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Approach toward enhancement of halophilic protease production by *Halobacterium* sp. strain LBU50301 using statistical design response surface methodology

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ABSTRACT

A new potent halophilic protease producer, *Halobacterium* sp. strain LBU50301 was isolated from salt-fermented fish samples (*budu*) and identified by phenotypic analysis, and 16S rDNA gene sequencing. Thereafter, sequential statistical strategy was used to optimize halophilic protease production from *Halobacterium* sp. strain LBU50301 by shake-flask fermentation. The classical one-factor-at-a-time (OFAT) approach determined gelatin was the best nitrogen source. Based on Plackett–Burman (PB) experimental design; gelatin, $MgSO_4 \cdot 7H_2O$, NaCl and pH significantly influenced the halophilic protease production. Central composite design (CCD) determined the optimum level of medium components. Subsequently, an 8.78-fold increase in corresponding halophilic protease yield (156.22 U/mL) was obtained, compared with that produced in the original medium (17.80 U/mL). Validation experiments proved the adequacy and accuracy of model, and the results showed the predicted value agreed well with the experimental values. An overall 13-fold increase in halophilic protease yield was achieved using a 3 L laboratory fermenter and optimized medium (231.33 U/mL).

1. Introduction

Haloarchaeal enzymes, known as extremozymes, are produced by halophilic archaea [1]. These enzymes are active and stable in extremely saline conditions and retain catalytic proficiency at very low water activity (a_w) [2]. They have adapted to this environmental pressure by acquiring a relatively large number of negatively charged amino acid on the solvent-exposed surfaces of the protein [3]. These negative charges attract water molecules and thereby keep the proteins hydrated so that they do not precipitate [4]. These enzymes remain active and stable in high salt environment and some are thermotolerant and alkaliphilic [5]. These properties make haloarchaeal extremozymes attractive for various industrial and biotechnological applications, e.g., as detergents and in the textile industry, fermented foods and in pharmaceutical industries [6,7]. Halophilic behavior could be observed in each of three taxonomic domains; Archaea, Eukarya and Bacteria [8]. Haloarchaea (halophilic archaea) are well adapted to saturated NaCl concentrations with many growing optimally above 20–30% NaCl [9]. They have been extensively considered as a rich source of useful salt-stable enzymes [10], which are of high potential for versatile industrial processes including a lipase [11], xylanases [12] and a glutathione *S*-transferase [13]. To maintain cell structure and function in high-salt environment, haloarchaea either accumulate molar concentrations of KCl, and/or exclude salt from the cytoplasm and to synthesize and/or accumulate organic compatible solutes or osmolytes which do not interfere with enzymatic activity [14].

Nevertheless, the medium components especially the nitrogen source and fermentation conditions greatly influence the growth of microbes, and physicochemical factors such as pH, temperature, NaCl, and inoculum size [15]. Since the growth rates of valuable enzyme producing microbes in fermentation medium is a limiting factor for enhanced yield. Therefore, optimized fermentation conditions and suitable medium components need to be determined. Optimization of conditions for growth and halophilic protease production by conventional methods such as a one-factor-at-a-time (OFAT) approach [16] is time-consuming. It ignores the interacting effects among factors and requires many experimental data sets [17,18]. Such limitations can be overcome

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by using response surface methodology (RSM) [19] which is routinely used for optimization studies in several biotechnological and industrial processes [20]. RSM combines mathematical and statistical techniques to study the effect of several different factors that influence the responses by varying them simultaneously and require only a limited number of experiments [21,22]. Plackett–Burman (PB) design is usually used as the first step to screen the most significant factors from a number of process variables [23]. Central composite design (CCD) is the second step, and estimates the relationship between the variables and responses [24].

The aim of this study was to optimize medium components using statistical tools for enhancing the halophilic protease production and yield from *Halobacterium* sp. strain LBU50301. The optimized conditions were then applied to scale up the halophilic protease production in a 3 L laboratory fermenter with a view to facilitate its application in the fish sauce industry.

2. Materials and methods

2.1. Chemicals used

Azocasein, tris-(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$), casein from bovine milk and silicon antifoam were procured from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). Skim milk powder and beef extract powder were purchased from HiMedia Laboratories (Mumbai, India). Yeast extract, potassium chloride (KCl) and sodium chloride (NaCl) were procured from Labscan (Bangkok, Thailand). Casamino acids, tryptone and peptone were purchased from Difco Laboratories (Becton Dickinson, Sparks, MD USA). Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), iron(II) chloride 4-hydrate ($FeCl_2{\cdot}4H_2O$) and gelatin were obtained from Ajax Finechem (Taren Point, NSW, Australia). The primers used to identify Archaea were purchased from Pacific Science Co., Ltd. (Bangkok, Thailand). All chemicals and medium components used were of analytical grade.

2.2. Microorganism

Halobacterium sp. strain LBU50301 was isolated from *budu*, a famous fermented fishery product in Southern Thailand or a traditional Malaysian salt-fermented fish sauce. *Budu* samples were collected from *budu* factories and different markets in Southern Thailand. Serial dilutions of *budu* samples were prepared and spread on the modified M73 (mM73) agar [25] containing (g/L) yeast extract 1.0, $MgSO_4{\cdot}7H_2O$ 10.0, KCl 5.0, $CaCl_2$ 0.2, agar 15 g, NaCl 250, skim milk final concentration 0.8% (w/v) in 1000 mL distilled water pH 8.0. Plates were incubated at 30 °C for 7 days and then zone of hydrolysis was observed around the colonies. The colonies showing high zone of hydrolysis were selected and subcultured on Sehgal and Gibbons Complex (SGC) agar [26] containing 25% (w/v) NaCl in order to attain a pure colony. For screening extracellular halophilic protease, the selected strains were inoculated into 80 mL M73 liquid medium [25] containing 25% (w/v) NaCl and incubated at 30 °C in a shaker incubator at 200 rpm, after 6 days incubation the cell-free supernatant was recovered by centrifugation at 8,000 rpm for 15 min at 4 °C and halophilic protease activity was measured as described below. After screening, the *Halobacterium* sp. strain LBU50301 showed the highest protease activity on skim milk agar plate, and in M73 liquid medium containing 25% (w/v) NaCl. Hence, it was considered as the most potent halophilic protease producer and used for further studies. The *Halobacterium* sp. strain LBU50301 was maintained on SGC agar slants [26] with the following composition (g/L): casamino acids 7.5, yeast extract 10.0, KCl 2.0, tri-sodium citrate 3.0, $MgSO_4{\cdot}7H_2O$ 20.0, $FeCl_2{\cdot}4H_2O$ 0.01, agar 15.0 and NaCl 250 (pH 8.0). After incubating at 30 °C for 7 days, the slants were stored at 4 °C and subcultured monthly period.

2.3. Identification of halophilic protease producing strain

To identify the halophilic protease producing strain, phenotypic and genotypic analysis was carried out. Phenotypic tests were performed according to the proposed minimal standards for description of new taxa in the order *Halobacteriales* [27]. Determination of morphology and growth characteristics and biochemical tests were performed as described by Cui et al. [28].

The gas vesicles in *Halobacterium* sp. LBU50301 were visualized using transmission electron microscope (TEM) according to the modified method of DasSarma et al. [29]. The strain was grown in SGC liquid medium containing 25% (w/v) NaCl and incubated at 30 °C in a shaker incubator at 200 rpm for 6 days. The cells were centrifuged at 8,000 rpm for 15 min at 4 °C and washed twice with 25% (w/v) NaCl. They were then fixed in 0.5 mL of 2.5% (v/v) glutaraldehyde containing 25% (w/v) NaCl for 4 h at room temperature and washed twice with 25% (w/v) NaCl. The cells were then fixed in 0.5 mL of 1% (w/v) Osmium tetraoxide (OsO_4) containing 25% (w/v) NaCl for 2 h and washed three times with 25% (w/v) NaCl. They were stained in 2% (w/v) Uranyl acetate containing 25% (w/v) NaCl and then, dehydrated by immersion in a series of ethanol solutions. After embedding in resin, thin sections were cut with a diamond knife on an RMC ultramicrotome (Model MTX, Tucson, Ariz., USA), stained with 1% (w/v) uranyl acetate followed by lead staining, and examined in a JEM 2010 TEM (JEOL Ltd., Tokyo, Japan) at 80–100 kV.

Genomic DNA of selected isolate was extracted and purified according to the method described by Saito and Miura [30]. The genomic DNA was used as template in PCR reaction using D30F (5′-ATTCCGGTTCATCCTGC-3′, positions 6–22) as the forward primer and D56R (5′-GYTACCTTGTTACGACTT-3′, positions 1492–1509) as the reverse primer [31]. The amplification of 16S rDNA gene was done in Bio-Rad PCR cycler (Hercules, CA, USA). The amplified PCR product was subjected to sequencing by automated DNA sequencer using ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, Foster, CA, USA) with the following primers: D30F, D33R (5′-TCGCGCCTGCGCCCCGT-3′, positions 344–360), D34R (5′-GGTCTCGCTCGTTGCCTG-3′, positions 1096–1113), and D56R [31]. The partial 16S rDNA gene sequence of selected strain was subjected to homology search with the standard 16S rDNA sequences in the GenBank database using basic local alignment tool (BLAST) [32] available in the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). Multiple alignments were performed using the CLUSTAL_X program. Gaps were edited using the BioEdit program [33]. Phylogenetic tree was constructed with the MEGA version 4 [34] using unweighted pair group method with arithmetic mean (UPGMA) algorithms method with a bootstrap value based on 1000 replications [35].

2.4. Inoculum preparation and halophilic protease production in shake-flask culture

For inoculum preparation, a full loop of cells was transferred from a slant culture into a 250 mL Erlenmeyer flask containing 50 mL of SGC seed culture medium [26]. The seed culture was grown at 30 °C in a shaker incubator (Innova 400, New Brunswick Scientific Co., NJ, USA) at 200 rpm for 3 days with illumination.

For halophilic protease production, an inoculum (5%, v/v) was added into 250 mL Erlenmeyer flask containing 80 mL of M73 fermentation medium [25] with the following composition (g/L): gelatin 10, yeast extract 1.0, $MgSO_4{\cdot}7H_2O$ 10.0, KCl 5.0, $CaCl_2{\cdot}2H_2O$ 0.2 and NaCl 250 (pH 8.0). The flasks were incubated at 30 °C in a shaker incubator at 200 rpm for 6 days. After incubation, the

culture broth was centrifuged at 8,000 rpm for 15 min at 4 °C using a Sorvall® RC-5C plus superspeed refrigerated centrifuge (Kendro Laboratory Products, Newtown, CT, USA). The cell-free supernatant was used for determination of halophilic protease activity.

2.5. Determination of halophilic protease activity

Halophilic protease activity was determined according to the modified method of Brock et al. [36] using azocasein as substrate. In this assay, 1 mL of reaction mixture consisting of 0.5 mL of crude enzyme (cell-free supernatant) and 0.5 mL of 0.8% (w/v) azocasein in 0.1 M Tris-HCl buffer (pH 8.0) containing 25% (w/v) NaCl. The reaction mixture was incubated at 40 °C for 2 h in a shaking water bath (model SW22, Julabo Labortechnik, Seelbach, Germany) with mild shaking. The reaction was terminated by adding 1.0 mL of 10% (w/v) trichloroacetic acid and the mixture was allowed to stand at room temperature for 30 min. The precipitate was removed by centrifugation at 10,000 rpm for 10 min. Subsequently, a volume of 600 μL of the initial supernatant fluid was transferred into a microcentrifuge tube and then mixed with 700 μL of 1.0 N NaOH. The absorbance was measured at 440 nm by a UV–vis spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan). One unit (U) of halophilic protease activity was defined as the amount of enzyme activity that produces a change in absorbance of 0.01 at 440 nm in 2 h at 40 °C under the standard assay conditions. The halophilic protease activity was calculated according to the following equation:

$$\text{Halophilic protease activity (U/mL)} = \frac{(A - B) \times 2}{0.5 \times 0.01}$$

where A and B are the optical densities of the crude enzyme and the control, respectively, 2 is the total reaction volume and 0.5 is the volume of crude enzyme.

2.6. Screening of nitrogen source by one-factor-at-a-time (OFAT) design

The nitrogen source affecting the halophilic protease production was selected by OFAT optimization approach. Various complex nitrogen sources (1%, w/v), including gelatin, yeast extract, beef extract, skim milk, casein, casamino acids, tryptone, casein + peptone were evaluated. These nitrogen sources were added as a substitute to additive nitrogen (10 g/L gelatin and 0.1 g/L yeast extract) in the M73 fermentation medium containing 25% (w/v) NaCl, while other components were kept constant at the original concentration. After incubation at 30 °C in a shaker incubator at 200 rpm for 6 days, the cell-free supernatant was used for determination of halophilic protease activity. The growth was measured at the indicated times by assay of the optical density (OD) at 600 nm. The nitrogenous source producing the highest activity was used as one of the variables in the Plackett–Burman experimental design.

2.7. Optimization of medium components and fermentation conditions for halophilic protease production by statistical designs

2.7.1. Plackett–Burman (PB) experimental design

The PB design was applied to screen the significant variables that influenced halophilic protease production. Eight variables of medium components and fermentation conditions were tested at low (−1) and high (+1) levels, including the nitrogen sources selected in the above experiment gelatin, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, KCl, NaCl, pH, temperature and inoculum size. The levels of each variable are listed in Table 1. A 12-run experiment was generated by the Stat-Ease software (Design-Expert 6.0.2 Trial, Stat-Ease Corporation, USA) (Table 2). The design was developed by

Table 1
Range of variables of the Plackett–Burman design.

Symbol code	Variables	Units	Experimental values	
			Low (−1)	High (+1)
X_1	Gelatin	g/L	10.0	20.0
X_2	$CaCl_2 \cdot 2H_2O$	g/L	0.1	0.2
X_3	$MgSO_4 \cdot 7H_2O$	g/L	5.0	10.0
X_4	KCl	g/L	2.5	5.0
X_5	NaCl	% (w/v)	25.0	28.0
X_6	pH		7.0	9.0
X_7	Temperature	°C	30.0	37.0
X_8	Inoculum size	% (v/v)	5.0	10.0

PB experimental design based on the following first-order model:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i \quad (1)$$

where Y represents the response (halophilic protease production), β_0 is the model intercept, β_i is the linear coefficient, x_i is the level of independent variable, and k is the number of involved variables.

All the trials were carried out in triplicates and the average halophilic protease production for each trial was used as the response variable. On the basis of regression analysis, the variables that showed a significant (95% confidence level) effect on halophilic protease production were evaluated in further optimization experiments.

2.7.2. Path of steepest ascent (or descent)

The initial estimates of operating conditions for the experiment are usually far from the actual optimum, a method is needed to move rapidly to the general vicinity of the optimum via experimentation. The steepest ascent (or descent) method is used to move rapidly toward the maximum increase in the response [37]. For a first-order model in PB experimental design, the contours of the response surface are a series of parallel lines [38]. The significant variables obtained from the PB design were further optimized to determine the path of maximum enhancement. The estimated path of steepest ascent (or descent) is given by the gradient Y and is normal (perpendicular) to the fitted response surface contours. The steps along the path are proportional to the regression coefficient β_i. The steepest ascent (or descent) path started from the center (zero level) of the chosen variables in the PB design until the response showed no further increase. The experimental design and results of the steepest ascent (or descent) method are shown in Table 3.

2.7.3. Central composite design (CCD)

To find the optimal fermentation conditions for halophilic protease production, CCD with five coded levels was used for locating the true optimum conditions of gelatin, $MgSO_4 \cdot 7H_2O$, NaCl and pH. The four significant variables studied at five coded levels (−2, −1, 0, +1, +2) are shown in Table 4. The CCD resulted in total of 30 experimental trials ($=2^k + 2k + 6$, where k is the number of factors) including 16 trials for factorial design, 8 trials for axial points (2 for each variable) and 6 trials for replications of the central points [39]. The experiments were formulated using the Stat-Ease software (Design-Expert 6.0.2 Trial, Stat-Ease Corporation, USA) (Table 5). Other media components and fermentation conditions were chosen at the low level concentrations from the PB design ($P > 0.05$). The results of the CCD were expressed by the following second order polynomial using a multiple regression technique according to the following equation:

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

Table 2
Plackett–Burman experimental design for screening of the medium components and fermentation conditions that affected the actual and predicted halophilic protease production (U/mL).

STD order	Coded variable level								Halophilic protease production (U/mL)	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Actual	Predicted
1	+1	−1	+1	−1	−1	−1	+1	+1	97.67	93.87
2	+1	+1	−1	+1	−1	−1	−1	+1	83.70	83.02
3	−1	+1	+1	−1	+1	−1	−1	−1	62.93	62.48
4	+1	−1	+1	+1	−1	+1	−1	−1	96.78	102.09
5	+1	+1	−1	+1	+1	−1	+1	−1	87.20	92.11
6	+1	+1	+1	−1	+1	+1	−1	+1	111.33	111.18
7	−1	+1	+1	+1	−1	+1	+1	−1	60.60	61.61
8	−1	−1	+1	+1	+1	−1	+1	+1	64.40	62.48
9	−1	−1	−1	+1	+1	+1	−1	+1	56.67	59.85
10	+1	−1	−1	−1	+1	+1	+1	−1	105.90	100.33
11	−1	+1	−1	−1	−1	+1	+1	+1	54.53	50.76
12	−1	−1	−1	−1	−1	−1	−1	−1	40.60	42.54

Table 3
Experimental design of the steepest ascent (or descent) and corresponding response[a] for halophilic protease production (U/mL).

Run	Gelatin (g/L)	$MgSO_4 \cdot 7H_2O$ (g/L)	NaCl (%)	pH	Halophilic protease production (U/mL)
Base point[b]	15.0	7.5	26.5	8.0	
Origin step unit[c]	5.0	2.5	1.5	1.0	
Slope[d]	40.48	10.85	9.09	8.22	
Proportion[e]	202.40	27.13	13.64	8.22	
New unit[f]	4.00	0.54	0.27	0.16	
Experiment 1	15.0	7.5	26.5	8.0	67.89
Experiment 2	19.00	8.04	26.77	8.16	147.34
Experiment 3	23.00	8.57	27.04	8.32	132.51
Experiment 4	27.00	9.11	27.31	8.49	48.96

[a] X_1, Gelatin; X_3, $MgSO_4 \cdot 7H_2O$; X_5, NaCl; X_6, pH.
[b] Zero level in the PB design in Table 1.
[c] Range of the unity level.
[d] Estimated coefficient ratio from Eq. (1).
[e] Origin step unit × slope.
[f] Proportion × 0.01976, where 0.01976 is a factor determined by the experimenter based on knowledge of the process or other practical considerations, and 0.01976 was appropriate in this example.

where Y is the predicted response, β_0 the intercept term, β_i the linear coefficients, β_{ii} the quadratic coefficients, β_{ij} the interactive coefficients, and x_i and x_j the coded independent variables.

2.7.4. Validation of the model

In order to validate the response surface model, two experimental combinations were conducted according to the conditions predicted by the model. The results were compared with the predicted values.

2.7.5. Statistical analysis

The Stat-Ease software (Design-Expert 6.0.2 Trial, Stat-Ease Corporation, USA) was used for the regression analysis of the experimental data, and also to plot the response surface graphs. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA) using the same software. Comparison of the mean values was carried out by the Turkey's multiple range test. A *T*-test was used for comparison of pairs and analysis was performed using the SPSS package (SPSS 16.0 for windows, SPSS Inc., Chicago, IL, USA).

2.8. Scale-up of halophilic protease production in the laboratory fermenter

Halophilic protease production from *Halobacterium* sp. strain LBU50301 was carried out in a 3 L laboratory fermenter equipped with a six-blade turbine for agitation (Bioflo 3000; New Brunswick Scientific Company, Edison, NJ, USA). A working volume of 2 L of the optimized production medium was determined by batch fermentation. Prior to fermentation, the optimized production medium was autoclaved at 121 °C for 15 min, and followed by inoculation with a 5% (v/v) inoculum. The fermentation was carried out at 30 °C with an agitation speed of 200 rpm and an aeration rate of 0.5 vvm. A pH of 7.88 was maintained using automatic additions of 1.0 N NaOH and 1.0 N HCl. Foaming was controlled with 5% (v/v) of commercial silicon based antifoam. The cultivations were continued up to 8 days, and samples (10 mL) were taken periodically every 24 h, and then centrifuged at 8,000 rpm for 15 min 4 °C. The cell-free supernatant was used as an enzyme solution for determination of halophilic protease activity.

3. Results and discussion

3.1. Isolation, identification and phylogenetic analysis

A new potent halophilic protease producer, *Halobacterium* sp. strain LBU50301 was isolated from *budu* samples collected from

Table 4
Levels of the factors chosen for the experimental design.

Factor codes	Factors	Coded variable levels				
		−2	−1	0	+1	+2
X_1	Gelatin (g/L)	10	15	20	25	30
X_2	$MgSO_4 \cdot 7H_2O$ (g/L)	1.50	5	8.5	12	15.50
X_3	NaCl (%)	22	24	26	28	30
X_4	pH	7.0	7.5	8.0	8.5	9.0

Table 5
Central composite design matrix for the experimental design along with actual and predicted responses for halophilic protease production (U/mL).

Std	X_1 (Gelatin)	X_2 ($MgSO_4 \cdot 7H_2O$)	X_3 (NaCl)	X_4 (pH)	Halophilic protease production (U/mL)	
					Actual	Predicted
1	0	−1	−1	−1	45.43	47.18
2	+1	−1	−1	−1	18.00	17.37
3	−1	+1	−1	−1	45.20	47.50
4	+1	+1	−1	−1	33.60	37.67
5	−1	−1	+1	−1	100.87	100.92
6	+1	−1	+1	−1	97.67	97.36
7	−1	+1	+1	−1	126.73	124.69
8	+1	+1	+1	−1	125.40	125.11
9	−1	−1	−1	+1	29.47	29.13
10	+1	−1	−1	+1	10.53	13.33
11	−1	+1	−1	+1	20.47	21.54
12	+1	+1	−1	+1	10.40	9.72
13	−1	−1	+1	+1	101.73	98.42
14	+1	−1	+1	+1	95.80	92.87
15	−1	+1	+1	+1	99.00	98.28
16	+1	+1	+1	+1	96.67	96.70
17	−2	0	0	0	39.13	40.51
18	+2	0	0	0	26.27	25.12
19	0	−2	0	0	56.47	58.15
20	0	+2	0	0	80.13	78.32
21	0	0	−2	0	1.33	-3.63
22	0	0	+2	0	148.27	153.10
23	0	0	0	−2	102.13	99.89
24	0	0	0	+2	67.33	69.44
25	0	0	0	0	126.67	124.47
26	0	0	0	0	124.40	124.47
27	0	0	0	0	126.40	124.47
28	0	0	0	0	122.60	124.47
29	0	0	0	0	123.00	124.47
30	0	0	0	0	124.13	124.47

budu factories and different markets in Southern Thailand. In order to find the most efficient isolate, mM73 containing 0.8% (w/v) skim milk and M73 liquid medium were used for initial screening and secondary screening halophilic protease-producing microorganisms, respectively. *Halobacterium* sp. strain LBU50301 showed the highest clear zone of hydrolysis around the colony on mM73 agar

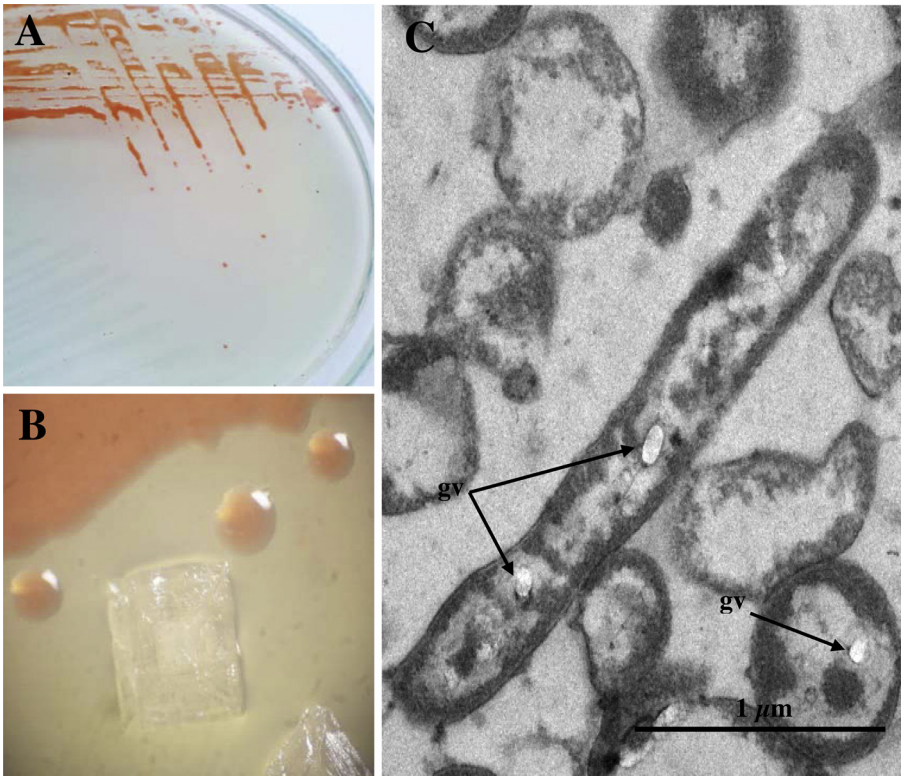

Fig. 1. Colonies of *Halobacterium* sp. strain LBU50301 on SGC agar plate containing 25% (w/v) NaCl after incubation at 30 °C for 10 days (A and B), Micrograph of *Halobacterium* sp. strain LBU50301 containing gas vesicles (gv) investigated by transmission electron microscope (C).

containing 0.8% (w/v) skim milk (Supplementary data Fig. S1) and also showed the best halophilic protease activity in M73 liquid medium containing 25% (w/v). Colonies grown on SGC agar containing 25% (w/v) NaCl for 10 days were smooth, circular and reddish orange (Fig. 1A and B). The halophilic archaebacteria such as *Halobacterium* are of a bright red-orange color due to a high content of carotenoid pigment (mainly the C50-carotenoid α-bacterioruberin) [40] but some strains are opaque, white, or pink colonies, depending on conditions such as *Halostagnicola alkaliphila* [41]. It must be noted that cells of *Halobacterium* sp. are extremely phenotypically variable. Colonies change their appearance from different shades of pink, red or orange to white, or from opaque to translucent, depending on conditions such as light or salt concentration [42]. The red pigmentation of halobacteria acts as a protein against damage by bright sunlight present at most location in which the extreme halophiles are found [43]. *Halobacterium* sp. strain LBU50301 also produced bright red-orange pigment when it was cultivated in M73 liquid medium (Supplementary data Fig. S2). Therefore, it probably synthesizes carotenoids. Cells of *Halobacterium* sp. strain LBU50301 are rod-shaped (0.4–0.9 × 2.5–3.2 μm). Gas vesicles were observed inside the cells (Fig. 1C), which were similar to *Halobacterium salinarum* DSM 3754^T (Table 6). They are often found in family *Halobacteriaceae*. The advantages of gas vesicles to halophilic Archaea such as buoying cells toward oxygen-rich surface layers in hypersaline water bodies to prevent oxygen limitation and reaching higher light intensities [44].

This microorganism grows in the temperature range of 25–45 °C with an optimum growth at 30 °C (Supplementary data Fig. S3). The microorganism grows over a wide pH range of 5–9 with an optimum growth at pH 8.0 (Supplementary data Fig. S4). The strain showed considerable growth between 20–30% (w/v) NaCl concentrations, with an optimum growth at 25% (w/v) NaCl (Supplementary data Fig. S5). *Halobacterium* sp. strain LBU50301 hydrolyzed gelatin and casein but did not hydrolyze starch and Tween 80, which was similar to *H. salinarum* DSM 3754^T [45]. The growth was observed in the presence of glycerol, which was similar with those *H. salinarum* DSM 3754^T and *Halobacterium piscisalsi* [46] (Table 6). However, it does not utilize other carbohydrates indicating its similarity with many *Halobacterium* species. For molecular identification of the microorganism, 16S rDNA (1500 bp) region was amplified, sequenced and analyzed with the NCBI database using BLAST program. The 16S rDNA gene sequencing analysis (Supplementary data Table S1) and alignment data of the strain LBU50301 were found to have 99% similarity with *H. salinarum* with accession number JQ015380 in GenBank database (Fig. 2).

Table 6
Differential characteristics between strain LBU50301 and recognized *Halobacterium* species.

Characteristic	1	2	3
Cell width (μm)	0.4–0.9	0.5–1.0	0.5–1.0
Cell length (μm)	2.5–3.2	1.0–6.0	1.0–2.0
Cell shape	Rods	Rods	Rods
Pigmentation	Red-orange	Red	Red
Motility	+	+	+
Gas vesicles	+	+	ND
NaCl (%, w/v)			
Range	20–30	20.5–30.4	15.2–29.8
Optimum	25	20.5–26.3	19.9–24.5
Temperature (°C)			
Range	25–45	20–55	20–60
Optimum	30	50	37–40
pH			
Range	5.0–9.0	5.5–8.0	5.0–8.0
Optimum	8.0	ND	7.0–7.5
Mg^{2+} required	+	+	+
Catalase and Oxidase	+	+	+
Starch hydrolysis	–	–	–
Gelatin hydrolysis	+	+	+
Casein hydrolysis	+	+	+
Tween 80 hydrolysis	–	–	+
Utilization of carbohydrates			
Glucose	–	–	–
Mannose	–	–	–
Galactose	–	–	–
Fructose	–	–	–
D-Xylose	–	–	–
Maltose	–	–	–
Sucrose	–	–	–
Lactose	–	–	+
Starch	–	–	–
Glycerol	+	+	+
Mannitol	–	–	–
Sorbitol	–	–	–

Taxa: 1, *Halobacterium* strain LBU50301; 2, *Halobacterium salinarum* DSM 3754^T (data from Boon and Castenholz [45]); 3, *Halobacterium piscisalsi* (data from Yachai et al. [46]). +, Positive; –, negative; ND, no data available.

3.2. Selection of nitrogen sources affecting cell growth and halophilic protease production

The various nitrogen sources affecting cell growth and halophilic protease production from *Halobacterium* sp. strain LBU50301 were screened through non-statistical methodology (one-factor-at-a-time experiments). Among the various nitrogen sources tested, gelatin showed the highest halophilic protease production (43.07 U/mL) and cell growth (0.48, OD_{600nm}), In the presence of casein, skim milk and M73, the halophilic protease production was 32.31 U/mL, 27.96 U/mL and 24.09 U/mL, respectively. However, yeast extract (5.96 U/mL), beef extract (4.98 U/mL) and casein + peptone (4.31 U/mL) could not produce any potential effect on halophilic protease production ($P > 0.05$) (Fig. 3). In the presence of 1% (w/v) gelatin, rapid and pronounced growth of *Halobacterium* sp. strain LBU50301 was observed as evidenced by increased turbidity during fermentation (Fig. 3). This might be due to efficient metabolization of gelatin probably due its proteinaceous nature [47]. Kanekar et al. [48] and Patel et al. [49] reported that increased protease production could be achieved after using gelatin as nitrogen source in a fermentation process with haloalkaliphilic *Bacillus* sp. and *Bacillus alcalophilus*, respectively. Manikandan et al. [50] also reported that gelatin could increase the cell density of the halophilic Archaea. Therefore, gelatin was selected as the best nitrogen source for further statistical optimization of halophilic protease production using the Plackett–Burman (PB) design.

3.3. Screening of significant variables by Plackett–Burman (PB) design

PB design was applied for screening and evaluating the significant variables that influenced halophilic protease production. Twelve runs were carried out to analyze the effect of 8 variables. The design matrix selected for screening of significant variables for halophilic protease production and the corresponding responses are shown in Table 2. The halophilic protease activity had a wide variation from 40.60 to 111.33 U/mL. The ANOVA of the PB design for halophilic protease production is shown in Table 7. The determinant of the coefficient R^2 of the first-order model was 0.9932 for halophilic protease production, and indicated that the data variability could be explained by the models very well. Usually, a model term is considered to be significant when its value of "*P*-value" is less than 0.05. In this case, gelatin, $MgSO_4 \cdot 7H_2O$, NaCl and pH were significant model terms, and indicated that

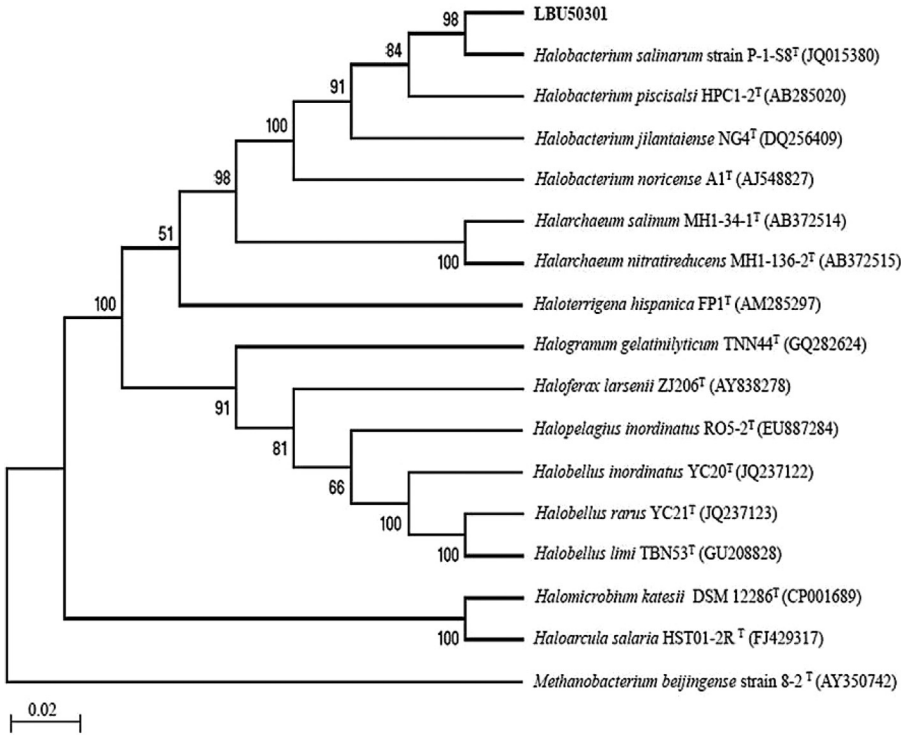

Fig. 2. Phylogenetic tree showing the relationships between strain LBU50301 and related archaeal species based on 16S rDNA gene sequences. The branching pattern was generated according to the UPGMA algorithms method. Bootstrap values were based on 1000 replicates. Bar, 0.02 substitutions per 100 nucleotide positions.

these four variables were the greatest important variables for halophilic protease production. The halophilic protease production increased with increase in concentration of gelatin, $MgSO_4 \cdot 7H_2O$, NaCl and pH low level to high level. This result was in accordance

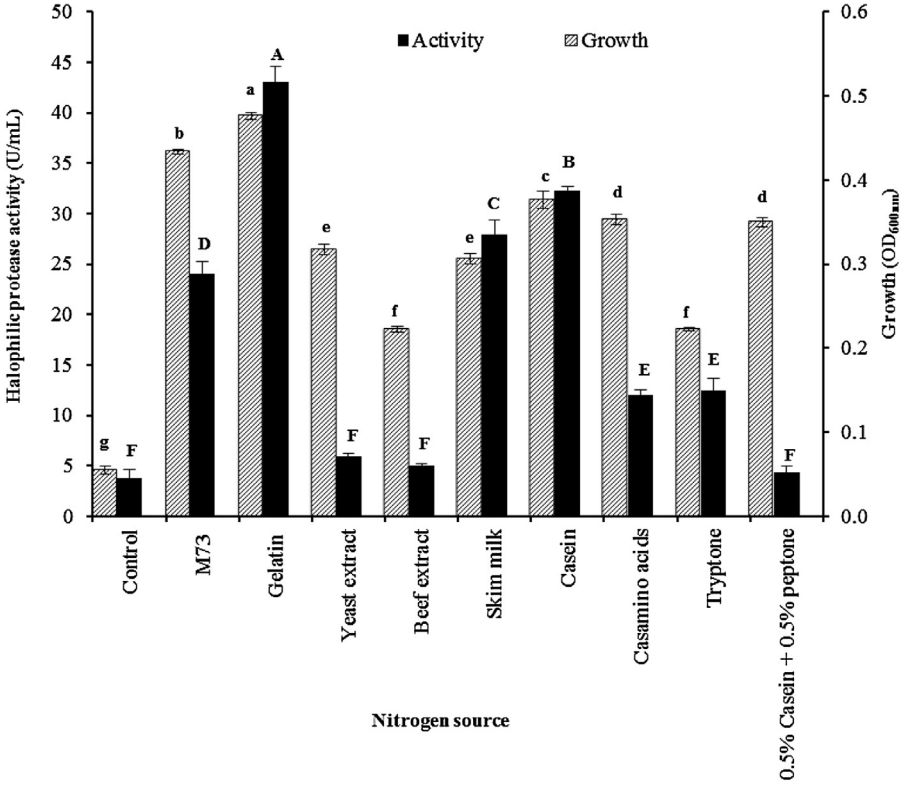

Fig. 3. Effect of different nitrogen sources on cell growth and halophilic protease activity by *Halobacterium* sp. strain LBU50301. Samples were taken after incubation at 30 °C on a shaker incubator at 200 rpm for 6 days. Bars represent the standard deviation ($n = 3$). Different lowercase letters on the bars indicating cell growth (OD_{600nm}) between different samples are significantly different ($P < 0.05$). Different uppercase letters on the bars indicating halphilophilic protease activity between different samples are significantly different ($P < 0.05$).

with Manikandan et al. [51] and Vidyasagar et al. [52], who reported that gelatin, $MgSO_4{\cdot}7H_2O$, NaCl and pH significantly influenced the halophilic protease using *Haloferax lucentensis* VKMM 007 and *Chromohalobacter* sp. TVSP101, respectively. PB design revealed a significant positive effect of NaCl on halophilic protease production, Therefore, based on PB design, increasing NaCl from low level (25%) to high level (28%), could increase the halophilic protease production. Similar results were reported by Litchfield [10] for *Chromohalobacter* sp. TVSP10. The extremely halophilic Archeae require NaCl or KCl for growth [10]. The isolated enzymes from halophilic Archeae frequently require high salt concentrations for their function and employ different adaptation mechanisms for stabilization [53]. Moreover, the contamination risk by undesired microorganisms could be minimized in the presence of high salt concentrations required for halophilic protease production. Magnesium in its sulfate form also supported maximal halophilic protease production. This could be attributed to the nature of halophilic habitats rich in $MgSO_4$ concentration such as Kelambakkam solar salterns [50]. The metal ions such as Ca^{2+} and Mg^{2+} are, in general, required for halophilic protein stability and activity of some halophilic enzymes [52].

Thus, gelatin, $MgSO_4{\cdot}7H_2O$, NaCl and pH were selected for further optimization using the path of steepest ascent (or descent) and CCD. By applying multiple regression analysis on the experimental data, the following first-order polynomial equation was established to explain the halophilic protease production:

$$Y(\mathrm{U/mL}) = 76.86 + 20.24X_1 + 5.43X_3 + 4.55X_5 + 4.11X_6$$

where Y was the halophilic protease production, X_1 (gelatin), X_3 ($MgSO_4{\cdot}7H_2O$), X_5 (NaCl), X_6 (pH).

3.4. Path of steepest ascent (decent) experiment

The method of steepest ascent (descent) is a procedure for moving sequentially along the path of steepest ascent (descent), that is, along the path of the maximum increase in the response. The path of steepest ascent started from the center of the PB design and moved along the path in which gelatin, $MgSO_4{\cdot}7H_2O$, NaCl and pH increased. Table 3 shows the design of the steepest ascent (or decent) and the corresponding response. The highest response was reached at the second step when the following medium concentrations were used in fermentation: 19.00 g/L gelatin, 8.04 g/L $MgSO_4{\cdot}7H_2O$, 26.77% (w/v) NaCl and pH 8.16. Regarding the results from the steepest ascent path (or decent), it was apparent that the halophilic protease production profile showed a maximum of 147.34 U/mL in experiment 2. Consequently, this point was near to the region of maximum halophilic protease production response. Thus, an appropriate center point for the further optimization step was chosen: 19.00 g/L gelatin, 8.04 g/L $MgSO_4{\cdot}7H_2O$, 26.77% (w/v) NaCl and pH 8.16.

Table 8
Results of the regression analysis of the second-order polynomial model for optimization of halophilic protease production.

Source	df	Sum of squares	Mean square	*F* value	*P*-value Prob > *F*
Model	14	58703.40	4193.10	451.16	<0.0001[a]
X_1	1	327.78	327.78	35.27	<0.0001[a]
X_2	1	600.39	600.39	64.60	<0.0001[a]
X_3	1	36262.26	36262.26	3901.62	<0.0001[a]
X_4	1	1369.02	1369.02	147.30	<0.0001[a]
X_1^2	1	14371.73	14371.73	1546.32	<0.0001[a]
X_2^2	1	5400.50	5400.50	581.06	<0.0001[a]
X_3^2	1	4224.28	4224.28	454.51	<0.0001[a]
X_4^2	1	2705.74	2705.74	291.12	<0.0001[a]
X_1X_2	1	14.08	14.08	1.52	0.2373
X_1X_3	1	93.43	93.43	10.05	0.0063[a]
X_1X_4	1	3.53	3.53	0.38	0.5471
X_2X_3	1	54.13	54.13	5.82	0.0291[a]
X_2X_4	1	558.46	558.46	60.09	<0.0001[a]
X_3X_4	1	0.20	0.20	0.022	0.8847
Residual	15	139.41	9.29		
Lack of fit	10	125.09	12.51	4.37	0.0587
Pure error	5	14.32	2.86		
Cor total	29	58842.81			

$R^2 = 0.9976$; Adj $R^2 = 0.9954$; CV = 3.93%.
[a] Model terms are significant.

3.5. CCD regression model analysis

The optimal levels of significant variables i.e., gelatin, $MgSO_4{\cdot}7H_2O$, NaCl and pH and their interactive effect on halophilic protease production were further evaluated using the CCD regression model (second order model). The design matrix and the corresponding experimental data to determine the effects of four independent variables are shown in Table 5. The coefficients and *P*-values on all the variables of linear (X_1,X_2, X_3, X_4), quadratic (X_1^2, X_2^2, X_3^2, X_4^2) and interactions (X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4) terms were determined and are shown in Table 8. Among the linear coefficients, X_1, X_2, X_3 and X_4 had significant effect on the halophilic protease production at $P < 0.05$. Similarly, quadratic coefficients, X_1^2, X_2^2, X_3^2 and X_4^2 were also significant ($P < 0.05$). However, only three of the interaction terms, X_1X_3, X_2X_3 and X_2X_4 were significant ($P < 0.05$), while the interaction terms, X_1X_2, X_1X_4 and X_3X_4 were non-significant ($P > 0.05$). After applying multiple regression analysis to the experimental data (Table 8), the three non-significant interactive variables were removed and following second order polynomial equation was established:

$$Y(\mathrm{U/mL}) = 124.53 - 3.85X_1 + 5.04X_2 + 39.18X_3 - 7.61X_4 - 22.91X_1^2 - 14.06X_2^2 - 12.43X_3^2 - 9.95X_4^2 + 2.56X_1X_3 + 1.86X_2X_3 - 5.98X_2X_4$$

Table 7
Identification of the significant variables for halophilic protease production by *Halobacterium* sp. strain LBU50301 using the Plackett–Burman design.

Variables	Sum of squares	Degree of freedom	Mean square	*F*-value	*Prob* > *F*
Model	5810.15	8	726.27	54.94	0.0036[a]
Gelatin (X_1)	4914.68	1	4914.68	371.80	0.003[a]
$CaCl_2{\cdot}2H_2O$ (X_2)	0.25	1	0.25	0.019	0.8994
$MgSO_4{\cdot}7H_2O$ (X_3)	353.28	1	353.28	26.73	0.0140[a]
KCl (X_4)	46.45	1	46.45	3.51	0.1575
NaCl (X_5)	247.98	1	247.98	18.76	0.0227[a]
pH (X_6)	202.62	1	202.62	15.33	0.0296[a]
Temperature (X_7)	27.88	1	27.88	2.11	0.2424
Inoculum size (X_8)	17.02	1	17.02	1.29	0.3390

$R^2 = 0.9932$.
[a] Model terms are significant.

where Y was the halophilic protease production, X_1 (gelatin), X_2 ($MgSO_4 \cdot 7H_2O$), X_3 (NaCl) and X_4 (pH).

The fit of the model was checked by the coefficient of determination R^2, which was 0.9976, and indicated that 99.76% of the variability in the response could be explained by the model. The statistical significance of the second-order model equation was determined with the F-test analysis of variance. The "Model F-value" was significant, and there was only a 0.01% chance that

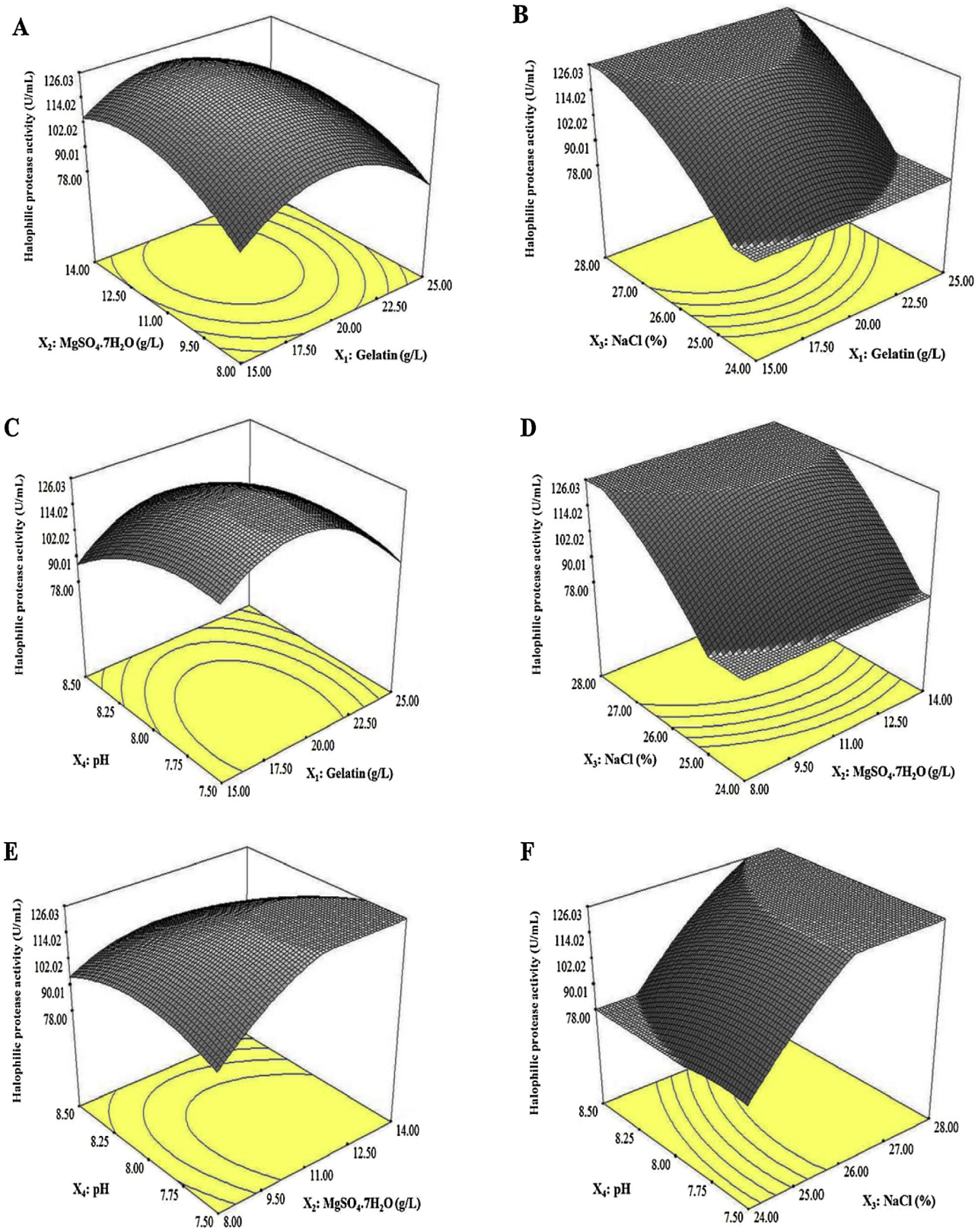

Fig. 4. Response surface 3D contour plots of halophilic protease production from *Halobacterium* sp. strain LBU50301; changing components are (A) gelatin and $MgSO_4 \cdot 7H_2O$, (B) gelatin and NaCl, (C) gelatin and pH, (D) $MgSO_4 \cdot 7H_2O$ and NaCl, (E) $MgSO_4 \cdot 7H_2O$ and pH and (F) NaCl and pH. Other variables were kept constant.

"Model *F*-value" could vary due to noise ($P < 0.0001$). In addition, the non-significant value 0.0587 for lack of fit showed that the quadratic model was valid for the present study. The coefficient of variation (CV) is the ratio of the standard error of estimate to the mean value of the observed response, and as a general rule a model can be considered reasonably reproducible if the CV is not greater than 10% [38]. Here, the low values of the CV (3.93%) indicated good reliabilities of the experiments performed.

Three dimensional (3D) response surface plots are generally used to demonstrate relationships between the response and experimental levels of each variable are shown in Fig. 4. Each figure presents the effect of two variables while the other factor was held at zero level. It showed that the mutual interactions between gelatin and NaCl (Fig. 4B), $MgSO_4{\cdot}7H_2O$ and NaCl (Fig. 4D), $MgSO_4{\cdot}7H_2O$ and pH (Fig. 4E) were significant. However, the interaction of gelatin and $MgSO_4{\cdot}7H_2O$ (Fig. 4A) indicated no positive interaction from the shape of the response surface. The maximum halophilic protease production was recorded in the middle levels of both the variables while further increase in the levels resulted in a gradual decrease in yield. In addition, the 3D response surface plots showed that the halophilic protease production increased significantly at higher levels of NaCl concentrations (Fig. 4B, D and F). In case of $MgSO_4{\cdot}7H_2O$ and NaCl (Fig. 4D), the halophilic protease production was increased with the concomitant increase in NaCl; whilst increase in $MgSO_4{\cdot}7H_2O$ did not produce any significant increase in halophilic protease production ($P > 0.05$). From Fig. 4C, it can be observed that the maximum halophilic protease production was found at pH

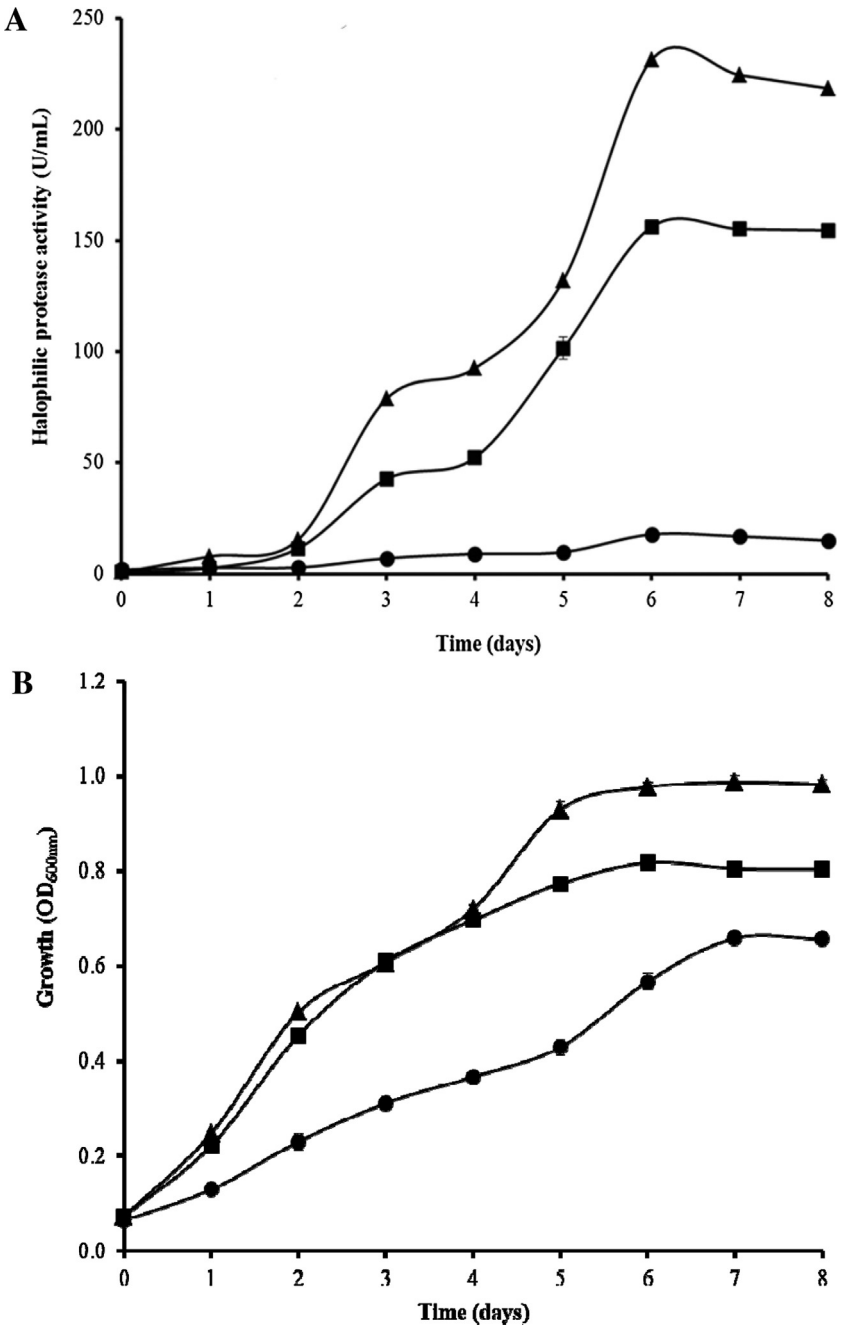

Fig. 5. Time courses of halophilic protease production (A) and cell growth (B) from *Halobacterium* sp. strain LBU50301 in a 3 L bioreactor with 2 L optimized medium (▲), 80 mL optimized medium in 250 mL Erlenmeyer flask (■) and 80 mL unoptimized medium in 250 mL Erlenmeyer flask (●).

8 along with increasing gelatin concentration; at high pH and high gelatin concentration, the yield was lower than the average condition. The maximum halophilic protease production was observed at high gelatin concentration (20 g/L) and alkaline pH (8.2). Fig. 4E showed that the significant halophilic protease production was observed along with the increase in pH of fermentation medium and the concentration of $MgSO_4 \cdot 7H_2O$. However, the enzyme production slightly decreased as the pH and $MgSO_4 \cdot 7H_2O$ increased beyond 8.2 and 7.25 g/L, respectively. By solving the inverse matrix using Expert-Design software, the optimal values for halophilic protease production of the four variables in uncoded units were 18.62 g/L for gelatin, 9.13 g/L for $MgSO_4 \cdot 7H_2O$, 27.95% (w/v) for NaCl and 7.88 for the pH, respectively. Under the optimum condition, the predicted maximum halophilic protease production was 151.68 U/mL. According to the CCD experiment, NaCl 27.95% (w/v) was optimal for maximum halophilic production by *Halobacterium* sp. strain LBU50301. This result is in agreement with Lanyi [54] who reported that the enzymes from extremely halophilic bacteria perform their functions in vivo and in vitro at 4–5 M NaCl. The results obtained in this work indicated that RSM is a reliable method for developing the model, optimizing factors, and analyzing interaction effects, before using protease in fermentation industry.

3.6. Experimental validation of the optimized condition and scale up production

In order to confirm the optimization results, the suggested medium components and fermentation conditions were tested. Under these suggested conditions, the mean value of the halophilic protease production was 150.16 U/mL, which was in agreement with the models of prediction. This result therefore validated the predicted values and the effectiveness of the model, which indicated that the optimized medium was favorable for the production of halophilic protease from *Halobacterium* sp. strain LBU50301.

After optimization studies in shake-flasks, production was scaled-up under controlled conditions in a 3 L laboratory fermenter containing 2 L working volume. The maximum halophilic protease production of 231.33 U/mL was obtained in 6 days in the presence of optimized medium and fermentation conditions (Fig. 5A). However, the halophilic protease production of 156.22 U/mL and 17.80 U/mL under optimized and unoptimized medium in a 250 mL Erlenmeyer flask was obtained in 6 days, respectively, an 8.78-fold increase. The halophilic protease production (Fig. 5A) and cell growth (Fig. 5B) were highest under optimized medium in laboratory fermenter. The halophilic protease production was produced by *Halobacterium* sp. LBU50301 in exponential phase and reached maximal in early stationary phase (Fig. 5). The results showed that enzyme production after using the optimized conditions in the laboratory fermenter increased the yield 13-fold. Similar results were reported by Reddy et al. [55], in which improved protease production was obtained in RSM based fermentation medium using *Bacillus* sp. RKY3. The halophilic protease production increased after optimization of medium components and fermentation conditions using RSM. The results revealed that, inexpensive and simple medium compositions under efficient cultivation conditions can be manipulated to increase the enzyme production gave insight for further studies regarding large scale production that is useful in food manufacture industry. In addition, based on the results obtained in these experiments, RSM has been used for improving product yield, reducing time, evaluating the relative significance of several affecting factors. The RSM has been recently used on cultivation condition [56], fermentation media [57] and enzyme production such as lipase [58], xylanase [59] and keratinase [60].

4. Conclusions

In this study, a new potent halophilic protease producer, *Halobacterium* sp. strain LBU50301 was isolated from salt-fermented fish samples (*budu*). The strain was identified on the basis of phenotypic and genotypic characteristics. Statistically based experimental designs proved to be effective for optimizing the medium components for halophilic protease production by *Halobacterium* sp. strain LBU50301, which resulted in about 8.78-fold increase in the yield relative to the original medium. The optimal medium components for halophilic protease production were determined as: 18.62 g/L gelatin, 9.13 g/L $MgSO_4 \cdot 7H_2O$, 27.95% (w/v) NaCl and an initial pH 7.88. Under such condition, the halophilic protease production was increased from 17.80 U/mL to 156.22 U/mL in the shake-flask fermentation. However, scaled up fermentation in 3 L laboratory fermenter produced a remarkable yield (231.33 U/mL) which was considerably higher (13-fold) than the results obtained using the unoptimized conditions. The halophilic protease production obtained in the bioreactor is greater than the one obtained in the shake-flasks fermentation, because the bioreactor systems provide a more precise control of parameters such as pH, aeration and agitation speed. Moreover, most of *Halobacterium* strains grow in aerobic condition. Hence, aeration rate and agitation speed are important parameters for their growth rate and production. We consider the results of this study useful for in large-scale applications in food industry especially for fish sauce fermentation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2016.02.004.

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