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Advances in Bioresearch

Functionalization of a *Bacillus Oryzae corticis strain R1* for novel haloalkaliphilic protease production and process optimization using a complete factorial design approach

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ABSTRACT
In this work, the functionalization of an isolated bacterium in the presence of NaCl was explored using a full factorial design methodology for determining the best conditions to produce an evaluated amount of haloalkaliphilic protease units. Optimization of fermentation conditions for maximum novel haloalkaliphilic protease production using a bacterium Bacillus Oryzaecorticis strain R1. The process components, NaCl, inoculum size, incubation temperature, and glucose, were elected to optimize enzyme production. The optimization experiments were designed using 2^4 factorial composite experimental design and response surface methodology, and results were analyzed. This statistical approach produces little experiments while allowing for interaction between the four components. The optimum variables for the maximum haloalkaline protease production were glucose 6%, inoculum size 5% (10^8 cells/ml), temperature 60°C, and NaCl 20% at pH 11. The maximum haloalkaline protease production was 16,624.5U. In this method, the model adequacy was very significant as the coefficient of determination was 0.991 and showed 1.3 fold increase after medium optimization. A smaller and less time-consuming experimental design suffices to optimize many fermentation processes.
***Keywords**: Haloalkaline protease, Factorial design, Counter plot, Multiple regression, Growth kinetics.*

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INTRODUCTION

Halophile is one category of extremophiles. Haloalkaliphiles have adapted to a few unique and unusual strategies [17]. Proteases are significant industrial biocatalysts because they are environmentally favorable. Widely being used in industries, haloalkaliphiles also reduce toxicity in the atmosphere and can be gained from various sources like microbes, plants, and animals. Biostatistical optimization is a commonly used approach for strain improvement that can be induced randomly for site specificity. This process leads to increased harvest production compared to unoptimized fermentation production [11]. Nutrition factors and fermentation conditions greatly influence the nature and yield of the secondary metabolites produced by the bacteria. So, the bioactive metabolites can be increased by several folds by applying optimization studies of the factors affecting the production process [3]. The exo-enzymes of haloalkaliphiles exhibit unique structural, biochemical characteristics that are stable under hypersaline saline conditions [2]. They are also crucial in many industrial and biotechnological applications for their stability and activity at low water levels. Factorial designs are more efficient than one-factor-at-a-time (OFAT) method experiments. They provide more information at a similar or lowest cost. They can find optimal conditions faster than OFAT experiments. Factorial design is a closed system for the optimization of the medium. This method involves the study of variation in factors or parameters at two or more levels [10]. It is highly efficient in providing interaction among many elements factors and allows us to study the effect of each factor, and their interaction gives maximum yield [15]. Factorial designs enable additional factors to be examined at no extra cost.

MATERIAL AND METHOD

1) **Hypersaline soil sample collection and isolation of bacteria:**
The novel strain of Haloalkaliphilic bacterial isolate was isolated from the hypersaline soil of Bhavnagar city, Ghogha beach coast in Bhavnagar (21.6767° N Latitude and 72.2845° E Longitude).

2) **Haloalkaliphilic protease production and assay:**
The haloalkaliphilic isolate was isolated and selected based on higher REA (zone of casein hydrolysis) on a haloalkaliphilic agar medium having 5% casein and pH 10.5 [21]. It was also analysed for higher haloalkaliphilic protease production in production broth medium (6% glucose, 2% peptone, 1% malt extract, 1% yeast extract, 0.5% $MgSO_4.7H_2O$, 0.5% K_2HPO_4, 0.1% FeSO4, 1% Casein, NaCl 10%, pH 10.5) [19]. The production medium was inoculated with 1% v/v inoculum (10^8 cells/ml) and incubated at room temperature (34±4ºC) with shaking at 180 rpm in a 250 ml Erlenmeyer flask. The cells were harvested every 24 h interval by centrifugation at 10000×*g* for 10 min. The cell-free extract was used for haloalkaliphilic protease production. The alkaline protease activity was measured using the slightly modified Anson-Hagihara method [6] [16]. The enzyme reaction mixture of 1 ml was taken after centrifugation at 10000×*g* for 15 min and diluted in 2 ml Na-bicarbonate buffer (0.1 M, pH 10.8) [18] and supplemented with 1% casein and 1% NaCl. The reaction was then incubated for 15 min at 60ºC. The response was stopped by adding 3 ml 10% (w/v) Trichloroaceticacid. After 10-15 min, the reaction mixture was centrifuged at 15000×*g* for 15 min to remove residual precipitates. The free enzyme was estimated by standard tyrosine curve (10-200 µg/ml tyrosine standards) using Folin Lowry spectrophotometric method. One unit of enzyme liberated was defined as the enzyme quantity required releasing 1 µg of tyrosine/ml of the substrate per minute under the assay conditions using 1% w/v Hammerstein casein as the substrate [8].

3) **Identification of bacteria**
DNA of the isolate was isolated from the culture, and its quality was evaluated on 1.0% agarose gel for a single band of high molecular weight. PCR amplified fragments of the 16S rRNA gene. DNA sequencing reaction of PCR amplicon was carried out with 357F and 1391R primers using BDT v3.1 cycle sequencing kit on ABI 3500xl Genetic Analyser. The 16S rRNA gene sequence was used to carry out BLAST with the database of NCBI GenBank. Based on the maximum identity score first fifteen sequences were selected and aligned using multiple sequence alignment software programs [11] [12].

4) **Statistical-based optimization of production medium**
Significant medium components and factors affecting haloalkaliphilic protease production are the pivotal steps for process optimization. 24 factorial designs optimized the considerable concentration of factors affecting enzyme production. In addition, factorial design helps assess multiple independent variables in a single study [21].

5) **Significant component screening by 2^4 factorial designs:**
For optimal production of haloalkaline protease, selected medium components and factors were used for the 24-factorial design. This fractional factorial design sets the essential elements of the production medium and parameters for the maximum production of haloalkaline protease [14]. As shown in Table 1, the four variables affecting the haloalkaliphilic protease production selected were namely temperature, inoculum concentration (%), NaCl (%), and glucose (Each component was individually considered in two concentrations of high and low (-)). The matrix of the factorial design was designed using 'Minitab 20 software [20]. The experiment's factorials design contains 16 flasks of all four different variables. The effect of all variables on haloalkaline protease production was separately calculated and analyzed by one way analysis of variance (ANOVA). Based on the *p*-value ($p < 1$) and the highest confidence level of the significant variance was selected from other independent variables. All these factors were studied in triplicate. The magnitude of selected components was interpreted in terms of different graphical presentations [5].

6) **Statistical analysis of the data and models:**
The data calculated from the factorial experiment were analysed for multiple regression analysis using least squares to construct the regression model design. A second-order (quadratic) function was used to fit the obtained data. Experimental design, data analysis, interaction plotting, and optimization of factor conditions were done with MINITAB 20 statistical software. In contrast, Excel 2007 confirmed model fits where predicted responses were plotted against experimentally derived data [5]. All hypotheses were tested at a 99% confidence level. For the three accepted variables as per the model considered in the optimization experiment, the quadratic model regression equation took the form below:

Enzyme Activity (U/ml) = 690647-159160 Inoculum size (%) - 29646 NaCl - 160100 Glucose +6880 Inoculum size (%)*NaCl+ 37643 Inoculum Size (%)*Glucose +6975 NaCl*Glucose-1641 Inoculum size (%)*NaCl*Glucose.

7) Illumination of nutritional factors leads to haloalkaline protease production:

In the medium optimization experiment, the most critical factors affecting haloalkaliphilic protease production were illuminated by a two-level fractional 24-factorial design. The cellular activity was estimated for the determination of growth. The broth aliquots were centrifuged at 10000×*g* for 15 min. The residual bacterial growth was estimated at OD 660 nm [7].

RESULTS AND DISCUSSION

Isolation and identification of potential haloalkaliphilic protease-producing bacterial isolate: 11 different morphotypes was isolated from the Ghogha coastline. All isolates were bacterial strains. The dominant genera of the isolates were haloalkaline bacillus. The morphotype was isolated from hypersaline soil on a haloalkaliphilic medium with a slight modification having a pH of 10.5, containing 5% casein and 10% NaCl and characterized by its morphological and biochemical properties.

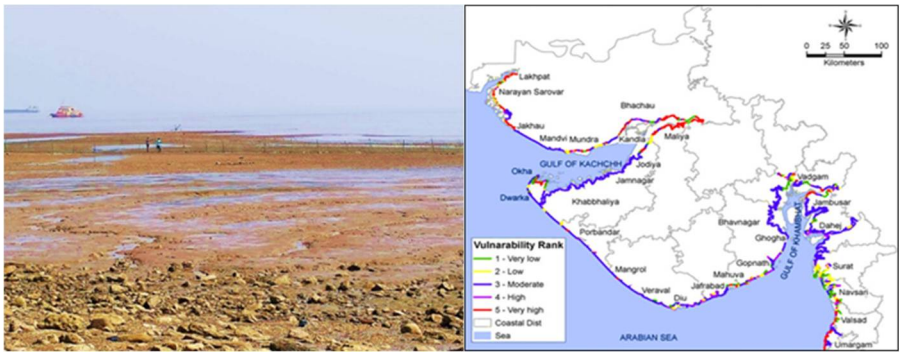

Figure 1 Ghogha Beach coastal area

The novel *Bacillus Oryzaecorticis* ***strain R1*** was isolated on a haloalkaliphilic agar medium. The morphotype utilized a wide range of complex and straightforward molecules and protease isolated on the haloalkaliphilic agar medium. In addition, the zone of casein hydrolysis showed the presence of substrate degradation and confirmation of an extracellular enzyme. Therefore, the strain was identified as ***Bacillus*** *Oryzaecorticis strain R1* (accession number NR_133977.1), and the isolate was designated as *Bacillus Oryzaecorticis strain R1* with 98.75% of the 16S rRNA gene sequence similarity with its closest homology (Fig. 2). The selected and identified strain ***Bacillus Oryzaecorticis strain R1*** was assessed for the haloalkaliphilic protease production under the SmF (submerged fermentation) conditions in the fermentative production medium where casein was utilized as a core source of protein.

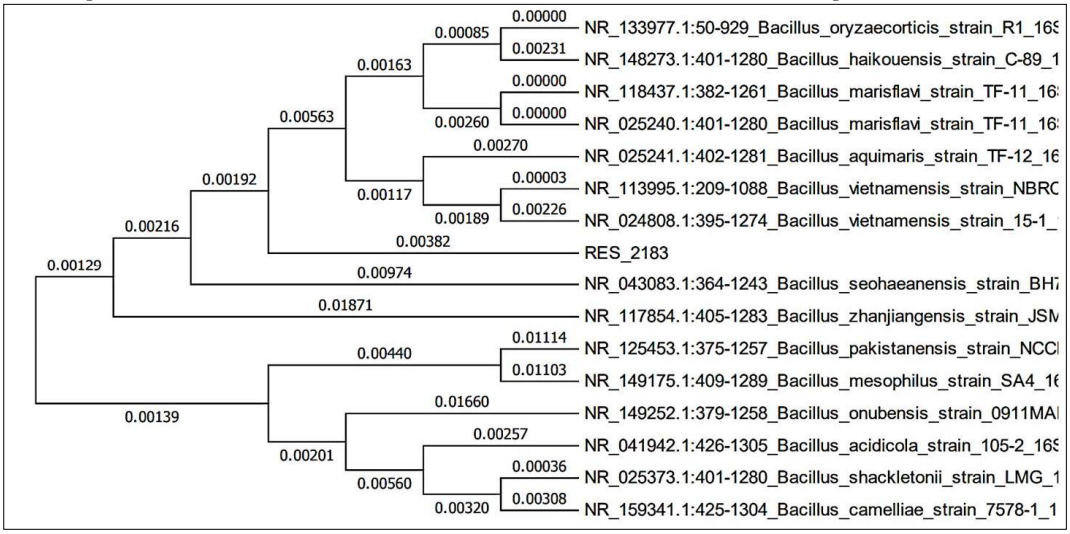

Figure 2 Phylogenetic tree of isolate

2^4 Factorial Design (FD) and selection of the significant variables:

Run flask number 14 shows the best result in the FD design. It was shown 16624.5 U/ml haloalkaliphilic protease production with a combination of variables temperature 60°C, Inoculum size 3%, NaCl

concentration 25%, and Glucose concentration 5%, which was highest with compared all other variables combinations (Table-1).

Table 1: 2^4Factorial design

Run	Bulk	Temperature (°C)	Inoculum size (%)	NaCl (%)	Glucose (%)	ResponseR1 (U/ml)
1	1	2	1	2	1	___
2	1	2	2	1	2	14558.3
3	1	2	2	2	2	3.5
4	1	1	1	2	1	637.7
5	1	2	2	2	1	327.6
6	1	1	1	2	2	244.1
7	1	1	1	1	2	4.5
8	1	1	2	2	2	11205.3
9	1	1	2	1	1	3811.9
10	1	1	2	2	1	676.9
11	1	2	1	1	1	192.3
12	1	1	1	1	1	14341.9
13	1	1	2	1	2	334.9
14	1	2	1	2	2	16624.5
15	1	2	1	1	2	2.2
16	1	2	2	1	1	461.0

After flask no 14, the other flasks 2, 12, and 8 also showed higher haloalkaliphilic protease production.

Pareto charts and coded-coefficient studies:

In the FD for 2^4 variables, with constant pH 11, lower and higher variables accordingly temperature 37±2°C and 60°C, inoculum 3% and 5% v/v containing 10^8 cells/ml, NaCl 20% and 25%, glucose 3% and 5%. The response was measured in terms of haloalkaliphilic protease production. Pareto plot focuses on problems with a higher potential for improvement by showing different issues. Relative frequency can be observed in descending bar graph showing the cumulative impact of the problems (Figure 3). Here inoculum size*NaCl*Glucose variables combination led to the highest effect on enzyme production. Then Inoculum size, NaCl, NaCl*Glucose, Inoculum size*NaCl, Inoculum size*Glucose, and glucose different variable combinations showed an impact on enzyme production in decreasing cumulative order.

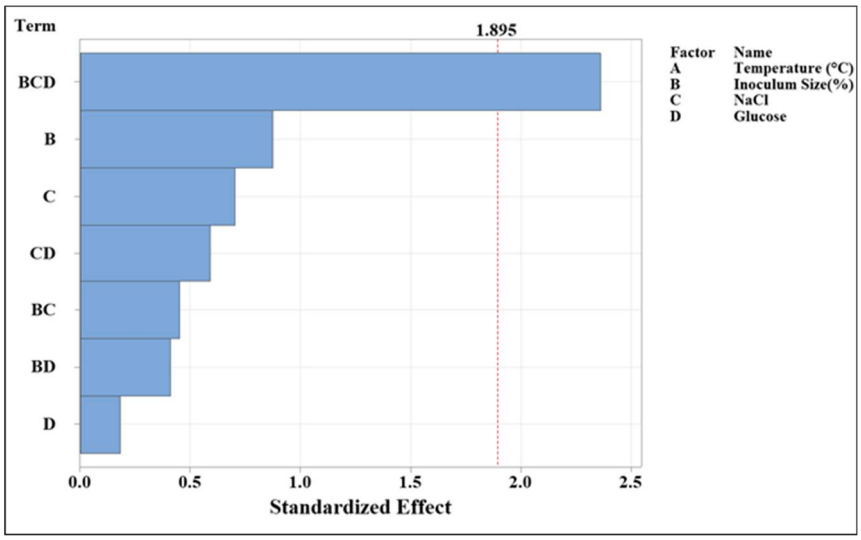

Figure 3 Pareto Chart illustrating the effects of various factors on protease production

Table 2: 2^4 Factorial design coded coefficients

Term	Effect	Co-efficient	SE-Coefficient	T-Value	P-Value	VIF
Constant	-	4874	1738	2.80	0.026	-
Inoculum Size (%)	-3036	-1518	1738	-0.87	0.411	1.05
NaCl	-2447	-1224	1738	-0.70	0.504	1.05

Glucose	-637	-318	1738	-0.18	0.860	1.05
Inoculum Size(%)*NaCl	1571	786	1738	0.45	0.665	1.05
Inoculum Size(%)*Glucose	1423	712	1738	0.41	0.694	1.05
NaCl*Glucose	2047	1023	1738	0.59	0.574	1.05
Inoculum Size(%)*NaCl*Glucose	-8207	-4103	1738	-2.36	0.050	1.05

The coded regression coefficient describes the size and direction of the relationship between a term in the model and the response variable. Minitab can fit linear models using a variety of coding schemes for the continuous variables in the model. These coding schemes can improve the estimation process and the interpretation of the results. Coded units can change the results of the statistical tests used to determine whether each term is a significant predictor of the response [1]. When a model uses coded units, the analysis produces coded coefficients. Coded coefficient variables NaCl*Glucose, Inoculum Size*NaCl, and Inoculum size*Glucose variables in pair showed significant enzyme production in decreasing order accordingly. As here we have taken the same isolate the standard error of the coefficient estimates the variability, the precision of the estimate of the coefficient remains the same (Table 2). T-value was used to calculate the P-value in Minitab-20, which was used to test whether the coefficient was different from '0'. The P-value was a probability that measures the evidence against the null hypothesis. Lower chances provide more substantial evidence against the null hypothesis [4]. Here (table 2) NaCl*Glucose, Inoculum size (%)*NaCl (%), and Inoculum size (%)*Glucose were the significant coefficient variables. The VIF (Variance inflation factor) indicated how much multicollinearity existed in a regression analysis [13]. The VIF value 1<VIF<5 suggested a moderately correlated value of regression coefficient, so as per values of VIF in Table 3, the estimated values were moderately correlated with multicollinearity in regression [9]. Here R^2 value of the model was 0.793, which resembled the 0.75% of the model validity. The R^2 value was reported ted 0.5593% in the haloalkaliphilic amylase enzyme-producing model validation for medium optimization [19].

Study of Analysis of Variance and multiple regression:

Analysis of variance (ANOVA) is a statistical method used to compare variances across the means or averages of different groups.

Table 3: Analysis of Variance (ANOVA)

Source	Degree of Freedom	Adjusted Sum of Squares	Adjusted Mean Sum of Squares of Data	F-Statistic Value	P-Value
Model	7	266561692	38080242	0.89	0.561
Linear	3	48825279	16275093	0.38	0.771
Inoculum Size (%)	1	32772986	32772986	0.76	0.411
NaCl (%)	1	21291597	21291597	0.50	0.504
Glucose (%)	1	1441455	1441455	0.03	0.860
2-Way Interactions	3	25498552	8499517	0.20	0.895
Inoculum Size (%)*NaCl	1	8780648	8780648	0.20	0.665
Inoculum Size (%)*Glucose	1	7202860	7202860	0.17	0.694
NaCl*Glucose	1	14898266	14898266	0.35	0.574
3-Way Interactions	1	239477634	239477634	5.57	0.050
Inoculum Size(%)*NaCl(%)*Glucose(%)	1	239477634	239477634	5.57	0.050
Error	7	300734055	42962008	-	-
Total	14	567295747	-	-	-

A range of scenarios allows it to determine if there is any difference between the means of different groups. ANOVA helps to determine the significance or randomness of the results of an experiment. ANOVA (Table 3) shows how the sum of squares is distributed according to the source of variation and hence the mean sum of squares. Here the one-way, two-way, and three-way ANOVA table is depicted. Sources which were depicting the significant variation in the data were Inoculum size (%), NaCl (%), and Glucose (%) which were analysed in coded coefficient studies. The larger the F-value, the greater the variation between the sample means relative to the variation within the samples. The P-value determines if the difference between group means is statistically significant, we can look at the P-value corresponding to the F-statistic. $0.05<p\leq0.10$ shown moderate evidence of variables whereas, $0.01<p\leq0.05$ shown strong evidence as per null hypothesis. Here, (Table 3) 3-way interaction of variables showed a significant combination level as per the null hypothesis. Inoculum size (%)*NaCl (%)*Glucose (%) combination of variables was significant

for higher haloalkaliphilic protease production. The rest of the other combinations of variables were also less significant for enzyme production.

Factorial plots for enzyme units:

Main Effect plot:

The main effect plot showed the results of all the variables with a comparison to haloalkaliphilic protease production in higher and lower concentrations of the variables. Variables shown in black in Figure 4 were non-significant for a model that showed a parallel line. The remaining variables showed the mean of the haloalkaliphilic protease enzyme unit production in a decreasing manner: inoculum size 3%, NaCl 20%, and Glucose 3% accordingly.

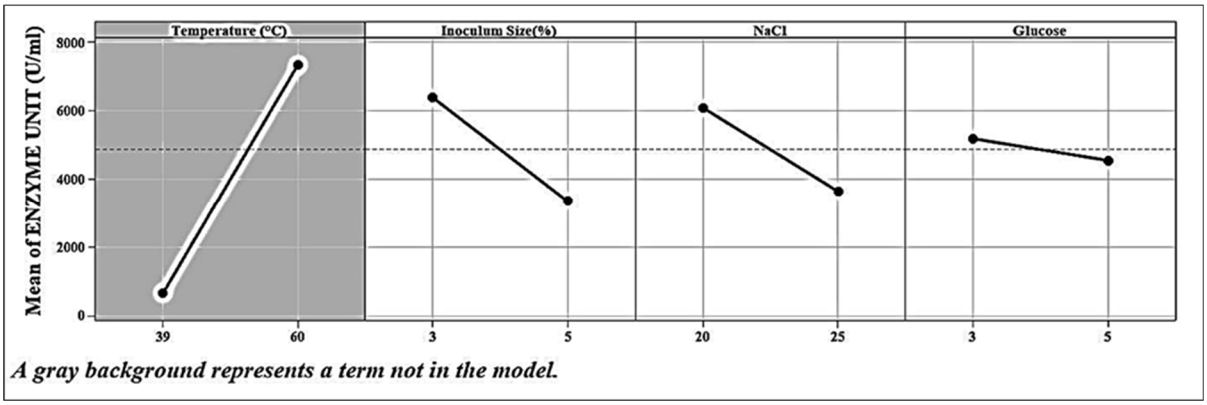

Figure 4: Main effect plot for enzyme production study

Interaction Plot:

The interaction plot shows how the relationship between one categorical factor and a continuous response depending on the value of the second definite factor. Those factors showed haloalkaliphilic enzyme activity, which was the specificity of the enzyme action with response to those specific factors. In addition, it showed the ability of the enzyme to discriminate between two compacting substrates (Figure 5).

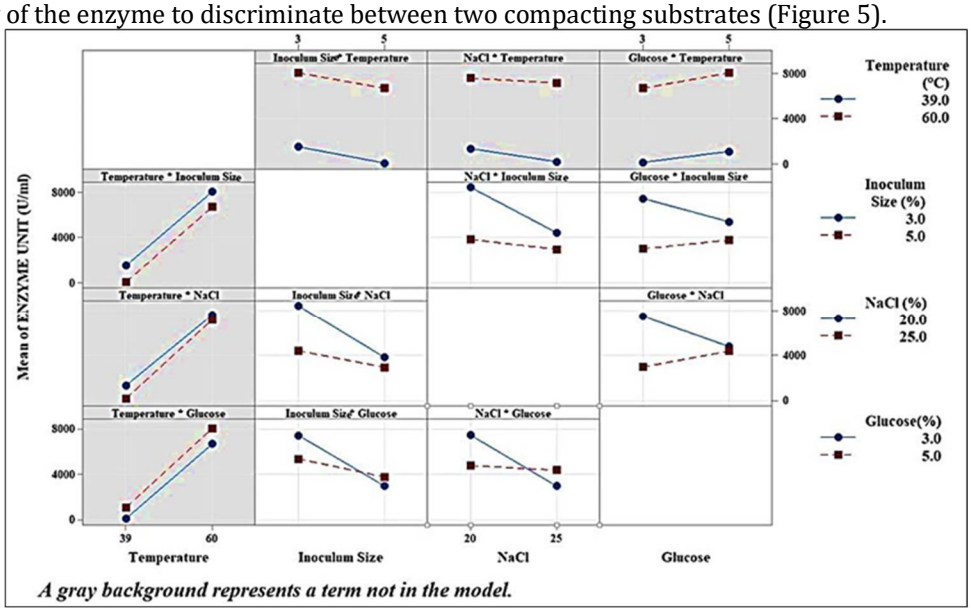

Figure 5: Interaction Plot

Glucose(%)*Temperature(°C), NaCl(%)*Temperature(°C), Inoculum size(%)*Temperature(°C), Temperature(°C)*Inoculum size(%), Temperature(°C)*NaCl(%) and Temperature(°C)*Glucose(%) were non-significant variable interactions. They were showing parallel lines. The parallel line in the graph means no interaction. The more non-parallel lines, the greater the strength of the interaction (Figure 5). As per the significant variables shown in Figure 5, NaCl in 20% concentration 8000 and 4000 U/ml haloalkaliphilic enzyme production combined with Inoculum size and glucose. Variable inoculum size gives 8000 and 4000 U/ml enzyme production at 3 (%) concentrations. The glucose variable also showed higher enzyme unit production at 5 (%) concentrations than the other two variables.

Counter Plot:

The counterplot shows the relationship between a fitted response and continuous variables. In addition, the counterplot offered a two-dimensional view in which points with the same response value was connected to produce counter lines. The counterpoints of counter lines showed enzyme production's efficiency to the variables it interacts with.

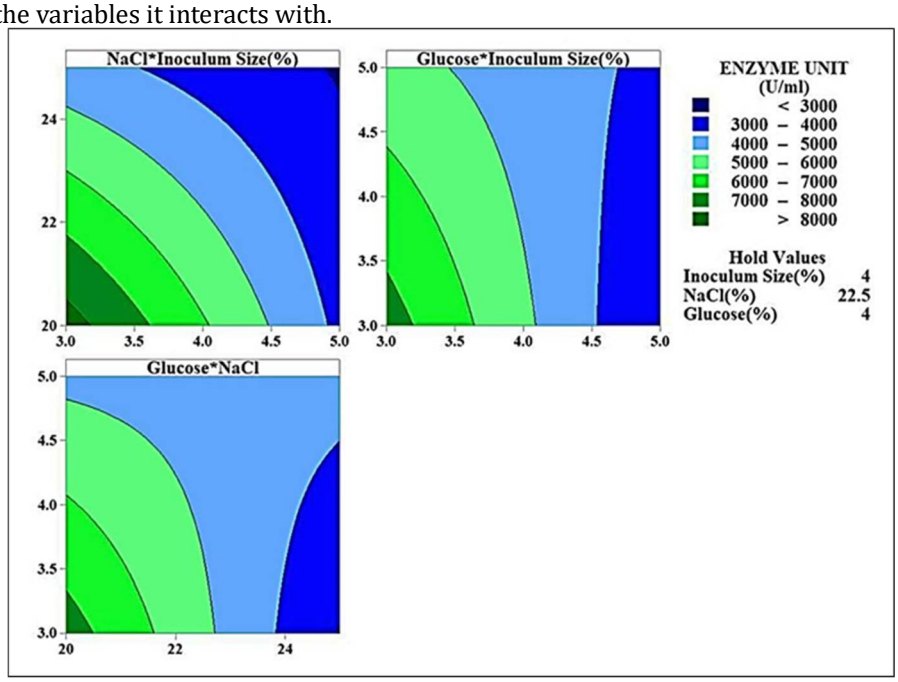

Figure 6: Counter plot of selected variables

The darker green region in Figure 6 indicates higher quality—these higher response values are from the upper to the lower of the graph. For example, the figure showed NaCl 22.5 % and inoculum size 4% produced more than 8000 U/ml of haloalkaliphilic protease enzyme. The remaining variable combinations NaCl*Inoculum Size, Glucose*Inoculum size, and Glucose*NaCl varying combinations also showed 5000-8000 U/ml of haloalkaliphilic protease production.

Surface Plot:

A surface plot displays the three-dimensional relationship in two dimensions, with a smooth surface representing the X- and Y- axes variables and the response variable (Z). A response variable relates to two predictor variables in a 3D surface plot (Figure 7). A 3D surface plot helps investigate desirable response values and operating conditions. Surface plot shows multiple regression analysis of variables Inoculum size (%), Glucose (%), and NaCl (%). The darker color showed a higher response of the variable for haloalkaliphilic protease enzyme production. In Figure 7, surface plot, two continuous variables were shown at once, and one additional variable, enzyme unit, was kept at a constant level.

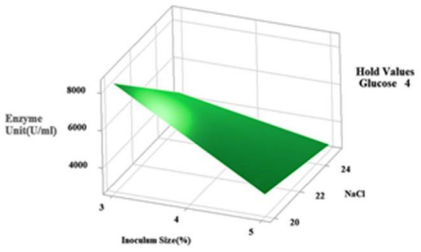

Figure-7(a) Enzyme Unit(U/ml) vs NaCl (%), Inoculum size (%)

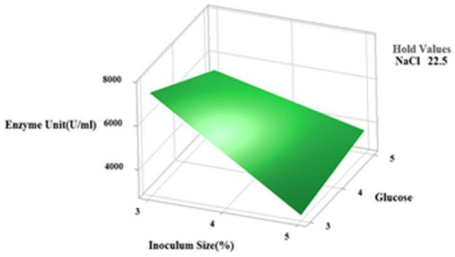

Figure-7(b) Enzyme Unit(U/ml) vs Glucose (%), Inoculum size (%)

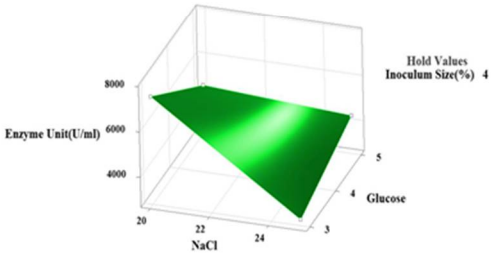

Figure-7(c) Enzyme Unit(U/ml) vs Glucose (%), NaCl(%)

Figure 7: Surface plots of selected variables

In Figure 7(a), NaCl 22.5(%) concentration showed 8000 U/ml enzyme production, whereas Inoculum size 3.5 (%) concentration showed more than 4000 U/ml enzyme production. Figure 7(b) Glucose (3%) concentration showed more than 7500 U/ml enzyme production, whereas inoculum size (5%) concentration showed more than 7500 U/ml enzyme production. Figure 7(c) Glucose 3(%) shown more than 7500 U/ml enzyme production, whereas NaCl 22.5(%) shown 8000U/ml enzyme production. over all NaCl 22.5(%), Glucose 3(%) and Inoculum size 5(%) were showed higher haloalkaliphilic protease production.

Cube Plot:

The cube plot shows the relationship between the factors and response. Figure 8 cube plot showed a connection between the NaCl (%), Inoculum size (%), and glucose (%) factors concentration and response. Figure 8 shows that NaCl 20(%) and Inoculum size 3(%) were associated with the highest fitted mean of haloalkaliphilic protease enzyme value 14,558.3 U/ml.

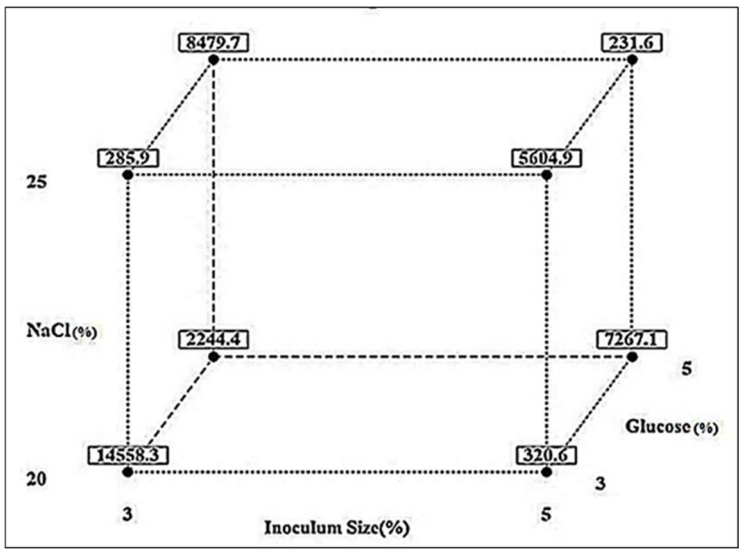

Figure 8: Cube plot (Fitted means)

Then NaCl 25(%) and Glucose 5(%), Glucose 5(%) and Inoculum size 5(%), Inoculum size 5(%) and Glucose 3(%) were showed 8479.7, 7267.1 and 5604.9 U/ml haloalkaliphilic protease production accordingly.

Comparative study of haloalkaliphilic protease production in unoptimized and optimized production medium:

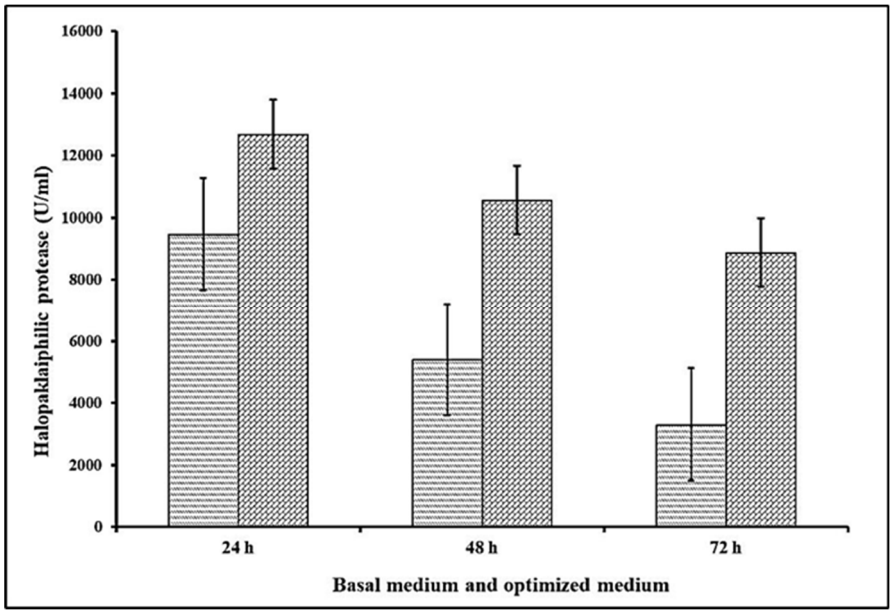

Figure 9: Enzyme production in modified medium variables amount

After completion of the FD design, the media formulated was showed 1.3 fold increases in the haloalkaliphilic protease production units. This can be useful for higher-scale enzyme production.

Growth cycle analysis:

The kinetic parameters of growth and enzyme production of the ***Bacillus Oryzaecorticis strain R1*** showed that maximum turbidity was observed in optimized conditions compared to the basal medium. The increase in the specific growth rate (μ) suggests that the increase in the cellular turbidity during the exponential phase reduces the time for enzyme production, resulting in higher enzyme accumulation at the early stage of growth. Furthermore, the increase in the specific growth rate and turbidity of maximal cell mass production notably increased the enzyme production rate and total volumetric enzyme.

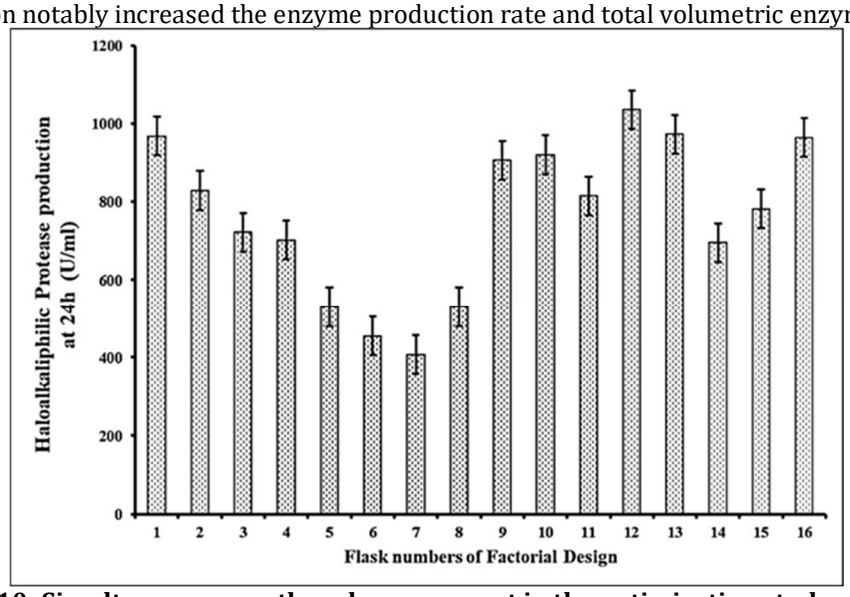

Figure 10: Simultaneous growth cycle assessment in the optimization study using FD

Figure 10 shows the growth kinetics of ***Bacillus Oryzaecorticis strain R1*** in the Factorial Design experiment flasks. Flask number 12 showed the highest haloalkaliphilic protease production units. All different bars indicate an increase in bacterial isolate growth. The medium physiological conditions drive the doubling time of the bacterial strain. Therefore, the doubling time in the optimized medium was slightly less than in the basal medium. The growth-dependent production of the enzyme in the exponential phase suggests a significant role of this enzyme in primary metabolism.

CONCLUSION

The work marks out the statistical optimization of the medium by a novel haloalkaliphilic ***Bacillus Oryzaecorticis strain R1*** isolated from hypersaline soil of Ghogha beach coastline, Bhavnagar, Gujarat, India. The study encompassed the effect of different variables like temperature, inoculum size, NaCl, and Glucose with an examination of growth kinetics and enzyme production. After statistical optimization by factorial design, 1.3 fold increases was achieved in haloalkaliphilic protease production. The economic survey of the lab-scale mass production can suggest its scale-up feasibility. The prescribed design model suggested 16624.5 enzyme unit production in the 1 number flask having variable condition temperature 60°C, inoculum size 4(%), NaCl 25%, and Glucose 4(%) as per model design.

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