels according to BBD in five variables with a total of 46 experimental runs. The flask was sterilized at 121 °C for 15 min at 15 lbs pressure, allowed to cool and inoculated with varying (w/v) inoculum. The contents of the flasks were mixed thoroughly and incubated at various temperatures for 24 h. Protease activity (U/ml) resulting from the combined effects of five variables was studied in their specified ranges as shown in Table 1(a, b, c). The flasks were analysed for protease activity.

2.5.1. Statistical optimization of protease production

Box-Behnken Design (BBD) by RSM was employed to develop a mathematical correlation model between different independent variables such as pH, temperature, maltose concentration, beef extract concentration and inoculum density on the production of protease. The software Design-Expert version 11 (Stat-Ease Inc., Minnesota, United States.) was used to generate data and to analyse the experimental design of BBD in RSM. BBD at three levels (+1, 0, and −1) designated as high, medium and low was used for this study. Based on this design, a set of 46 experimental setup were suggested by the software. All the setup was validated in the laboratory in triplicate and the results were analysed by fitting to a second-order polynomial Equation (1). Each experimental trial was set up and protease was harvested at 24 h to measure protease activity as per the design.

Equation (1): where Y represents the response variable; β0 is the interception coefficient; βi is the coefficient of the linear effect; βii is the coefficient of quadratic effect; βij is the coefficient of interaction effect when i < j; and k is the numbers of involved variables.

$$Y = \beta_0 + \Sigma\beta i\ xi + \Sigma\beta ii\ xi^2 + \Sigma\Sigma\ \beta ij\ xi\ xj$$

Table 1a
Experimental range and coded levels of process variables for protease production by Bacillus cereus ABBA1.

PROCESS VARIABLES	RANGE AND LEVELS		
	−1	0	+1
Initial pH (X_1)	6.5	7.5	8.5
Temperature (X_2)	30	40	50
Maltose concentration (X_3)	1%	1.5%	2%
Beef extract concentration (X_4)	0.5%	1%	2%
Inoculum density (X5)	2%	2.5%	3%

Table 1b
Experimental range and coded levels of process variables for protease production by *Bacillus subtillis* RD7.

PROCESS VARIABLES	RANGE AND LEVELS		
	−1	0	+1
Initial pH (X_1)	6.5	7.5	8.5
Temperature (X_2)	30	35	40
Maltose concentration (X_3)	1%	1.5%	2%
Beef extract concentration (X_4)	0.5%	1%	2%

Table 1c
Experimental range and coded levels of process variables for protease production by *Bacillus subtillis* NRD9.

PROCESS VARIABLES	RANGE AND LEVELS		
	−1	0	+1
Initial pH (X_1)	7	7.5	8
Temperature (X_2)	35	40	45
Maltose concentration (X_3)	1%	1.5%	2%
Beef extract concentration (X_4)	0.5%	1%	2%

2.5.2. Validation experiment

To check the validity of quadratic model, five fermentation variables as predicted by Design-Expert prediction software were performed. Experiments were carried out in shake flasks under predicted conditions to validate the model. Protease activity was estimated and compared with predicted values. The crude protease enzyme from each flask was extracted via centrifugation at 13,000 rpm for 15 min at 4 °C. The supernatant was decanted and used to determine enzyme activity.

2.6. Protease enzyme assay

Proteolytic activity was measured following the method of Kole et al. (1988) using azocasein (Sigma, USA) as a substrate. The reaction mixture contains 120 µL of enzyme extract, 480 µl of azocasein (1% w/v) dissolved in 0.2 M Tris HCl buffer pH 7.2 and incubated at 37 °C in a heating block for 30 min. The reaction was terminated by addition of 600 µL of Trichloro acetic acid (TCA), kept on ice for 30 min and centrifuged at 13,200 rpm for 15 min. 800 µl of the supernatant was neutralised by addition of 200 µl of 1.8N NaOH, mixed properly and absorbance was read at wavelength 440 nm. One unit of enzyme activity was defined as the amount of enzyme required to cause an absorbance change of 1.0 at 440 nm in a cuvette of 1.0 cm path length under the conditions of the assay.

2.7. Statistical analysis

Statistical analysis of all results was carried out using Duncan multiple range test, Standard deviation and ANOVA accordingly using IBM SPSS Statistics Ver.22.0 (IBM Corporation, United States of America.). Statistically significant at ($p \leq 0.05$).

3. Results

3.1. Isolation and screening of protease-producing bacteria

A total number of forty-eight (48) *Bacillus* strains were isolated from the soil samples. Table 2a shows the ten (10) isolates that gave clear zones when screened on casein agar and hence were regarded as the potential producers of protease. Isolates RD 7, ABBA 1 and NRD 9 gave

Table 2a
Zones of clearance (MM) Of *Bacillus* isolates.

S/N	ISOLATES CODE	ZONE OF CLEARANCE (MM)		AVERAGE ± SD
		Z1	Z2	
1	RD 7	48	50	49 ± 1.414^{f}
2	ABBA 1	47	47	47 ± 0.000$^{e, f}$
3	RD 9	44	46	45 ± 1.414^{e}
4	ABBA 6	41	43	42 ± 1.414^{d}
5	RD 16	39	41	40 ± 1.414$^{c, d}$
6	NRD 6	37	39	38 ± 1.414$^{b, c}$
7	NRD 9	39	37	38 ± 1.414$^{b, c}$
8	RD 5	36	38	37 ± 1.414$^{b, c}$
9	ABBA9	34	36	35 ± 1.414$^{a, b}$
10	RD 10	33	33	33 ± 0.000^{a}

Means followed by the same letter along the rows are not significantly different at $P < 0.05$ (Duncan multiple range tests). S.D (Standard deviation). Z1 and Z2 are replicates 1 and 2 respectively.

Table 2b
Identification of Bacteria isolated from soil samples in Lagos, Nigeria.

Organism ID	Identity	% Similarity	Max Score
ABBA1	97	*Bacillus cereus*	1352
RD7	99	*Bacillus subtilis*	1504
NRD9	100	*Bacillus subtilis*	1478

the highest zones of clearance (49 ± 1.414, 47 ± 0.000 and 38 ± 1.414) respectively from the different soil samples.

3.2. Identification of bacterial isolates and phylogenetic study

Table 2b shows the identity of these bacterial isolates identified via the amplification of the 16S rRNA gene sequence in this study. Most of the isolate from the soil samples were of the genus *Bacillus* with 97-100% similarity. The identity of these bacterial isolates was identified by API test and confirmed by comparing the sequence of the amplified 16S rRNA gene against sequences deposited in GenBank database. The isolates were identified as *Bacillus cereus* (ABBA1) and *Bacillus subtilis* (RD7 and NRD9). Figs. 1–3shows the phylogenetic tree based on these sequences. Therefore, the isolates ABBA1, RD7 and NRD9 were identified as a strain of *Bacillus* and named *Bacillus cereus* strain ABBA1, *Bacillus subtilis* strain RD7 and *Bacillus subtilis* strain NRD9. The obtained sequences were deposited in the GenBank database with accession number: MG255316, MG255317 and MG255318 respectively.

3.3. Selection of carbon and nitrogen source

The one factor at a time optimization of carbon and nitrogen sources for protease production shows that maltose gave the highest protease activity of (681.71, 693.41 and 671.21) U/ml for the three *Bacillus* isolates as compared to the other sources of carbon tested (Table 3a). Beef extract gave the highest protease activity of (766.37, 785.25 and 748.31) U/ml as shown in (Table 3b) when compared with other nitrogen sources during protease production by the *Bacillus* isolates.

3.4. Optimization of protease production by Box-Behnken Design (BBD)

Five parameters (temperature, pH, maltose concentration, beef extract concentration and inoculum density) were considered for Box-Behnken Design (BBD) analysis by RSM to find out the optimum conditions for maximising the production of protease by *Bacillus cereus* ABBA1. A set of 46 experiments were conducted according to BBD. The highest protease activity in the experimental design was found to be 159.43 U/ml at 40 °C, pH 8.5, maltose conc. (1.5%), beef extract powder (2%), inoculum density (2.5 ml) after 24 h of incubation (Table 4b) and the results showed that the predicted and experimental values for protease activity did not show significant difference and was statistically significant ($p \leq 0.05$) (Table 4a). A second order polynomial equation was fitted to the experimental protease activity which resulted in the following regression Equation (2a).

$$Y = +91.76 + 0.58X_1 + 7.68X_2 + 8.30X_3 + 6.02X_4 + 8.39X_5 + 10.37X_1^2 + 9.02X_2^2 + 8.05X_3^2 + 3.33X_4^2 + 0.055X_5^2 + 6.28X_1X_2 + 9.93X_1X_3 + 3.29X_1X_4 + 0.72X_1X_5 + 3.41X_2X_3 + 6.89X_2X_4 + 8.63X_2X_5 + 8.72X_3X_4 + 8.46X_3X_5 + 2.37X_4X_5$$

Where X1 - initial pH of the medium, X2 - temperature, X3 - maltose concentration, X4 - beef extract concentration and X5 - inoculum density. The model was used to generate response surfaces based on the results obtained for the analysis of the variable effect on the production of protease. The response surface plots obtained using equation (2a) is depicted in Fig. 4a.

3.4.1. Validation of protease production

Fig. 2a shows the optimum value of the combination of the five

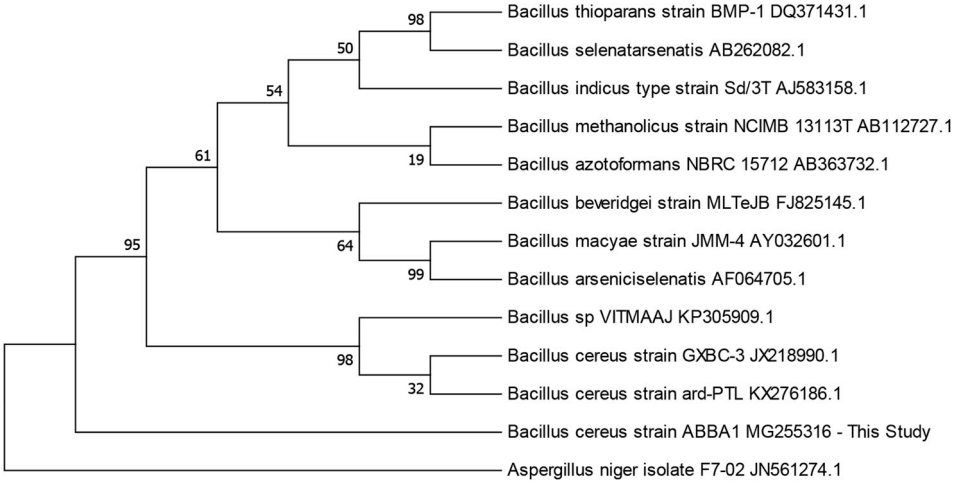

Fig. 1. Phylogenetic dendrogram based on the 16S rRNA sequence of Bacillus cereus strain ABBA1. Number in parenthesis are accession numbers of published sequences. Bootstrap values were based on 1000 replicates (Saitou and Nei, 1987).

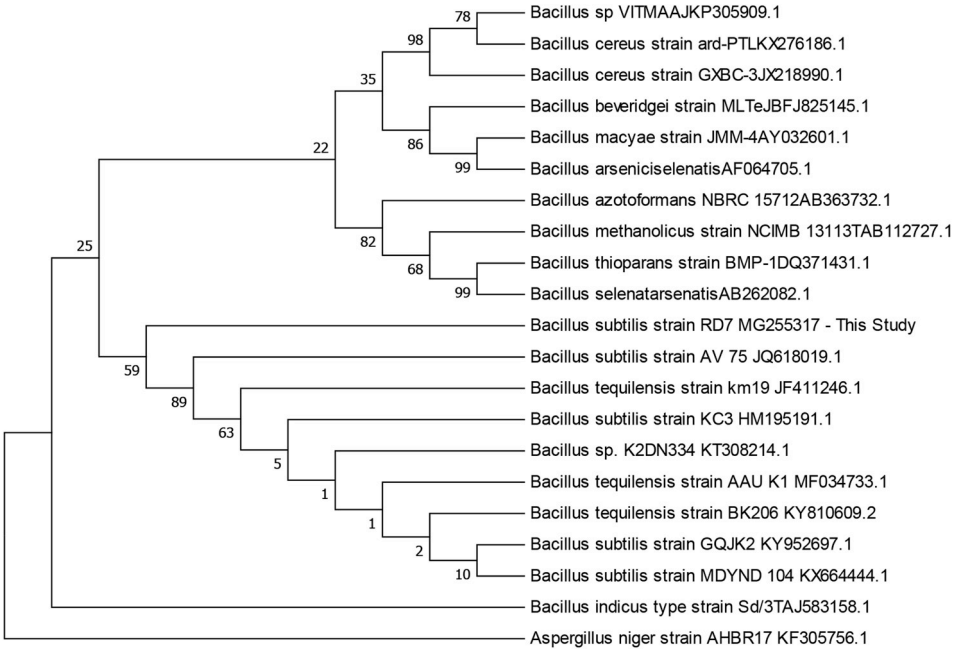

Fig. 2. Phylogenetic dendrogram based on the 16S rRNA sequence of Bacillus subtilis strain RD7. Number in parenthesis are accession numbers of published sequences. Bootstrap values were based on 1000 replicates (Saitou and Nei, 1987).

fermentation variables determined for maximum production of protease by *Bacillus cereus* ABBA1. There was high degree of similarity between the predicted and experimental values, showing the validity of the RSM (Fig. 5a). The model prediction was in good agreement with the experimental data and correlation coefficient was found to be 0.97 (Table 4a). Correlation coefficient was close to 1, suggesting the significance of the model. The optimum production of protease in the experimental validation was found to be 200.56 U/ml (at 42 °C, pH 8.5, maltose conc. 1.96 %w/v, beef extract powder 1.96 %w/v, inoculum density 2.99 ml). Thus, the statistical optimization resulted in 33.54 folds of protease activity over the un optimized condition.

Table 3a
Optimization of carbon sources for protease production by *Bacillus* isolates.

Carbon sources	Protease activity ± SD (U/ml)		
	Bacillus cereus ABBA1	*Bacillus subtilis* RD7	*Bacillus subtilis* NRD9
GLUCOSE	564.40 ± 5.42	554.60 ± 4.72	570.14 ± 3.02
SUCROSE	486.38 ± 5.38	457.36 ± 4.08	453.64 ± 3.35
FRUCTOSE	600.37 ± 2.74	620.28 ± 2.53	612.93 ± 4.37
LACTOSE	555.82 ± 5.84	520.42 ± 3.44	505.84 ± 4.54
MALTOSE	681.71 ± 4.10	693.41 ± 2.15	671.21 ± 4.16
STARCH	517.64 ± 3.20	523.94 ± 5.08	530.64 ± 3.70

3.5. Insilco-optimization of protease production

Four parameters (temperature, pH, maltose concentration and beef extract concentration) were considered for BBD analysis, followed by RSM to find out the optimum conditions for maximising the production of protease by *Bacillus subtilis* RD7. A set of 29 experiments was conducted according to. BBD. The optimum protease activity in the experimental design was found to be 141.28 U/ml at 40 °C, pH 8.5, maltose conc. (1.5 %w/v), beef extract powder (1.25 %w/v), inoculum density (2.5 ml) at 24 h of incubation and the results showed that the predicted and experimental values for protease activity did not show

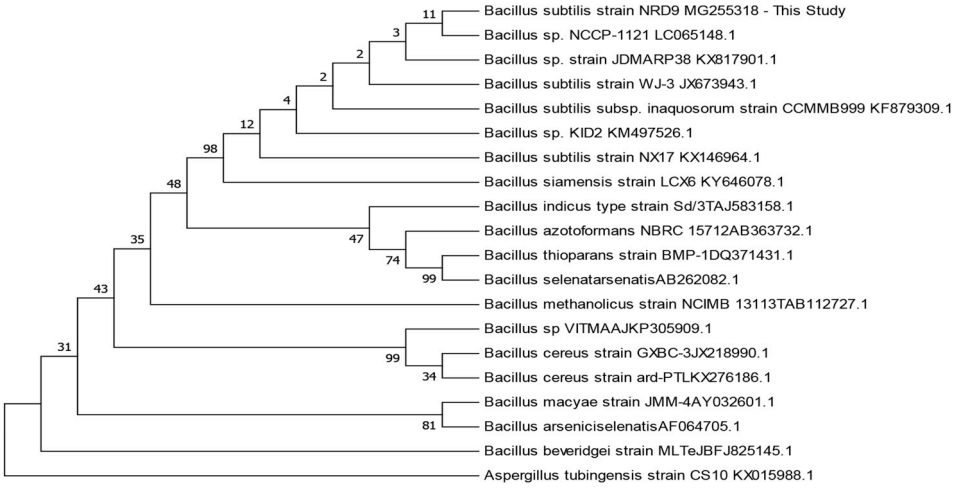

Fig. 3. Phylogenetic dendrogram based on the 16S rRNA sequence of Bacillus subtillis strain NRD9. Number in parenthesis are accession numbers of published sequences. Bootstrap values were based on 1000 replicates (Saitou and Nei, 1987).

Table 3b
Optimization of nitrogen sources for protease production by *Bacillus* isolates.

Nitrogen sources	Protease activity ± SD (U/ml)		
	Bacillus cereus ABBA1	*Bacillus subtilis* RD7	*Bacillus subtilis* NRD9
YEAST EXTRACT	659.57 ± 2.82	643.51 ± 2.42	649.09 ± 3.52
BEEF EXTRACT	766.37 ± 2.74	785.25 ± 3.54	748.31 ± 3.44
MALT EXTRACT	541.99 ± 3.11	563.25 ± 2.45	561.39 ± 4.60
PEPTONE	686.41 ± 2.95	653.59 ± 4.05	636.73 ± 2.05
GELATIN	516.81 ± 3.06	524.01 ± 3.36	509.22 ± 2.25
AMMONIUM CHLORIDE	488.32 ± 2.07	463.49 ± 2.39	459.12 ± 3.37

significant difference and was statistically significant ($p \leq 0.05$) (Tables 5a and 5b) i.e.., the R^2 value was 0.98 close to unity. A second order polynomial equation was fitted to the experimental protease activity which resulted in the following regression Equation (2b).

$$Y = +82.44 + 4.56X_1 + 7.95X_2 + 7.46X_3 + 1.05X_4 + 1.31X_1^2 + 7.65X_2^2 + 2.91X_3^2 + 7.34X_4^2 + 11.07X_1X_2 + 5.69X_1X_3 + 8.47X_1X_4 + 0.67X_2X_3 + 0.30X_2X_4 + 8.71X_3X_4$$

Where X1 - initial pH of the medium, X2 - temperature, X3 - maltose concentration and X4 - beef extract concentration. The model was used to generate response surfaces based on the results obtained for the analysis of the variable effect on the production of protease. The response surface plots obtained using equation (2b) is depicted in Fig. 4b.

3.5.1. Validation of protease production

Fig. 2b shows the optimum value of the combination of the five fermentation variables determined for maximum production of protease *Bacillus subtilis* RD7. There was high degree of similarity between the predicted and experimental values, showing the validity of the RSM (Fig. 5b). The model prediction was in good agreement with the experimental data and correlation coefficient was found to be 0.98 (Table 5a). Correlation coefficient was close to 1, suggesting the significance of the model. The optimum production of protease in the experimental validation was found to be 176.00 U/ml (at 39 °C, pH 8.3, maltose conc. 1.96 %w/v, beef extract powder 1.92 %w/v, inoculum density 2.99 ml). Thus, the statistical optimization resulted in 42.21 folds of protease activity over the unoptimized condition.

3.6. Optimization of protease production by Box-Behnken Design (BBD)

Four parameters (temperature, pH, maltose concentration and beef extract concentration) were considered for BBD analysis, followed by RSM to find out the optimum conditions for maximising the production of protease *Bacillus subtilis* NRD9. A set of 29 experiments was conducted according to BDD. The optimum production of protease in the experimental design was found to be 138.17 U/ml at 40 °C, pH 7.5, maltose conc. (2.0 %w/v), beef extract powder (2.0 %w/v), inoculum density (2.5 ml) after 24 h of incubation and the results showed that the predicted and experimental values for protease activity did not show significant difference and was statistically significant at ($p \leq 0.05$) (Tables 6a and 6b) i.e.., the R^2 value was 0.98 close to unity. A second order polynomial equation was fitted to the experimental protease activity which resulted in the following regression Equation (2c).

$$Y = +81.26 + 6.66X_1 + 4.57X_2 + 7.45X_3 + 1.46X_4 + 8.39X_1^2 + 5.42X_2^2 + 4.37X_3^2 + 5.41X_4^2 + 9.57X_1X_2 + 10.91X_1X_3 + 8.03X_1X_4 + 5.49X_2X_3 + 8.47X_2X_4 + 9.63X_3X_4$$

Where X1 - initial pH of the medium, X2 - temperature, X3 - maltose concentration and X4 - beef extract concentration. The model was used to generate response surfaces based on the results obtained for the

Table 4a
Statistical analysis of box benkhen design for protease production in Bacillus cereus ABBA 1.

ANOVA for Response Surface Quadratic model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	6901.14	20	345.06	53.75	< 0.0001	Significant
A-Initial pH	*5.42*	*1*	*5.42*	*0.84*	*0.3671*	
B-Temperature	*944.49*	*1*	*944.49*	*147.13*	*0.0001*	
C-Maltose concentration	*1102.07*	*1*	*1102.07*	*171.68*	*< 0.0001*	
D-Beef extract concentration	*579.61*	*1*	*579.61*	*90.29*	*< 0.0001*	
E-Inoculum concentration	*1126.44*	*1*	*1126.44*	*175.47*	*< 0.0001*	
AB	*430.15*	*1*	*430.15*	*67.01*	*< 0.0001*	
AC	*325.44*	*1*	*325.44*	*50.70*	*< 0.0001*	
AD	*259.53*	*1*	*259.53*	*40.43*	*< 0.0001*	
AE	*44.29*	*1*	*44.29*	*6.90*	*0.0145*	
BC	*0.012*	*1*	*0.012*	*1.885E-003*	*0.9657*	
BD	*157.50*	*1*	*157.50*	*24.53*	*< 0.0001*	
BE	*394.62*	*1*	*394.62*	*61.47*	*< 0.0001*	
CD	*43.36*	*1*	*43.36*	*6.75*	*0.0155*	
CE	*2.10*	*1*	*2.10*	*0.33*	*0.5722*	
DE	*46.58*	*1*	*46.58*	*7.26*	*0.0124*	
A^2	*439.84*	*1*	*439.84*	*68.52*	*< 0.0001*	
B^2	*682.90*	*1*	*682.90*	*106.38*	*< 0.0001*	
C^2	*696.09*	*1*	*696.09*	*108.43*	*< 0.0001*	
D^2	*655.52*	*1*	*655.52*	*102.11*	*< 0.0001*	
E^2	*58.28*	*1*	*58.28*	*9.08*	*0.0059*	
Residual	160.49	25	6.42			
Lack of Fit	*99.51*	*20*	*4.98*	*0.41*	*0.9710*	*Not significant*
Pure Error	*60.98*	*5*	*12.20*			
Cor Total	7061.63	45				

The Model F-value of 53.75 implies the model is significant. There is only a 0.01% chance that an F value this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant.

Table 4b
Box behnken experimental design matrix and result of optimization of protease production by *Bacillus cereus* ABBA1.

Std	Run	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Response 1	Observed Protease activity (U/ml)
		A: Initial pH	B: Temperature (°C)	C: Maltose conc. (% w/v)	D: Beef extract conc. (%w/v)	E: Inoculum density (ml)	Protease activity (U/ml)	
21	1	7.5	30	1	1.25	2.5	91.52	108.15
18	2	7.5	40	1.5	2	2	97.17	130.35
23	3	7.5	30	2	1.25	2.5	109.40	118.28
34	4	8.5	40	1.5	1.25	2	90.55	135.83
12	5	7.5	50	1.5	1.25	3	129.60	121.27
24	6	7.5	50	2	1.25	2.5	127.00	127.16
13	7	6.5	40	1	1.25	2.5	110.90	134.26
30	8	7.5	40	2	1.25	2	101.50	134.12
26	9	8.5	40	1.5	0.5	2.5	91.78	129.71
31	10	7.5	40	1	1.25	3	101.20	139.53
20	11	7.5	40	1.5	2	3	119.40	152.10
36	12	8.5	40	1.5	1.25	3	113.80	138.49
45	13	7.5	40	1.5	1.25	2.5	91.66	139.54
19	14	7.5	40	1.5	0.5	3	100.70	130.14
27	15	6.5	40	1.5	2	2.5	104.20	118.57
5	16	7.5	40	1	0.5	2.5	99.57	129.07
41	17	7.5	40	1.5	1.25	2.5	88.38	135.37
25	18	6.5	40	1.5	0.5	2.5	105.20	90.47
6	19	7.5	40	2	0.5	2.5	109.90	134.59
29	20	7.5	40	1	1.25	2	86.10	126.62
16	21	8.5	40	2	1.25	2.5	122.80	158.87
2	22	8.5	30	1.5	1.25	2.5	91.72	125.71
11	23	7.5	30	1.5	1.25	3	93.75	129.65
28	24	8.5	40	1.5	2	2.5	123.00	159.43
22	25	7.5	50	1	1.25	2.5	108.90	115.71
44	26	7.5	40	1.5	1.25	2.5	92.40	139.73
17	27	7.5	40	1.5	0.5	2	92.12	125.57
35	28	6.5	40	1.5	1.25	3	107.60	125.99
38	29	7.5	50	1.5	0.5	2.5	104.50	113.92
9	30	7.5	30	1.5	1.25	2	95.04	128.03
4	31	8.5	50	1.5	1.25	2.5	126.40	119.76
40	32	7.5	50	1.5	2	2.5	129.40	119.03
43	33	7.5	40	1.5	1.25	2.5	86.87	119.36
8	34	7.5	40	2	2	2.5	125.30	139.11
1	35	6.5	30	1.5	1.25	2.5	107.30	131.06
10	36	7.5	50	1.5	1.25	2	91.16	114.54
15	37	6.5	40	2	1.25	2.5	107.50	113.93
7	38	7.5	40	1	2	2.5	101.80	133.72
32	39	7.5	40	2	1.25	3	119.50	140.82
46	40	7.5	40	1.5	1.25	2.5	96.89	121.73
37	41	7.5	30	1.5	0.5	2.5	103.00	85.82
39	42	7.5	30	1.5	2	2.5	102.80	129.63
14	43	8.5	40	1	1.25	2.5	90.12	121.93
33	44	6.5	40	1.5	1.25	2	97.66	123.55
42	45	7.5	40	1.5	1.25	2.5	91.80	122.32
3	46	6.5	50	1.5	1.25	2.5	100.50	113.30

analysis of the variable effect on the production of protease. The response surface plots obtained using equation (2c) is depicted in Fig. 4c.

3.6.1. Validation of protease production

Fig. 2c shows the optimum value of the combination of the five fermentation variables determined for maximum production of protease *Bacillus subtilis* NRD9. There was high degree of similarity between the predicted and experimental values, showing the validity of the RSM (Fig. 5c). The model prediction was in good agreement with the experimental data and correlation coefficient was found to be 0.99 (Table 6a). Correlation coefficient was close to 1, suggesting the significance of the model. The optimum production of protease in the experimental validation was found to be 163.76 U/ml (at 41 °C, pH 7.9, maltose conc. 1.88 %w/v, beef extract powder 1.88 %w/v, inoculum density 2.99 ml) (Fig. 5c). Thus, the statistical optimization resulted in 36.64- folds of protease activity over the unoptimized condition.

4. Discussion

Characterization and optimization for each factor of growth and production yield are essential requirements before the selected bacteria strains are used for further investigation (Ferrero et al., 1996: Kumar et al., 1999; Kembhavi et al., 1993; Lee et al., 2000). The yield improvement of alkaline protease by any microbial system also depends on the physiological, nutritional and biochemical nature of the microbe employed and these factors vary from organism to organism (Elliaiah et al., 2002; Johnvesly and Naik, 2001; Josey et al., 1979). Microbial proteases have various commercial applications in industries like food, leather, meat processing, detergent and cheese making. A major commercial use is the addition of microbial proteases to detergents for the digestion of protein-based stains in fabrics. It has been reported that the production of extracellular proteases by different microorganisms can be strongly influenced by the culture conditions, so it becomes important to understand the nature of proteases under different conditions (Chinnasamy et al., 2011).

In this study forty-eight (48) *Bacillus* species were isolated from soil samples and screened for protease production. Ten (10) isolates gave clear zones when screened on casein agar and hence were regarded as the potential producers of protease. Three promising *Bacillus* strains were selected based on their zone of inhibition and their identity was

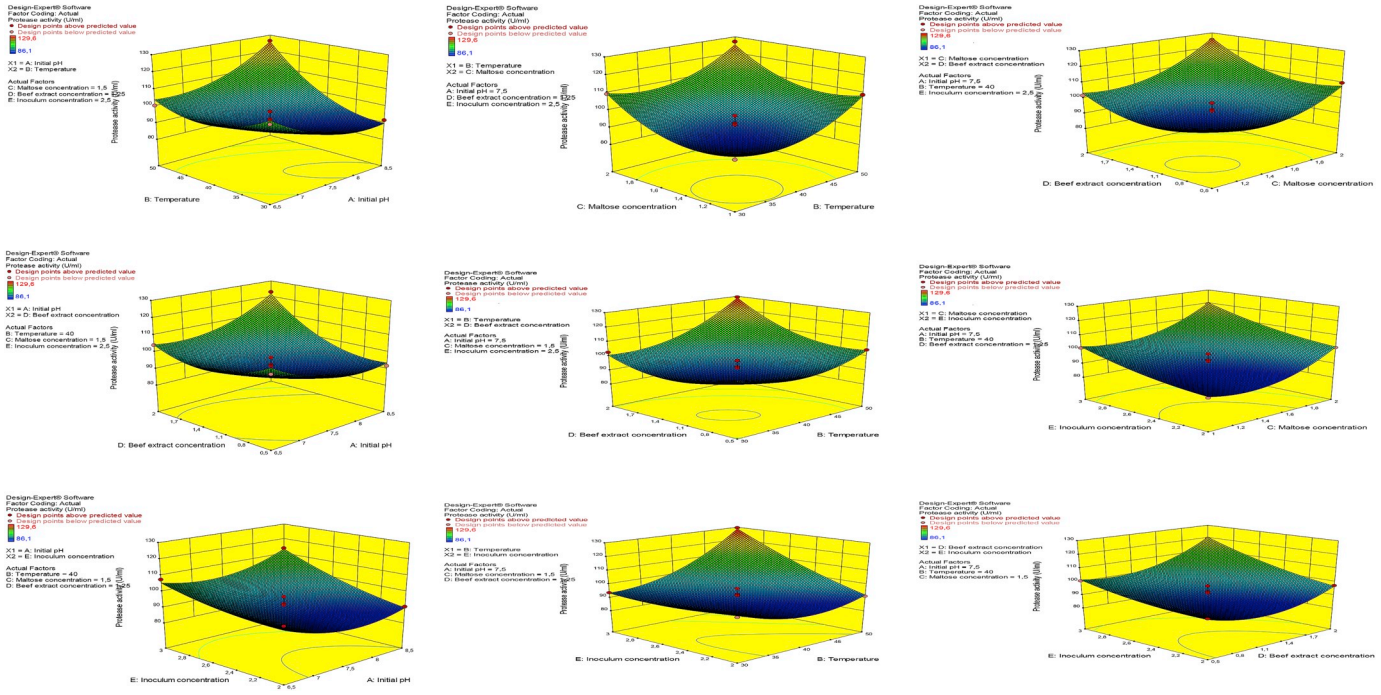

Fig. 4a. 3D surface interaction for protease production in *B*acillus cereus *A*BBA1.

revealed through comparison of their sequenced 16S rRNA gene with those in the NCBI data base. They belonged mainly to the genus *Bacillus* with sequence homology ranging between 97 and 100% (Table 2b). The optimum conditions for alkaline protease production in *Bacillus cereus* ABBA1, *Bacillus subtilis* RD7 and *Bacillus subtilis* NRD9 from this study revealed that various parameters investigated had varying effects on the protease activity of the *Bacillus* isolates. This result indicated that the optimum incubation period for alkaline protease production is 24 h at a temperature of 40 °C, using maltose and beef extract powder as the best carbon and nitrogen sources respectively.

Incubation time had varying effect on the production of alkaline protease from the *Bacillus* cultured by submerged fermentation. *Bacillus cereus* ABBA1, *Bacillus subtilis* RD7 and *Bacillus subtilis* NRD9 recorded highest protease activity at 24 h of incubation, this result agrees totally

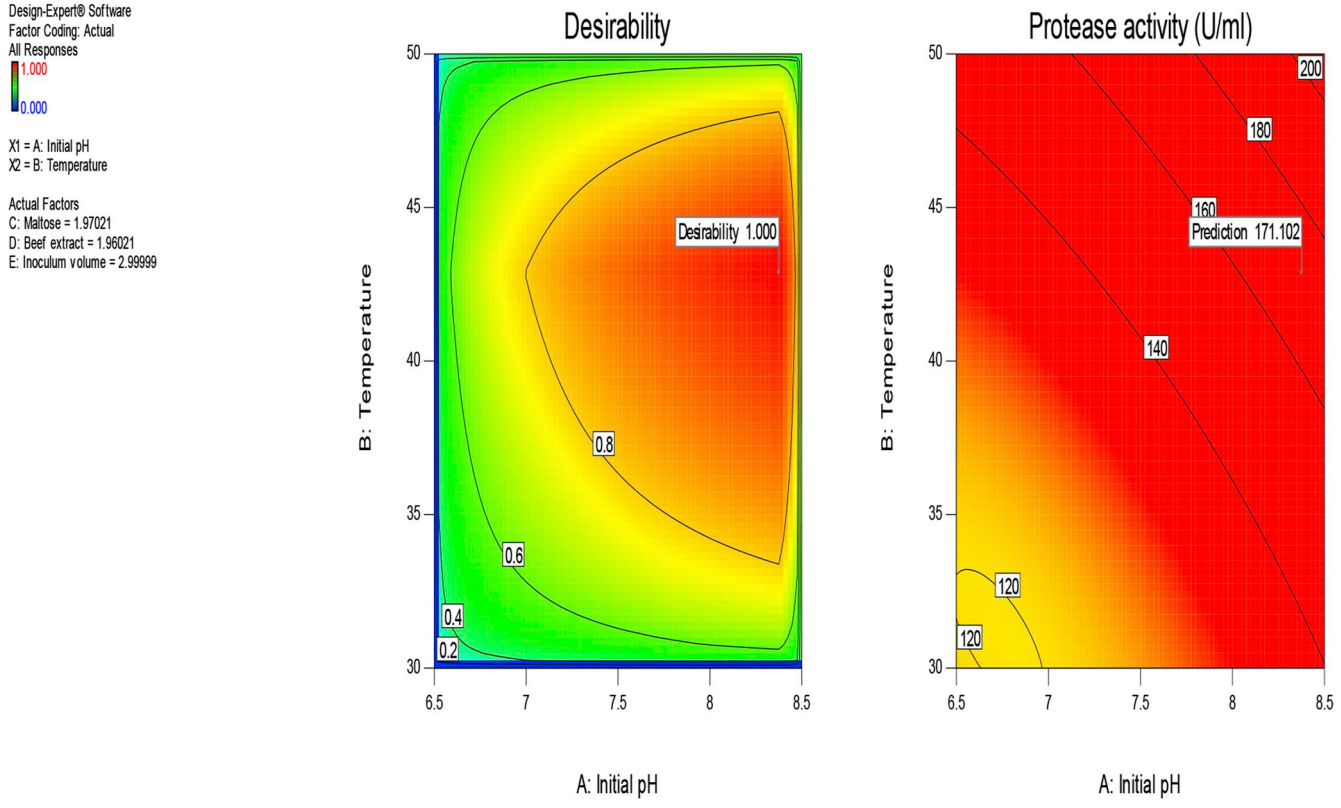

Fig. 5a. Contour graph of validation experiment for protease production by *Bacillus cereus* ABBA1.

Table 5a
Statistical analysis of box benkhen design for protease production in *Bacillus subtilis* RD7.

ANOVA for Response Surface Quadratic model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of		Mean	F	p-value	
	Squares	Df	Square	Value	Prob > F	
Model	3664.75	14	261.77	59.66	< 0.0001	Significant
A-Initial pH	*248.98*	*1*	*248.98*	*56.75*	*< 0.0001*	
B-Temperature	*759.07*	*1*	*759.07*	*173.01*	*< 0.0001*	
C-Maltose concentration	*668.12*	*1*	*668.12*	*152.28*	*< 0.0001*	
D-Beef extract agar	*13.15*	*1*	*13.15*	*3.00*	*0.1054*	
AB	*6.84*	*1*	*6.84*	*1.56*	*0.2323*	
AC	*233.94*	*1*	*233.94*	*53.32*	*< 0.0001*	
AD	*33.99*	*1*	*33.99*	*7.75*	*0.0147*	
BC	*215.50*	*1*	*215.50*	*49.12*	*< 0.0001*	
BD	*489.96*	*1*	*489.96*	*111.67*	*< 0.0001*	
CD	*129.39*	*1*	*129.39*	*29.49*	*< 0.0001*	
A^2	*464.83*	*1*	*464.83*	*105.95*	*< 0.0001*	
B^2	*2.87*	*1*	*2.87*	*0.65*	*0.4321*	
C^2	*0.54*	*1*	*0.54*	*0.12*	*0.7316*	
D^2	*490.72*	*1*	*490.72*	*111.84*	*< 0.0001*	
Residual	61.42	14	4.39			
Lack of Fit	*57.22*	*10*	*5.72*	*5.44*	*0.985*	*not significant*
Pure Error	*4.21*	*4*	*1.05*			
Cor Total	3726.18	28				

The Model F-value of 59.66 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant.

Table 5b
Box behnken experimental design matrix and result of optimization of protease production by *Bacillus subtilis* RD 7.

Std	Run	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Observed Protease activity (U/ml)
		A: Initial pH	B: Temperature (°C)	C: Maltose conc.(% w/v)	D: Beef extract conc. (%w/v)	Protease activity (U/ml)	
16	1	7.5	40	2	1.25	109.60	137.74
11	2	6.5	35	1.5	2	93.95	130.19
18	3	8.5	35	1	1.25	82.61	130.85
25	4	7.5	35	1.5	1.25	83.34	134.65
1	5	6.5	30	1.5	1.25	81.10	123.09
8	6	7.5	35	2	2	105.10	108.23
20	7	8.5	35	2	1.25	110.00	94.95
24	8	7.5	40	1.5	2	111.80	126.97
23	9	7.5	30	1.5	2	75.10	90.22
13	10	7.5	30	1	1.25	72.16	123.42
29	11	7.5	35	1.5	1.25	82.79	103.27
4	12	8.5	40	1.5	1.25	103.30	141.28
3	13	6.5	40	1.5	1.25	90.94	112.3
10	14	8.5	35	1.5	0.5	99.72	135.38
6	15	7.5	35	2	0.5	92.33	88.73
28	16	7.5	35	1.5	1.25	82.69	105.27
5	17	7.5	35	1	0.5	87.77	119.62
2	18	8.5	30	1.5	1.25	88.23	121.01
19	19	6.5	35	2	1.25	85.64	91.05
9	20	6.5	35	1.5	0.5	97.03	101.09
7	21	7.5	35	1	2	77.79	131.31
21	22	7.5	30	1.5	0.5	95.10	89.39
12	23	8.5	35	1.5	2	108.30	139.25
26	24	7.5	35	1.5	1.25	80.68	113.27
15	25	7.5	30	2	1.25	74.22	107.29
27	26	7.5	35	1.5	1.25	82.78	103.27
17	27	6.5	35	1	1.25	88.84	124.55
14	28	7.5	40	1	1.25	78.18	116.29
22	29	7.5	40	1.5	0.5	87.53	134.40

with the findings of Kumar et al. (2007) who reported that *Pseudomonas specie* 22 showed optimum activity for protease production at 24 h of incubation. Kalaiarasi and Sunitha (2009) also observed the same optimum activity for protease production at 24 h of incubation. Odu and akujobi, (2012) also worked on the production capabilities of *Micrococcus luteus* and *Bacillus* species isolated from Abattoir environment and they reported highest protease activity of 24 h and 48 h of incubation respectively in both organisms.

For the effect of carbon source on alkaline protease production, maltose gave the maximum alkaline protease activity of (681.71, 693.41 and 671.21) U/ml by *Bacillus cereus* ABBA1, *Bacillus subtilis* RD7 and *Bacillus subtilis* NRD9 respectively during production (Table 3a). There are general reviews that different carbon sources have different influences on extracellular enzyme production by various isolates. Enzyme production is regulated by physiological mechanisms and the catabolites of glucose (catabolite repression) in liquid culture often repress the production of hydrolytic enzymes (Zambare et al., 2011).

On the effect of Nitrogen on alkaline protease production, this result indicated beef extract powder as the best nitrogen source for alkaline protease production by *Bacillus cereus* ABBA1, *Bacillus subtilis* RD7 and *Bacillus subtilis* NRD9 after showing maximum protease activity of (766.37, 785.25 and 748.31) U/ml respectively (Table 3b). This result is in accordance with (Das and Prasad., 2010; Adinarayana and Ellaiah, 2002). Umayaparvathi et al. (2013) and Vonothini et al. (2008) that reported beef extract powder as the best nitrogen source.

The pH of the culture media is a strong contributing factor to many enzymatic processes and plays a vital role in the transport of compounds across the cell membrane which in turn support the cell growth and product production (Elliaiah et al., 2002). The protease activity of the *Bacillus* isolates was affected by varying the pH of the medium. It was observed that there was an increase in alkaline protease production of *Bacillus cereus* ABBA1, *Bacillus subtilis* RD7 and *Bacillus subtilis* NRD9 with increasing pH values up to pH 8.5 (Tables 4b,5b,6b). This means there was a stimulation of the enzyme production at alkaline pH for *Bacillus* isolates. This result agrees with the work of Hongxia et al. (2015) on production and characterization of alkaline protease from a high yielding and moderately halophilic strain of soil marine bacteria reporting that protease showed high activity in a broad pH range of 8.0–11.0.

Temperature is a critical factor for maximum enzyme activity and a prerequisite for industrial enzymes to be active and stable at higher temperature. The optimum temperature for protease activity by *Bacillus cereus* ABBA1, *Bacillus subtilis* RD7 and *Bacillus subtilis* NRD9. was observed at 40 °C (Tables 4b,5b,6b). This result is similar to the findings of Usharani and Muthura (2010) where they purified protease from *Bacillus laterosporus* which is found to be active at 40 °C. The stability was seen to decrease as the temperature increases from the optimum due to the breakage in the hydrogen bonds (Usharani and Muthura, 2010). This is also in agreement with the work of Ravindran et al. (2011a,b) in solid state fermentation to produce alkaline protease by *Bacillus cereus* 1173900 using proteinaceous tannery solid waste with optimum temperature for protease activity at 40 °C. Also, in accordance to our result is the work of Nazenin Ahmetoglu et al., 2015; Sousa et al. (2007) and Vijayan and Lakshmi., 2009 reporting the protease to be very active at 40–45 °C.

Optimization is a complex process which is done in two ways: conventional and modern methods. The conventional method of optimization employs one at a time strategy but modern multivariate RSM enables optimization of more than one parameter at a time (John et al., 2007) which is performed for assessing the relationship between environmental and cultural parameters to produce a 3D surface plots. This is more effective, easier, faster and economical than conventional methods.

To achieve maximal potential of microorganism for the synthesis of desired metabolite/product, it is very important to optimize the cultural parameters. Response Surface Methodology has been effectively used

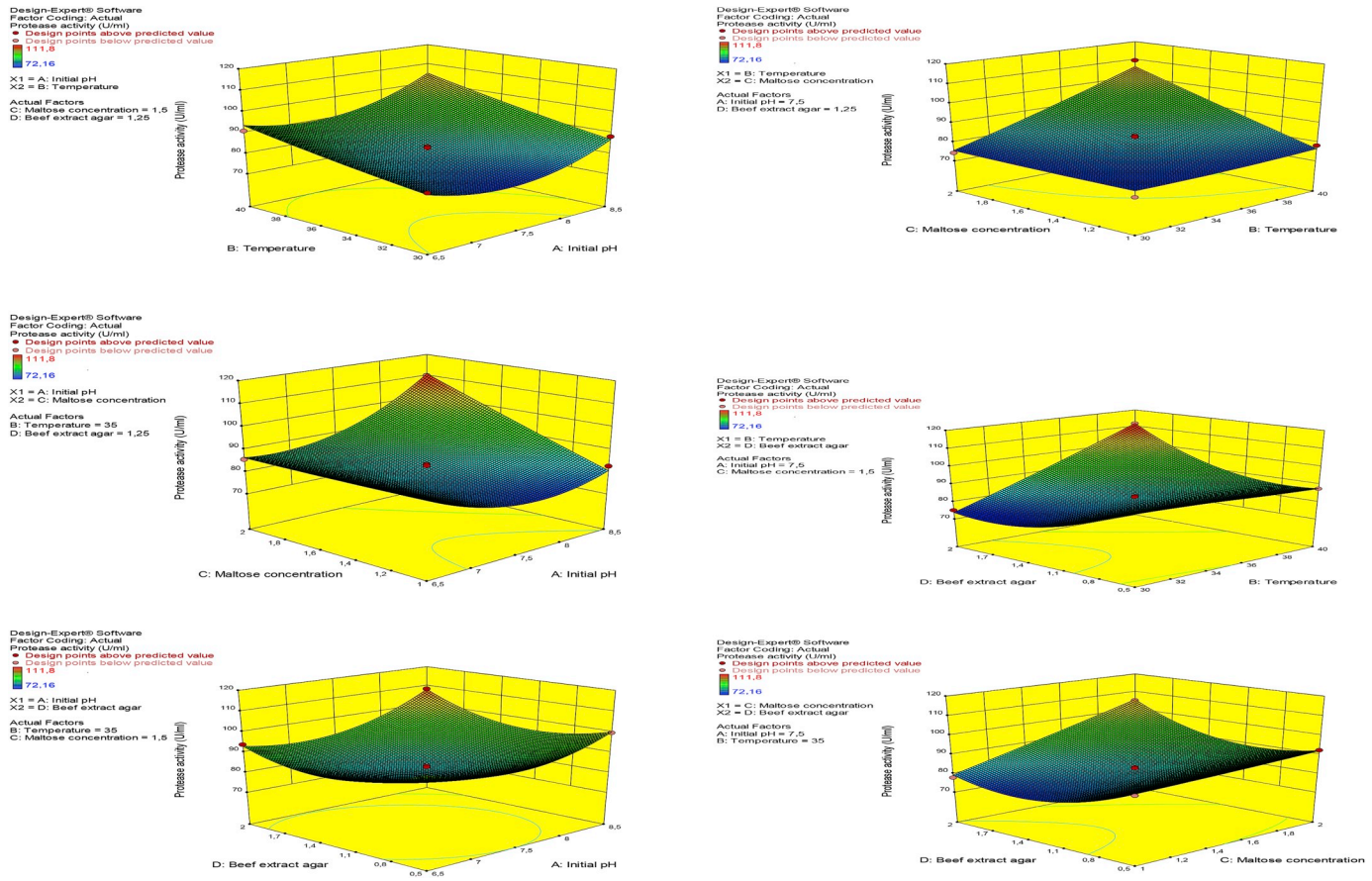

Fig. 4b. 3D surface interaction for protease production in *Bacillus subtilis* RD7.

for optimization of cultural parameters for industrial enzyme fermentation. It has been successfully applied in the optimization of fermentation medium components and conditions for enzymatic hydrolysis and fermentation processes (Dave et al., 2013; Zhang et al., 2012; Acharya et al., 2010; Chapla et al., 2010; Jeya et al., 2009). It gives the maximum enzyme production based on few sets of experiments in which all the factors are being varied within selected range and it also studies the interactive effects of various process parameters (Mohana

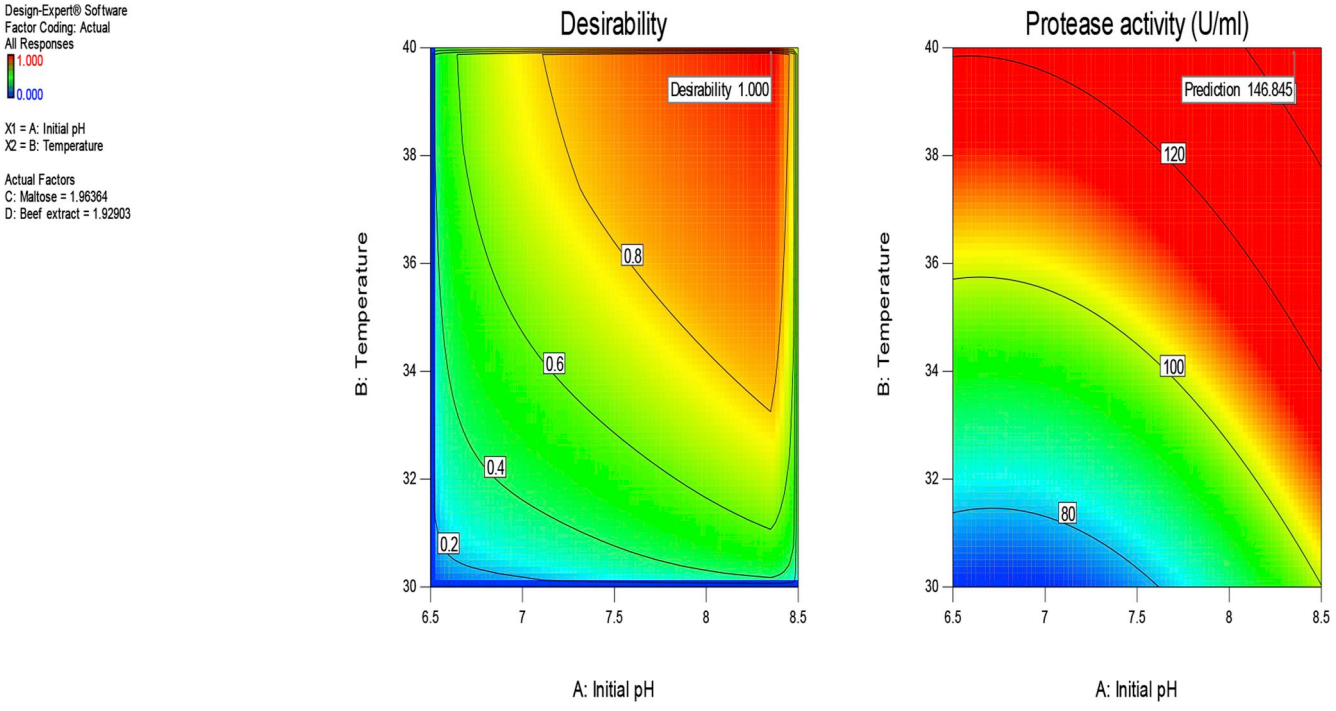

Fig. 5b. Contour graph of validation experiment for protease production by *Bacillus subtilis* RD7.

Table 6a
Statistical analysis of box benkhen design for protease production in *Bacillus subtilis* NRD9.

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of		Mean	F	p-value	
	Squares	df	Square	Value	Prob > F	
Model	4017.74	14	286.98	29.38	< 0.0001	Significant
A-Initial pH	*532.67*	*1*	*532.67*	*54.54*	*< 0.0001*	
B-Temperature	*250.44*	*1*	*250.44*	*25.64*	*0.0002*	
C-Maltose	*665.58*	*1*	*665.58*	*68.15*	*< 0.0001*	
D-Beef extract	*25.52*	*1*	*25.52*	*2.61*	*0.1283*	
AB	*281.90*	*1*	*281.90*	*28.86*	*< 0.0001*	
AC	*117.51*	*1*	*117.51*	*12.03*	*0.0038*	
AD	*76.48*	*1*	*76.48*	*7.83*	*0.0142*	
BC	*117.29*	*1*	*117.29*	*12.01*	*0.0038*	
BD	*366.72*	*1*	*366.72*	*37.55*	*< 0.0001*	
CD	*476.33*	*1*	*476.33*	*48.77*	*< 0.0001*	
A^2	*418.42*	*1*	*418.42*	*42.84*	*< 0.0001*	
B^2	*195.71*	*1*	*195.71*	*20.04*	*0.0005*	
C^2	*465.52*	*1*	*465.52*	*47.66*	*< 0.0001*	
D^2	*600.95*	*1*	*600.95*	*61.53*	*< 0.0001*	
Residual	136.73	14	9.77			
Lack of Fit	*38.05*	*10*	*3.80*	*0.15*	*0.9923*	*not significant*
Pure Error	*98.69*	*4*	*24.67*			
Cor Total	4154.48	28				

The Model F-value of 29.38 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant.

Table 6b
Box behnken experimental design matrix and result of optimization of protease production by *Bacillus subtilis* NRD 9.

Std	Run	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Observed Protease activity (U/ml)
		A: Initial pH	B: Temperature (°C)	C: Maltose conc. (% w/v)	D: Beef extract conc. (%w/v)	Protease activity (U/ml)	
14	1	7.5	45	1	1.25	87.62	114.37
4	2	8	45	1.5	1.25	113.40	134.38
11	3	7	40	1.5	2	88.38	116.97
3	4	7	45	1.5	1.25	85.34	100.93
24	5	7.5	45	1.5	2	112.90	130.15
22	6	7.5	45	1.5	0.5	89.50	103.00
23	7	7.5	35	1.5	2	84.68	104.55
19	8	7	40	2	1.25	94.13	127.17
12	9	8	40	1.5	2	110.20	130.69
27	10	7.5	40	1.5	1.25	79.60	102.69
25	11	7.5	40	1.5	1.25	86.55	126.85
16	12	7.5	45	2	1.25	111.40	120.68
1	13	7	35	1.5	1.25	93.52	114.68
20	14	8	40	2	1.25	120.60	103.25
10	15	8	40	1.5	0.5	99.55	115.14
26	16	7.5	40	1.5	1.25	76.79	96.85
18	17	8	40	1	1.25	91.13	117.48
6	18	7.5	40	2	0.5	93.98	125.16
8	19	7.5	40	2	2	118.40	138.17
13	20	7.5	35	1	1.25	88.72	133.38
9	21	7	40	1.5	0.5	95.22	131.96
29	22	7.5	40	1.5	1.25	75.73	106.85
5	23	7.5	40	1	0.5	102.7	121.92
21	24	7.5	35	1.5	0.5	99.58	118.61
2	25	8	35	1.5	1.25	88.00	123.38
17	26	7	40	1	1.25	86.34	127.70
28	27	7.5	40	1.5	1.25	78.72	106.85
7	28	7.5	40	1	2	83.47	127.76
15	29	7.5	35	2	1.25	90.84	127.08

et al., 2008).

Cultivation of *Bacillus cereus* ABBA1, *Bacillus subtilis* RD7 and *Bacillus subtilis* NRD9 resulted in protease activity of (5.98 ± 2.66, 4.17 ± 0.25 and 4.47 ± 1.41) U/ml respectively in the unoptimized medium. The production of protease is influenced by the type and concentration of carbon and nitrogen sources, culture pH and temperature (Du et al., 2007). Different operational variables interact and influence their respective effect on response, thus it is worthwhile to use an experimental design that could account for these interactions (Kumar et al., 2016; Kumar et al. 2009). Based on results obtained from this study, protease production was the result of a synergy of all the parameters on the microorganisms in the culture. Therefore, optimization of cultural conditions using RSM gave an increase of 33.54, 42.21 and 36.64-fold in the production of protease after optimization of culture conditions in the three *Bacillus* isolates according to the validation experiment.

The best result obtained from *Bacillus cereus* ABBA1 for protease production using the experimental design in medium containing: basal medium (KH_2P0_4, K_2HP0_4, NaCl, $MgS0_4$, $CaCl_2$), maltose (1.5) % and beef extract powder (2) % with pH 8.5 and temperature of 40 °C inoculated with an aliquot of 2.5 ml inoculum for 24 h is 159.43 U/ml (Table 4b) of protease activity. *Bacillus subtilis* RD7 recorded highest protease activity using the experimental design in medium containing basal medium, maltose (1.5) % and beef extract powder (1.25) % with pH 8.5 and temperature of 40 °C inoculated with an aliquot of 2.5 ml inoculum for 24 h gave 141.28 U/ml (Table 5b) and *Bacillus subtilis* NRD9 gave highest protease activity of 138.17 U/ml (Table 6b) in medium containing basal medium, maltose (2) % and beef extract powder (2) % with pH 7.5 and temperature of 40 °C inoculated with an aliquot of 2.5 ml of inoculum after 24 h of incubation.

The experimental results suggest that the variables selected for the fermentation process had strong effect on protease production. The initial pH of the medium is an important parameter considered in any production optimization process because any enzyme produced by microorganisms can be stable at a range of optimal pH values. *Bacillus* is generally able to grow over a wide range of pH values. The enzymes produced by these indigenous *Bacillus* species are also stable over a wide range of pH. (7–12). The pH values below and above the optimum value required for growth as well as enzyme production leads to slower growth rate along with low levels of enzyme secretion.

5. Conclusion

To meet the growing demand of industrial proteases (produced from indigenous *Bacillus* species) with potential biotechnology applications in industries like food, leather, meat processing, detergent and cheese making, the present study was conducted to produce industrially important proteases by *Bacillus cereus* ABBA1, *Bacillus subtilis* RD7 and *Bacillus subtilis* NRD9 by optimizing various cultivation parameters which are important in protease production. Optimization of fermentation conditions like initial pH value of the medium is an important parameter to be considered. Other Various physicochemical parameters which are important for the maximum production of this industrially important proteases includes temperature, inoculum density, nitrogen and carbon sources. These indigenous *Bacillus* species from soil samples are good potential sources of industrial proteases and its hydrolytic activity can be explored for various commercial applications.

Data availability

All sequencing data have been deposited in National Centre for Biotechnology Information (NCBI) under assertion number MG255316 - MG255318.

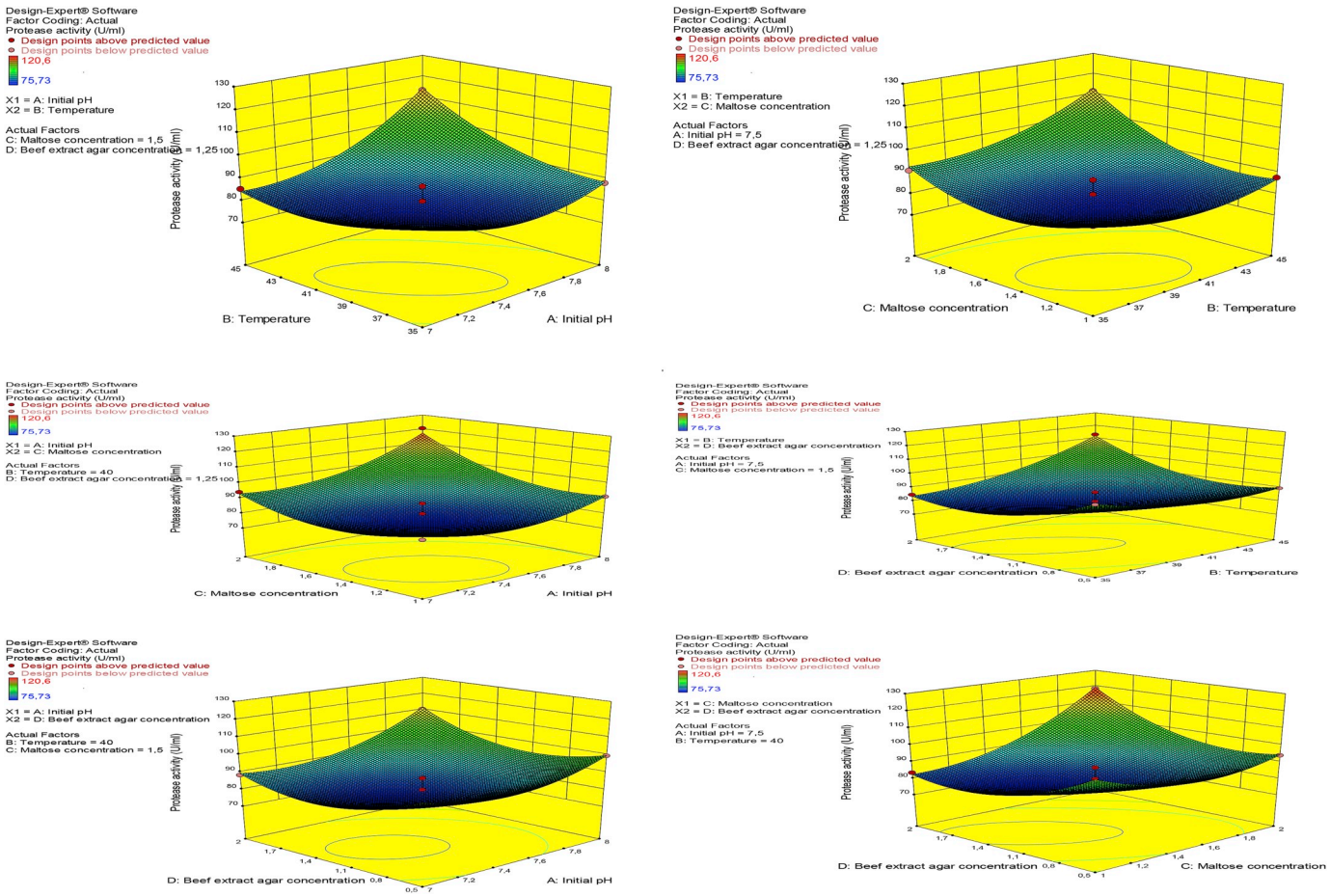

Fig. 4c. 3D surface interaction for protease production in *Bacillus subtilis* NRD9.

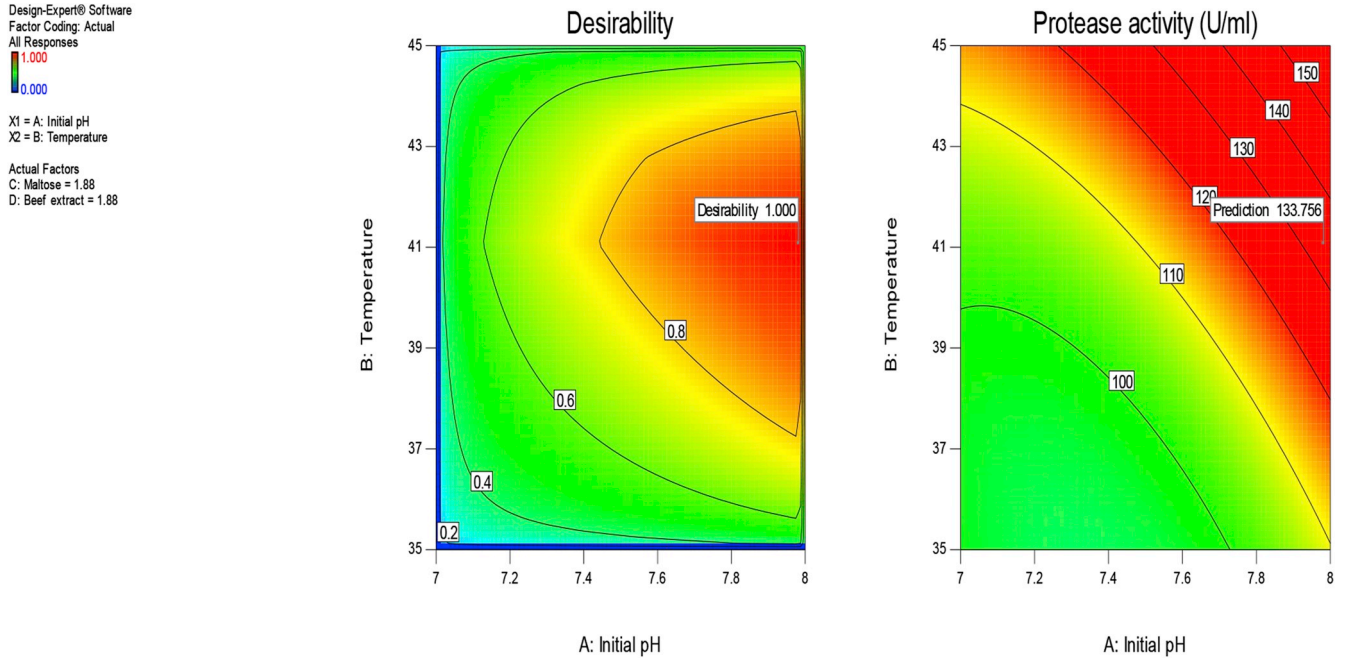

Fig. 5c. Contour graph of validation experiment for protease production by *Bacillus subtilis* NRD 9.

Declaration of interest

The authors declare no conflicts of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgments

We thank Mr S.O.A. Olatope, Dr Ajit Kumar and Dr Collins Odjadjare for their skillfull technical assistance. We would like to appreciate reviewers for helpful suggestions and comments to improve our manuscript.

References

Acharya, B.K., Mohana, S., Jog, R., Divecha, J., Madamwar, D., 2010. Utilization of anaerobically treated distillery spent wash for production of cellulases under solid-state fermentation. J. Environ. Manag. 91, 2019–2027.

Adinarayana, K., Ellaiah, P., 2002. Response surface optimization of the critical medium components to produce alkaline protease by newly isolated *Bacillus* Sp. J. Pharmacol. Sci. 5 (3), 272–278.

Ahmetoglu, N., Matpan Bekler, F., Acer, O., Guven, K., 2015. Production, purification and characterisation of thermostable metallo-protease from newly isolated *Bacillus* sp. KG5. Eurasia J Biosci 9, 1–11.

Alcaraz, L.D., Moreno-Hagelsieb, G., Eguiarte, L.E., Souza, V., Herrera-Estrella, L., 2010. Understanding the evolutionary relationships and major traits of Bacillus through comparative genomics. BMC Genomics 11, 332.

Aygan, A., Karcioglu, L., Arikan, B., 2011. Alkaline thermostable and halophilic endoglucanase from *Bacillus licheniformis* C108. Afr. J. Biotechnol. 10, 789–796.

Benjamin, S., Panichikkal, A., Erandapurthukadumana, S.H., Prakasan, P., Kizhakkepowathial, N.U., Sreedharan, S., Sasidharan, S., Moolakkariyil, S.J., 2014a. Optimization of parameters for the production of lipase from *Pseudomonas* sp. BUP6 by Solid State Fermentation Advances in Enzyme Research 2, 125–133.

Benjamin, S., Priji, P., Unni, K.N., Sajith, S., Binod, P., Benjamin, S., 2014b. Production, optimization and partial purification of lipase from *Pseudomonas* sp. Strain BUP6, a novel rumen bacterium characterized from malabari goat. Biotechnol. Appl. Biochem. 62 (1), 71–78. https://doi.org/10.1002/bab.1237.

Chapla, D., Divecha, J., Madamwar, D., Shah, A., 2010. Utilization of agro-industrial waste for xylanase production by *Aspergillus foetidus* MTCC 4898 under solid state fermentation and its application in saccharification. Biochem. Eng. J. 49, 361–369.

Chinnasamy, M., Duraisamy, G., Dugganaboyana, G.K., Ganesan, R., Manokaran, K., Chandrasekar, U., 2011. Production, purification and characterization of protease by *Apergillus flavus* under solid state fermentation. Jordan J. Biol. Sci. 4 (3), 137–148.

Chu, W.H., 2007. Optimization of extracellular alkaline protease production from species of *Bacillus*. J. Ind. Microbiol. Biotechnol. 34, 241–245.

Das, G., Prasad, M.P., 2010. Isolation, purification and mass production of protease enzyme from *Bacillus subtilis*. Int Res J Microbiol 1, 26–31.

Dave, B.R., Dave, P.P., Ankit Sudhir, K.P., Subramanian, R.B., 2015. Optimization of process parameters for cellulase production by Bacillus licheniformis MTCC 429 using RSM and molecular characterization of cellulase gene. J. Bioprocess. Biotech. 5, 3.

Dave, B.R., Sudhir, A.P., Parmar, P., Pathak, S., Raykundaliya, D.P., 2013. Enhancement of cellulose activity by a new strain of *Thermoascus aurantiacus*. Optimisation by statistical design response surface methodology. Biocatal Agr Biotechnol 2, 108–115.

Du, G.C., Zhang, S.L., Hua, Z.Z., Zhu, Y., Chen, J., 2007. Enhanced cutinase production with Thermobifida fusca by two-stage pH control strategy. Biotechnol. J. 2, 365–369.

Elliaiah, P., Adinarayana, K., Bhavani, Y., Padmaja, P., Srinivasula, B., 2002. Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergillus* species. Process Biochem. 38, 615–620.

Ferreira, S., Duarte, A.P., Ribeiro, M.H.L., Queiroz, J.A., Domingues, F.C., 2009. Response surface optimization of enzymatic hydrolysis of Cistus ladanifer and Cytisus striatus for bioethanol production. Biochem. Eng. J. 45, 192–200.

Ferrero, M.A., Castro, G.R., Abate, C.M., Baigori, M.D., Sineriz, F., 1996. Appl. Microbial. Biotechnol. 45, 327–332.

Hongxia, C., Liping, W., Yang, Y., 2015. Production and characterization of alkaline protease from a high yielding and moderately halophilic strain of SD11 Marine Bacteria. J. Chem, 798304 8 pages.

Jeya, M., Zhang, Y.W., Kim, I.W., Lee, J.K., 2009. Enhanced saccharification of alkali treated rice straw by cellulase from Trametes hirsuta and statistical optimization of hydrolysis conditions by RSM. Bioresour. Technol. 100, 5155–5161.

John, R.P., Sukumaran, R.K., Nampoothiri, K.M., Pandey, A., 2007. Statistical optimization of simultaneous saccharification and l (+)-lactic acid fermentation from cassava bagasse using mixed culture of Lactobacilli by response surface methodology. Biochem. Eng. J 36, 262–267.

Johnvesly, B., Naik, G.R., 2001. Studies on production of thermostable alkaline protease from thermophilic and alkalophilic *Bacillus* sp. JB-99 in a chemically defined medium. Process Biochem. 37, 139–144.

Josey, P., Beyho, J.L., Johnson, A.W.B., Beringer, J.E., 1979. J. Appl. Bacteriol. 343–350.

Kalaiarasi, K., Sunitha, P.U., 2009. Optimization of alkaline protease production from *Pseudomonas* fluorescens isolated from meat waste contaminated soil. Afr. J. Biotechnol. 8, 7035–7041.

Kembhavi, A.A., Kulkarni, A., Pant, A., 1993. Appl. Biochem. Biotechnol. 28, 409–413.

Kole, M.M., Draper, I., Gerson, D.F., 1988. Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. J. Chem. Technol. Biotechnol. 41 (3), 197206.

Kumar, C.G., Tiwari, M.P., Jany, K.D., 1999. Novel alkaline serine proteases from alkalophilic Bacillus sp. purification and characterization. Process Biochem 34, 441–449.

Kumar, A.G., Swarnalatha, S., Sairam, B., Sekaran, G., 2007. Bioresour. Technol. 99, 1939–1944.

Kumar, S., Pakshirajan, K., Dasu, V.V., 2009. Development of medium for enhanced production of glutaminase-free L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. Appl. Microbiol. Biotechnol. 84, 477–486. https://doi.org/10.1007/s00253-009-1973-0.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Lee, E.G., Eun, H.P., Hyung, H.H., 2000. J. Microbiol. Biotechnol. 10, 677–684.

Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J., Dymock, D., Wade, W.G., 1998. Design and evaluation of useful bacterium- specific PCR primers that amplify genes coding 16S rRNA. Appl. Environ. Microbiol. 64, 795–799.

Mohana, S., Shah, A., Divecha, J., Madamwar, D., 2008. Xylanase production by *Burkholderia* sp. DMAX strain under solid state fermentation using distillery spent wash. Bioresour. Technol. 99, 7553–7564.

Ninghoujam, D.S., Kshetri, P., 2010. A thermostable alkaline protease from a moderately halo-alkali thermotolerant *Bacillus subtilis*strainSH1. Australian J. Basic Appl.Sci. 4, 5126–5134.

Ningthoujam, D.S., Kshetri, P., Sanasam, S., Nimaichand, S., 2009. Screening, identification of best producers and optimization of extracellular proteases from moderately halophilic alkali thermotolerant indigenous Actinomycetes. World Appl. Sci. J. 7, 907–916.

Odu, N.N., Akujobi, C.O., 2012. Protease production capabilities of *Micrococcus Luteus* and *Bacillus* species isolated from abattoir environment. J. Microbiol. Res. 2 (5), 127–132.

Proteases market by source 2016. Available from: http://www.marketsandmarkets.com/Market-Reports/proteases-market184780427.html.

Rai, S.K., Roy, J.K., Mukherjee, A.K., 2010. Characterization of a detergent-stable alkaline protease from a novel thermophilic strain *Paenibacillus tezpurensis* sp. Nov. AS-S24-II. Appl. Micrbiol.Biotechnol. 85, 1437–1450.

Ravindran, B., Kumar, A.G., Bhavani, P.S., Sekaran, G., 2011a. Solid-state fermentation for the production of alkaline protease by Bacillus cereus 1173900 using proteinaceous tannery solid waste. Curr. Sci. 100 (5), 726–730 26.

Ravindran, B., Ganesh, A., Kumar, P.S., Aruna, B., Ganesan, S., 2011b. Solid-state fermentation for the production of alkaline protease by *Bacillus cereus* 1173900 using proteinaceous tannery solid waste. Curr. Sci. 100 (5).

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Singh, R., 2016. Microbial enzymes: industrial progress in 21st century. 3 Biotech 6 174. 2.

Sousa, F., Ju, S., Erbel, A., Kokol, V., Cavaco-Paulo, A., Gubitz, G.M., 2007. A novel metalloprotease from *Bacillus cereus* for protein fibre processing. Enzym. Microb. Technol. 40, 1772–1781.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739. https://doi.org/10.1093/molbev/msr121.

Umayaparvathi, S., Meenakshi, S., Arunmugam, M., Balasubramanian, T., 2013. Purification and characterization of protease from Bacillus cereus SU12 isolated from oyster Saccostrea cucullate. Afr. J. Biotech 12 (40), 5897–5908.

Usharani, B., Muthuraj, M., 2010. Production and characterization of protease enzyme from Bacillus laterosporus. Afr. J. Microbiol. Res. 4 (11), 1057–1063.

Vijaya, L.M., Lakshmi, N.M., 2009. Optimization of production protocol of alkaline protease by *Streptomyces pulvereceus*. Inter JRI Sci Technol 1 (2), 79–82.

Vonothini, G., Murugan, M., Sivakumar, K., Sudha, S., 2008. Optimization of protease production by an actinomycete Strain, PS-18A isolated from an estuarine shrimp pond. Afr. J. Biotechnol. 7 (18), 225–3230.

Zambare, V., Nilegaonkar, S., Kanekar, P., 2011. A novel extracellular protease from *Pseudomonas aeruginosa* MCM B-327: enzyme production and its partial characterization. N. Biotech. 28, 173–181.

Zhang, H., Sang, Q., Zhang, W., 2012. Statistical optimization of cellulases production by *Aspergillus Niger* HQ-1 in solid-state fermentation and partial enzymatic characterization of cellulases on hydrolysing chitosan. Ann. Microbiol. 62, 629–645.