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Screening, selection and development of *Bacillus subtilis* apr-IBL04 for hyper production of macromolecule alkaline protease

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ABSTRACT

Bacillus subtilis microbe is commonly found in soil and produces proteases on nitrogen and carbon-containing sources and increases the fertility rate by degrading nitrogenous organic materials. The present study was aimed to develop hyper producing mutant strain of *B. subtilis* for the production of proteases, to improve the process variables by the response surface methodology (RSM) under central composite design (CCD) and the production of protease by the particular mutant strain in a liquid state fermentation media. The mutation of the strain was carried out using ethidium bromide. Pure *B. subtilis* strain was collected and screened for hyper-production of protease. The production of protease by mutant *B. subtilis* strain was optimized by varying temperature, inoculum size, pH and incubation time under liquid state fermentation. The CCD model were found to be reliable with r^2 of 0.999. The maximum enzyme activity of *B. subtilis* IBL-04 mutant with 3 mL/100 mL inoculum size, 72 h fermentation time, pH 8, and 45 °C temperature was developed with enzyme activity 631.09 U/mL, indicates 1–7-fold increase in enzyme activity than the parent strain having 82.32 U/mL activity. These characteristics render its potential use in industries for pharmaceutical and dairy formulation.

1. Introduction

The proteases are biological macro-molecules known as simple destructive enzymes due to their broad range of scientific, catalytic, analytic, and industrial applications (Neurath and Walsh, 2011). These proteolytic enzymes are universal and commonly found in all types of microorganisms and are vital for the growth and development of a cell and are preferred over inorganic catalyst (Sharma et al., 2004). They are hydrolytic in their mode of action and are of most importance due to their significant demand in pharmaceuticals, food and other related industries for different formulations (Razzaq et al., 2019; Ikram-Ul-Haq and Umber, 2006). Catalysis of the peptide bond in protein is catalyzed by the protease enzyme (P. Singhal et al., 2012). It has been seen that these enzymes act as a bioactive catalyst in living biological processes such as blood clotting, regulation of apoptosis, as well as in transcriptional regulation moreover it is a compulsory reactant for dehairing process of leather, detergents, for removing silver over the x-ray surfaces, food, medicine, and textile industry and (Walsh and Remigio, 2011). Alkaline proteases are separable from the immense range of sources and many types of these enzymes are present in plants, animals, bacteria, fungi, viruses and archaea (Jeongn et al., 2004).

Proteases found in plants and animals are not sufficient to fulfill the industrial demand due to the increasing demand and it is compulsory to produce this enzyme at a low cost and in a shorter period (S. Kumar et al., 2005). *B. subtilis* among the Bacillus species is a best alkaline protease producer at commercial level (Oda, 2012), its molecular mass varies from 40 to 130 kDa (M.I. Gimenez et al., 2000; C.A. Studdert et al., 2001). Alkaline proteases produced by *B. subtilis* have physiochemical properties (Mitchell et al., 2007). *B. subtilis* found in different sources like wastewater and soil are diverse these microbes enhanced the soil fertility as well, isolated

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and used to produce the protease at a commercial level (H.S. Alnahdi, 2012). The mutagenicity is the induction of the genetic variation in the cell through the changes in the genetic makeup of the cell (usually deoxyribonucleic acid DNA) (Hubar et al., 2017). These variations or changes can subsequently be inherited from one cell to another (Deng et al., 2010). The mutagenesis is a significant procedure where DNA mutations are deliberately intended to yield mutant strains or genes of the organism. Numerous components of a gene, for example, its control components and its gene product can be changed so that the functioning of the genes or protein can be observed in detail (Uyar et al., 2011). The mutation yields mutant proteins with interesting, new or improved properties.

B. subtilis gene apr-IBL-04 (1149 bp) is responsible for the production of alkaline protease (Xu et al., 2019). Different mutagens are commonly used to cause mutation in the DNA likewise: chemical mutagens (ethyl methyl sulfonate, ethidium bromide, nitrous oxide (NO_2), hydroxylamine (HA), etc.) and radioactive X-ray and UV radiations (Justin et al., 2001). These mutagens are highly carcinogenic and can cause mutation at different concentrations. RSM is a statistical and mathematical techniques useful in to determining the effect of a variety of independent variables on the yield. RSM is also helpful to estimate the interaction between variables and also helpful to indicate the optimum conditions according to set variables with a minimal number of observations and experiments (S. Puri et al., 2002; V.V.R. Bandaru et al., 2006). To check the gene expression optimized conditions with different variables are required to select the maximum activity producing strain. In this research, Ethidium bromide was used to cause mutation in the *B. subtilis* strain to develop a hyper producing microbial strain for alkaline protease production in liquid state fermentation followed by the optimized conditions by applying the screening, selection, and optimization by applying RSM under CCD. The present study was constructed with the following objectives; (1) To develop hyper producing mutant strain of *B. subtilis* for the production of proteases. (2) To improve the process variables by the RSM under CCD. (3) To improve the production of protease by the particular mutant strain in a liquid state fermentation media.

2. Material and methods

2.1. Microbial strain collection and materials

Pure line *B. subtilis* strain was collected from the Industrial Biotechnology Lab 04, department of biochemistry. The culture was grown on agar-agar medium slants for 72 h at pH ranges from 7 to 7.5 at 37 °C. The cultured slants were preserved at 4 °C for further use in inoculum preparation.

Ethidium bromide, Luria Bertania, peptone, 2-Deoxy-D glucose, triton X-100, agar, skim milk, calcium chloride, sodium chloride, glucose, dibasic-potassium phosphate, phosphate buffer, hydrochloric acid, follin-ciocalteau reagent, magnesium sulfate, sephadex G-75, sodium hydroxide, potassium dihydrogen phosphate, trichloroacetic acid, methylated spirit, distilled water. The chemicals were purchased from sigma-USA.

2.2. Screening and selection of bacterial strain for protease activity

B. subtilis suspensions were pre-cultured in agar-agar medium plates at pH 7 and 30 °C temperature. Pre-cultures were agitated on shaker bath at 150 rpm for 24 h. This homogenized culture was inoculated in fermentation media; (g/L) glucose, 10; peptone 5; KH_2PO_4, 2; $CaCl_2$, 0.4; $MgSO_4.7H_2$ 0.02; in double distilled water. The medium was autoclaved for 15 min at 121 °C and inoculated with *B. subtilis*. The inoculated media was incubated for 24 h at 37 °C temperature. After that the fermented broth was centrifuged to harvest the enzyme at 4 °C for 20 min at rotating speed of 10,000 rpm and supernatant was collected and used for estimation of enzyme activity (Essam et al., 2012). After enzyme assay absorbance was checked by using UV/Visible Spectroscopy at 660 nm (A. K Sharma et al., 2015). The activity of pure lines are given in the Table 1, and best strain optimized was used for further experiments and mutagenic treatment.

2.3. Protease assay

Activity of protease was analyzed by the standard procedure of enzyme assay (Gopiriya et al., 2014). 1 mL of 0.5% casein phosphate buffer solution of pH 8 was taken in each 0.2 mL supernatant which was taken from the culture medium. The mixture solution was incubated at 37 °C for 30 min. Then 3 mL of 10% TCA (Trichloroacetic acid) was poured in that mixture and then incubated for 10 min at 40 °C for precipitation, and centrifuged for 15 min at 12,000 rpm. The supernatant was collected and 500 mM Na_2CO_3, 1 mL of FC reagent was added and was further incubated for 30 min in dark to produce blue color. Then characterization was done under UV/Visible spectroscopy at 660 nm to check the optical density.

Protease Activity = Liberated Tyrosine × Total assay volume/ Used Protease volume × incubation Time

2.4. Tyrosine standard curve

A stock solution of an amino acid tyrosine (100 μg/mL) was prepared and aliquots in the range of 5–350 μM. A standard curve was plotted between the increasing concentrations of tyrosine against absorbance at 660 nm.

2.5. Mutagenic treatment

B. subtilis suspension was treated with different concentrations of ethidium bromide (EB) for different periods and was incubated at almost 37 °C for different time intervals. The sample solution at different time intervals were taken serially diluted and centrifuged immediately at 12,000 rpm for 15 min. Pellet obtained was re-suspended in 5 mL phosphate buffer (S.S. Mehmod et al., 2015) to stop the further reaction of EB. The cell suspension was washed in phosphate buffer solution by serial dilution procedure (Fig. 1).

2.6. Screening and selection of mutant strain

The mutant strain was grown on agar plates. The petri dishes were incubated at 37 °C for 24 h. The number of colonies formed in each skim milk, agar containing dish was expected to be formed by a single spore cell. A hyper producing mutant for the production of protease was detected by analyzing its growth pattern (Mukhtar and Haq, 2012). The screening of a mutant strain was performed in 25 mL of petri dish1% skim milk and 2% agar, peptone, triton x-100; a colony restrictor, and 2-deoxy D glucose; an antibiotic marker shown in (Fig. 2). After incubation of 24 h at 37 °C, all the dishes were over-flood with TCA 10% solution for 5 min which removed the mild protease layer over the colonies and the circular and transparent zones around the colonies in an opaque white background was picked up and analyzing the absorbance of protease.

2.7. Purification and enzyme assay

Protease produced by mutagenic strains of various concentrations and time-periods at skim milk agar plates were collected

Table 1
Activity of pure line *Bacillus subtilis*.

S. No.	Incubation time (h)	Temperature (°C)	Inoculum size (mL)	pH	Activity
1	24	37	1	7	82.32
2	24	37	1	7	80.73
3	24	37	1	7	82.23

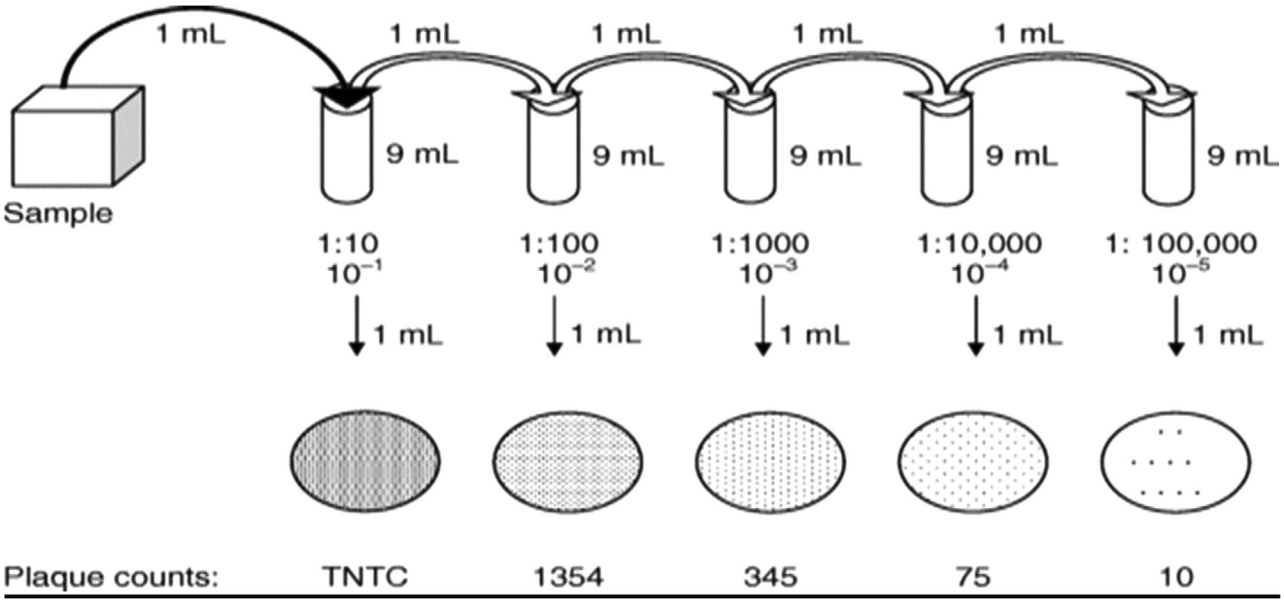

Fig. 1. Serial dilution of *Bacillus subtilis* after mutagenic treatment.

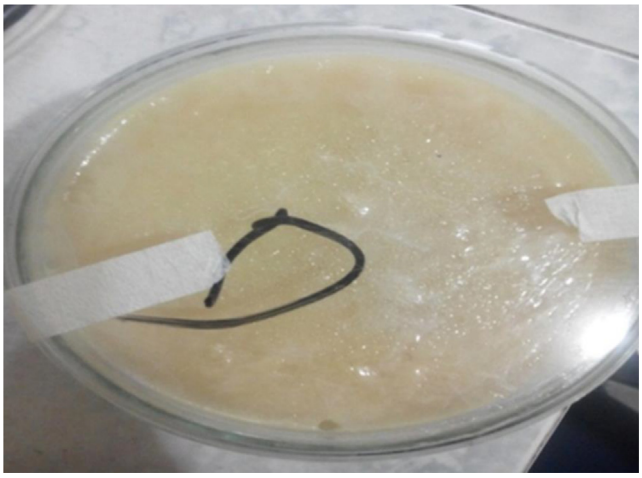

Fig. 2. Selection of mutant strain on 2-deoxy D-glucose skim milk plates.

by adding 10% TCA solution which stops the reaction of protein hydrolysis, after leaving for 15–20 min, centrifuged, 1 mL supernatant was taken and filtered through Whatman filter paper no.1. Enzyme filtrate (2 mL) was taken in a test tube and 4 mL of 0.1 M NaOH and 0.5 mL of diluted Folin Ciocalteau reagent was added. The residues of tyrosine released by enzymatic break down of protein were determined under spectrophotometry under 660 nm wavelength (A.K Sharma et al., 2015). The strain showed greater activity was selected for further optimization for fermentation media.

2.8. Optimization parameters of liquid state fermentation media and RSM

The fermentation media for protease production was prepared by 1% carbon source, 0.5% organic or inorganic nitrogen sources, 2% K_2HPO_4, 0.02% $MgSO_4$, and 0.04% $CaCl_2$. The different ranges of pH 6–10 shown in (Fig. 3) of the media were adjusted with NaOH/1N HCl and then autoclaved for 15–20 min at 121 °C. After the selection of a best mutated bacterial strain various parameters for the fermentation process were optimized in triplicate flasks for hyper protease production with selected *B. subtilis* mutated and parent strains and in liquid state fermentation (LSF) medium at varying temperatures, fermentation time, pH, and inoculum size by applying RSM.

RSM is a modest design to evaluate the effect of several parameters affecting the response by varying them. The CCD model were used to study the interaction effects of protease using Design expert statistical software (version 7.1.1). The tests consists of measured responses of groups from experimental and controlled factors, according to 1 or more chosen criteria were used. For the current analysis, designs of four factors were applied to make fit the model of 2nd order polynomials which shows that the 30 experimental runs were compulsory for the optimization process. By solving the regression equation, the optimized standards of the variables were selected. The fermentation runs of 30 test runs were carried out in triplicates (Mukhtar, 2013). The media having varying pH was added in flasks of 250 mL, cotton plugged and aluminum foiled. The specifically selected substrate was inoculated with mutated *B. subtilis* strain with different inoculum sizes in each run and were incubated with continuous shaking for different fermentation periods at different temperatures (Table 2).

Fig. 3. Fermentation media at different pH for protease production.

Table 2
Values of experimental independent variables.

Independent variables	Coded levels	Coded levels
Ranges	−1	+1
pH	6	10
Temperature (°C)	35	55
Inoculum size (mL)	1	5
Incubation period (h)	24	120

3. Results and discussion

3.1. Wild type strain growth

The wild type of strain was grown on skim milk agar plates to optimize the parent and mutant strain activity. The physiological growth of wild type strain *B. subtilis* is shown in Fig. 4A.

3.2. Mutagenesis of B. subtilis

Procedures of mutagenesis were optimized in terms of dose and type of mutagen. Mutagen specificity can be taken into account and mutagenesis itself can be enhanced to screen out the best mutants. Chemical mutagenesis was carried out by using Ethidium bromide (EB), independently

3.3. Chemical mutagenesis of B. subtilis by EB

EB is known as a mutagenic and carcinogenic organic and aromatic intercalating compound. EB causes a shift of frame type mutation in the genetically makes up. EB is a widely used chemical that is used as a fluorescent dye in biotech experiments. It is a mutagenic compound that interacts with RNA and double-stranded DNA. EB is water-soluble and shows great stability under normal pressure and temperature (Kumar et al., 2012).

3.4. Optimization of mutant strain

The best selected mutant strain for further optimization of physical and nutritional parameters were found at 60 min (Fig. 4B) among 4 different time intervals expressed in Table 3 incubation period of mutagenic treatment which produces 484 (IU/mL) of protease at 24 h fermentation time.

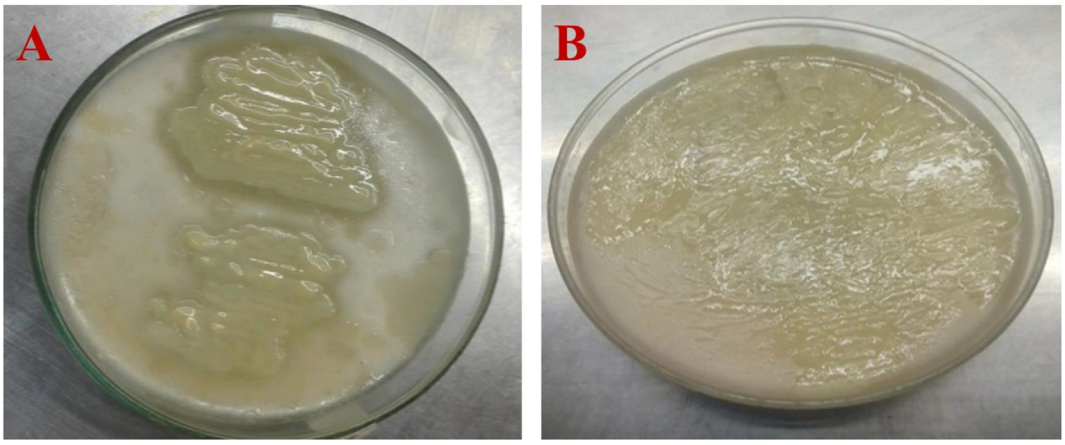

Fig. 4. Wild type strain growth (A). The growth pattern of chemically mutated *Bacillus subtilis* by EB 60 min on a Petri plate (B).

Table 3
Effect of mutagen on protease production by *Bacillus subtilis* at different time intervals.

S. No.	Incubation time (min)	Enzyme activity IU/mL
1	30	437
2	60	484
3	90	435
4	120	426

3.5. Response-surface methodology

RSM is a mathematical and statistical technique which is used to analyze various parameters in which effective and valuable response is affected by different kind of parameters and the basic aim is to optimize that response (Beg Q.k. and R. Gupta, 2009) and to develop the best growth medium for *B. subtilis*. The activity of protease enzyme was taken as dependent and response variables were selected for further study (Table 4). The *P-value* and *F- value* in the ANOVA were studied which shows lesser the *P-value*, and grater the importance of the corresponding coefficient (Mhamdi et al., 2017). The *F-value* of 97.57 indicates the model is significant (Table 5) and minimum chances of errors in the model. The p-value of 0.05 demonstrates the model is significant. The model terms will not be significant if the values are greater than 0.1000. The present model were significant corresponding to its p-values.

Table 4
CCD (Central Composite Design) for optimization of protease production by EB.

Run	Temperature	pH	Inoculum Size (mL)	Fermentation time (h)	Protease activity (IU/mL)
1	55	6	5	120	70.49
2	40	8	3	72	566.64
3	35	6	5	120	274.73
4	45	8	3	72	630.86
5	45	8	2	72	585.15
6	45	7	3	72	563.15
7	35	10	5	24	287.89
8	45	9	3	72	542.11
9	45	8	3	72	631.09
10	35	6	1	120	274.06
11	55	6	1	24	269.60
12	35	10	1	24	283.43
13	45	8	4	72	582.92
14	55	10	5	120	276.74
15	45	8	3	96	517.13
16	35	10	1	120	282.14
17	45	8	3	48	583.14
18	45	8	3	72	630.84
19	45	8	3	72	630.89
20	35	10	5	120	283.43
21	55	10	5	24	283.40
22	45	8	3	72	630.86
23	50	8	3	72	493.49
24	45	8	3	72	630.89
25	35	6	1	24	274.51
26	35	6	5	24	278.97
27	55	6	1	120	269.60
28	55	6	1	120	270.05
29	55	6	1	120	281.64
30	55	6	1	120	275.62

Table 5
ANOVA for response surface quadratic model.

Source	Sum of squares	df	Mean square	F-Value	P-value Prob > F	
Model	4010.75	14	17922.66	97.57	<0.0001	*Significant*
A-Temperature	829.64	1	829.64	4.52	0.0506	
B-Inoculum size	336.38	1	336.38	1.83	0.196	
C-Fermn Temp.	330.49	1	330.49	1.8	0.1998	
D-pH	489.6	1	489.6	2.67	0.1234	
AB	44.72	1	44.72	0.24	0.6289	
AC	455.5	1	455.5	2.48	0.1362	
AD	101.15	1	101.15	0.55	0.4695	
BC	168.29	1	168.29	0.92	0.3537	
BD	78.9	1	78.9	0.43	0.5222	
CD	201.43	1	201.43	1.1	0.3116	
A^2	9135.67	1	9135.67	49.73	<0.0001	
B^2	66.17	1	66.17	0.36	0.5573	
C^2	0.56	1	0.56	3.06E-03	0.9566	
D^2	146.8	1	146.8	0.8	0.3855	
Residual	2755.4	15	183.7	0.68		
Lack of Fit	955.1	10	95.51		0.916	*Non-significant*
Pure Error	802.23	5	173.22			
Cor Total	5764.59	29				

The comparison of observed response variability values with the variables under experiment and their particular interaction was calculated by the coefficient of determination R^2. The best analysis of fitting of the model was calculated by R^2, its values range from 0 to 1 and the model is found to be good if predicted value is near or equivalent to 1 (Olajuyigbe and Ajele, 2005). In the present study the coefficient of determination value was found to 0.9891 for the production of alkaline protease. This can be interpreting as up to 98.91% variability response. The adjusted R^2 was 0.9790 which has a close relationship with R^2. The predicted R^2 was 0.9491 which shows close resemblance with R^2 and adjusted R^2. This shows the goodness of the model and the parameters optimized. The value 0.9491 of Pred R-Square is in agreement with the value of 0.979 of Adj R-Square with precision (*CV%:* 2.59). The Adeq-Precision calculates the ratio of signal to noise and this should be greater than 4 (Ahmad et al., 2020; Bagewadi et al., 2011). In the present investigation Adeq-precision was found to be 22.64 which shows a satisfactory signal. Hence the model can be used to direct the space of this design (Pant et al., 2015; Ahmad et al., 2015).

The BBD model resulted in six response plots for the optimization of alkaline protease and these were studied. The relationship of dependent and independent variables of protease were studied graphically by 3D response surface (RS) plots which were used to

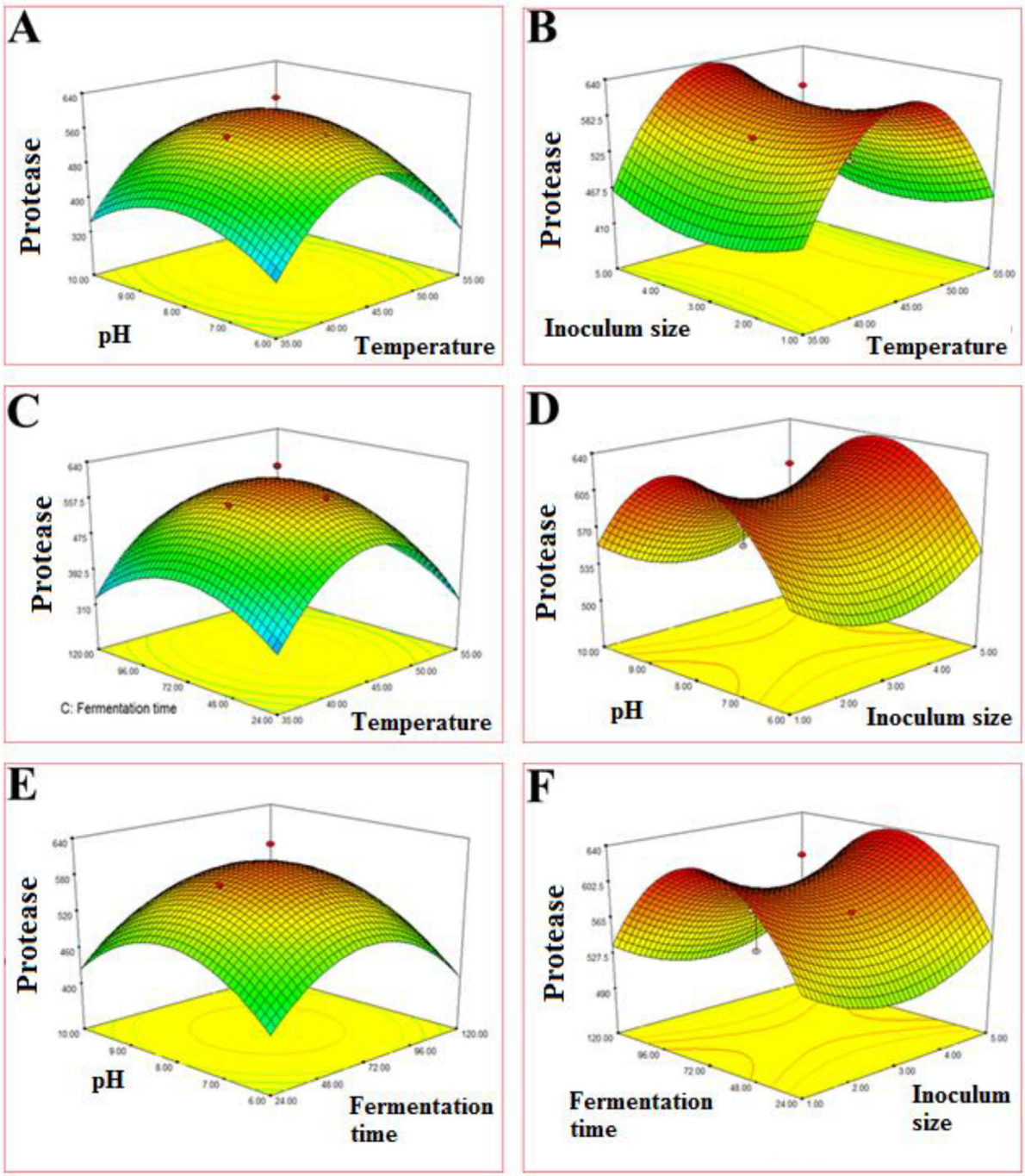

Fig. 5. Response surface graphs showing (A) Interaction of pH 8–9 *vs* temperature 49 °C (B) Inoculum size (5 mL) *vs* temperature 49 °C; (C) Fermentation time 72 h *vs* temperature 45 °C (D) Inoculum size 5 mL *vs* pH 8–9 (E) Fermentation Time 72 h *vs* pH 8 (F) Fermentation time 72 h *vs* Inoculum size 3 mL.

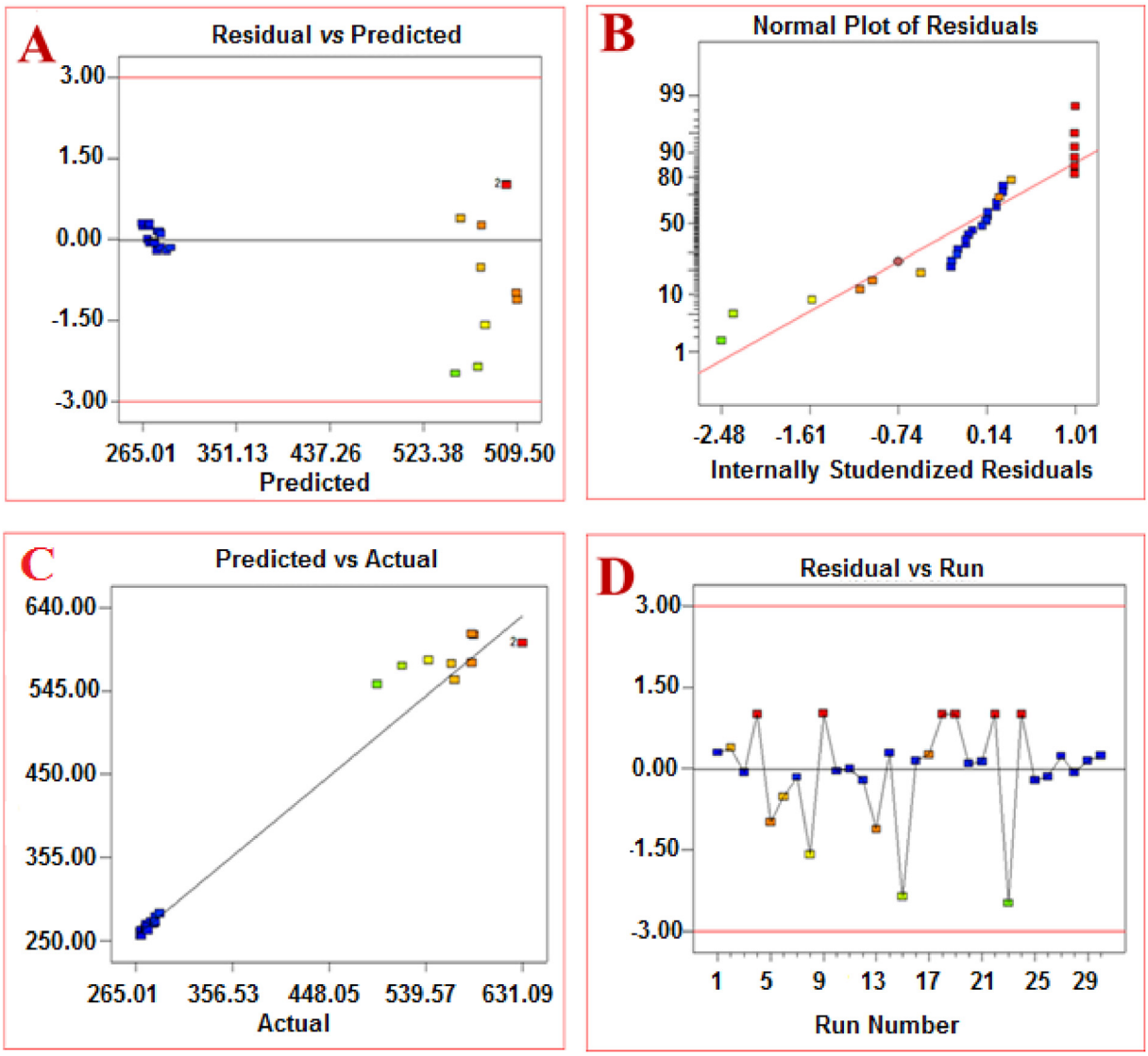

Fig. 6. Diagnostic plots (A) Normal % of Probability Plot (B) Correlation between Actual and Predicted value (C) Correlation between Residuals and Predicted values (D) Correlation between Residuals and Run represents adequacy of the model analyzed by residuals from the least square fit.

explain the optimum circumstances significantly. The optimum pH for protease enzyme synthesis by *B. subtilis* was 8–9 and temperature of 45 °C (Fig. 5A). In the case of protease enzyme produced by *B. subtilis* inoculum size and temperature have opposed effect. There is an increase in the production of an enzyme with an initial increase in inoculum size and temperature. RS plot showed that at 5 mL inoculum size at 45 °C temperature yields maximum protease production (Fig. 5B). Initially increase in fermentation time (72 h) and temperature (45 °C) increases the production of protease (Fig. 5C). 3D graphs indicates the pH range of 7–9 (alkaline) and inoculum size of 5 mL were optimum for protease production (Fig. 5D). Protease production was affected by fermentation time and pH at their most mid-level. The response surface graphs shows that at pH 8 and fermentation time 72 h protease enzyme production was found to be maximum (Fig. 5E). Conversely, the protease enzyme production also showed an optimum yield at 3 mL of inoculum size and 72 h fermentation time (Fig. 5E).

Adequacy of the model is analyzed by residuals from the least square fit. To assume the probable normality a diagnostic check of the design were plotted for the production of protease by *B. subtilis* (Fig. 6A). For the production of protease by *B. subtilis* in liquid state fermentation, a very close relationship in predicted and actual values were observed and a strong correlation was used to demonstrate the accuracy and precision of CCD (Fig. 6B). Between residual and predicted values a strong correlation was used to demonstrate the accuracy and precision of CCD (central composite design) (Fig. 6C-D). For the production of protease by selected chemically mutated strain, optimized parameters were 3 mL/100 mL of inoculum size, 72 h fermentation time, pH 8, and 45 °C temperature. The activity of the protease enzyme were increased 1–7 folds than the parent strain or the enzyme showed 631 IU/mL activity produce by mutant strains while the wild type strain exhibit 82.32 IU/mL activity.

4. Conclusion

Alkaline bacterial proteases play an essential role in different pharmaceutical, food and industries and their future use is likely to be increased. The investigators are using advancing strategies such as computer biology, protein/genetic engineering, and molecular biology for the production of improved strains of protease. In

the current investigation mutagenesis of *B. subtilis* apr-IBL-04 was done for hyper protease producing strain by using EB as a mutagen. A random chemical mutation was preceded for different periods and at different chemical concentrations. Selection and screening were done to select the strain that ability of hyper producing protease at minimum nutritional elements. Further on various physical parameters, inoculum size, fermentation time, temperature, and pH was optimized by applying RSM under CCD yielded maximum protease production. The maximum enzyme activity of *B. subtilis* IBL-04 mutant under optimized conditions were found to be 1–7-fold increase in enzyme activity than the parent strain. Overall the model was able to calculate the alkaline protease yield and provide valuable tools for optimization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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