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Optimization of Alkaline Protease Production by Solibacillus silvestris, Isolated from Gir Region of Gujarat

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Abstract

Objectives: Microbes from Mangrove ecosystem have the ability to produce useful primary and secondary metabolites of biotechnological significance and hence the objective of this study is to optimize the production of bacterial alkaline protease from a Mangrove isolate *Solibacillus silvestris*. **Methodology:** The bacterium (named as Madhwad 10^{-3} Summer Zobell) used in the present study was isolated from the soil collected from Madhwad, Gir, India in summer season and was identified using MALDI-TOF (Mass spectrometry). It had shown the highest alkaline protease enzyme activity amongst the other isolates and hence was further analyzed using different physical and chemical factors. Selected parameters i.e. Sucrose, Peptone, and Salinity were further used for the optimization using Response Surface Methodology (RSM). **Findings:** The bacterium (named as Madhwad 10^{-3} Summer Zobell) was identified as *Solibacillus silvestris*. Among the physical parameters, the results proved that highest enzyme production can be obtained at pH 8.5, 40 **°C** of temperature, 48 h of incubation period, and agitation speed of 120 rpm while in the chemical parameters, Sucrose and Peptone with 0% salinity were found to give highest enzyme activity i.e. 221.7 U/ml and 246.7 U/ml, respectively. In the statistical analysis done using RSM, all the parameters found to be significant and gave the highest result of 258.750 U/ml at 2.5% Sucrose, 10% Peptone, and 0% Salinity level. **Applications/improvements:** The enzyme produced due to its efficiency in working at alkaline pH can be utilized in industries such as detergent and textile or in Bioremediation.

Keywords: Mangroves, Alkaline Protease, Response Surface Methodology, *Solibacillus silvestris*.

1. Introduction

Mangrove ecosystem is most unique as it is exposed to the periodic flooding of seawater and fresh water in case of the estuarine area, salinity variation, a lack of oxygen due to muddy soils and these frequent changes influence the transformation of organic matter into nutrients [1]. The change in organic matter and nutrients as well as extreme environments as a result has impact on biodiversity of mangrove ecosystem. These conditions altogether make microbes to produce enzymes and other metabolites which can survive in extremely variable environment and thus had biotechnological and industrial interest [2].

In mangrove ecosystem, hydrolases produced by microbes play an important role in degrading various types of biomass *viz.* leaves, branches, fruits, shells, etc. Among different types of enzymes produced by microbes, protease which is capable of hydrolyzing the peptide bond in protein molecule represents one of the three largest groups of industrial enzymes. Protease enzymes have wide applications in various industries such as in the detergents, cheese maturation, beer production, meat softening, functional hydrolysate production, and in bioremediation [3–5]. The enzyme also has a significant role in many physiological and pathological processes i.e. protein catabolism, blood coagulation, zymogen activation and transport and secretion of proteins across membranes [6–7]. The largest application of proteases is in the detergent industry to remove protein-based stains from the cloths. The enzyme used in the detergent had to be stable in a wide range of temperature and had to be active in the presence of various surfactants, bleaching agents, and various other additives. Currently, a large proportion of commercially available protease is derived from *Bacillus* strains and most of them were isolated from coastal regions [7–11]. From the study of the previous literature, it can be seen that due to the longest coastal region of Gujarat the possibility of getting the isolate with very good alkaline protease production is high [12–16].

Microbes isolated from mangrove ecosystem are ideal source of protease enzymes because of their rapid growth rate in limited space and they are easy to manipulate genetically to produce new modified protease enzymes with new/altered properties which are suitable for various applications [17–18]. Production of enzymes from these microbes responds differently to physical (e.g. pH, temperature, etc.) and chemical factors (e.g. carbon source, nitrogen source, etc.) and therefore optimization of the factors is essential to improve the protease yield [1–20]. A number of statistical experimental designs with response surface methodology (RSM) have been employed for optimizing enzyme production from microorganisms [21]. RSM includes regression analysis and factorial design, which is used to study the interaction and to select the optimum conditions of variables for desirable response by evaluating the effective factors and building models [22].

In the current study, alkaline protease producing isolates were screened and the highest alkaline protease producing bacterium was then analyzed under various physical and chemical factors. Selected chemical factors i.e. Sucrose as a carbon source, Peptone as a nitrogen source and % salinity were further optimized for alkaline protease production using RSM which is resulted in a statistically significant increase in the enzyme activity after the optimization.

2. Materials and Methods

2.1. Sample Collection

The soil samples were collected from Madhwad, Gir, Gujarat (Latitude – 20.706353 and Longitude – 70.833123) which had presence of mangroves which were quickly identified as species *Avicennia marina* from its morphology [23]. The sampling was done from the surroundings of rhizospheric region of mangroves in triplicates from the depth of 10 cm in summer season and the collected soil samples were stored in sterile plastic bag. All plastic bags were maintained at 4°C till the soil was utilized in laboratory.

2.2. Isolation of Bacteria from the Soil Samples

The soil samples were enriched in Zobell marine broth [24] and incubated at 30 °C, 120 rpm for 72 h. After enrichment all the samples were serially diluted from 10^0 to 10^{-4} and were inoculated in Zobell marine agar and incubated at 30 °C for 72 h. Morphologically different and unique colonies were isolated and stored in glycerol stock for further analysis.

2.3. Screening of Protease Producing Bacteria

For the screening of protease, nutrient agar with 10% skimmed milk was used. Positive protease producers were identified by clear zone surrounding the colony due to consumption of milk as a substrate. From the results, the highest protease producing isolate was selected and optimization of protease production was done.

2.4. Growth Medium

For the determination of enzyme activity and optimization of culture conditions, Kathiresan and Manivannan's protocol with some modification was followed [25]. The composition of the growth medium (Starch casein agar medium) was starch – 15 g, casein – 5 g, peptone – 5 g, beef extract – 3 g, NaCl – 5 g, Nalidixic acid – 10 µl/ml, Nystatin – 25 µl/ml, Cyclohexamide – 10 µl/ml, agar – 15 g, aged seawater – 500 ml, and distilled water – 500 ml.

2.5. Production Medium

Protease production medium was modified and composed of 5% starch, 5% yeast extract and 50 ml salt solution (KH_2PO_4 – 0.25%, $MgSO_4$ – 0.1%, K_2HPO_4 – 0.25%, and $FeSO_4$ – 0.1%), 500 ml aged seawater and 500 ml distilled water with pH of 8.5 (25). The 1000 ml medium was sterilized in an autoclave for 15 min at 121 °C. The sterilized medium was inoculated with a loopful culture of highest alkaline protease producer and then the medium was incubated at 30 °C in an orbital shaker at 100 rpm for 72 h. At the intervals of every 24 h, 5 ml of the culture filtrate was taken from the culture medium and centrifuged at 7000 rpm for 15 min at 4 °C and the supernatant was used to assay for protease activity.

2.6. Enzyme Assay

To determine the protease activity, 200 µl of supernatant from the centrifuged culture filtrate was collected and mixed with 500 µl of 0.5% (w/v) casein solution (pH 9.0, prepared in 10 mM Tris-HCl buffer), and the reaction mixture was incubated at room temperature for 10 min. The reaction of the enzyme was terminated by adding 1 ml of 5% (w/v) Trichloroacetic acid [26] and then the reaction mixture was centrifuged at 7000 rpm for 15 min at 4 °C to separate the reaction mixture and unused substrate. From the centrifuged reaction mixture, 1 ml of supernatant was collected and mixed with 5 ml of 0.4 M Na_2CO_3 and 1 ml of 3× diluted Folin Ciocalteu's reagent. The resulting solution was incubated in dark for 30 min at room temperature and the absorbance was measured at 660 nm in UV-Spectrophotometer. Bovine Serum Albumin (BSA) was used as a standard for enzyme assay using Standard protocol [27].

2.7. Effect of Various Physical and Chemical Factors on Alkaline Protease Activity

The effect of different physical and chemical factors on production of alkaline protease were carried out in production medium, examining one factor at a time, keeping all the other factors constant. Once the optimization was done with the respective factor, it was incorporated in the optimization experiment of the next factor. pH, temperature, incubation period, and agitation rate were selected as physical factors while carbon source *viz.* Glucose, Maltose & Sucrose; Nitrogen source *viz.* Peptone, Meat Extract & Casein and different concentrations of salinity were used for the optimization of alkaline protease production. The selected Carbon and Nitrogen source combination was further optimized by the Box–Behnken design.

2.8. Optimization of Various Physical Parameters for the Production of Protease

The effect of parameters *viz.* pH (7 to 11), temperatures (30 to 60 °C), incubation period (24 to 96 h), agitation rate (0 to 160 rpm) was evaluated in modified protease production media. The growth was measured as optical density at 600 nm.

2.9. Optimization of Various Chemical Parameters for Protease Production

The effect of Carbon source (C-source) *viz.* Glucose, Maltose, and Sucrose (at 5% concentration in media); Nitrogen source (N-source) *viz.* Peptone, Meat Extract, and Casein (at 5% concentration in media); and Salinity (0–40% of seawater) was evaluated in modified protease production media. The growth was measured as optical density at 600 nm.

2.10. Statistical Optimization of Alkaline Protease Production

The selected C and N-source combination *viz.* Sucrose and Peptone with % salinity were further optimized for the production of alkaline protease by using Box–Behnken design. A design with 15 experiments, including three centre points was generated by Minitab 16, Minitab Ltd., and UK. The run no. 4, 8, and 9 were centre points, which were repeated three times for estimation of error in the design. Each variable was studied at low, middle, and high concentration levels and was designated as −1, 0, and +1 (coded values), respectively (Table 1). Prediction of parameter and generation of response counter plot by the model was also done by the same software. ANOVA was used to establish the significance of the model parameters.

TABLE 1. Experimental range and levels of the independent variables to optimize media components for alkaline protease production by Solibacillus silvestris

Variables		0	1
Sucrose	0	2.5	5
Peptone	0	5	10
Salinity (in sea water)	0	10	20

3. Results and Discussions

3.1. Isolation and Screening

$$Index\ of\ relative\ enzyme\ activity = \frac{Clear\ zone\ diameter}{Colony\ Diameter}$$

From the soil samples collected from Madhwad, Gir, total 17 morphological different bacterial colonies were isolated and screened for their protease activity. The enzyme activity of these isolates was recorded as the Index of Relative Enzyme Activity and calculated using the below mentioned formula [28–29]:

The results of the extracellular enzyme activity of each isolated were represented in Table 2.

TABLE 2. Results of protease activity of bacteria isolated from Madhwad, Gir

Sr. no.	Isolate name	Protease
1	Madhwad 10-3 Summer Zobell	1.2
2	Madhwad 10-3 Summer Zobell	8.0
3	Madhwad 10-3 Summer Zobell	0.0
4	Madhwad 10-0 Summer Zobell	2.8
5	Madhwad 10-0 Summer Zobell	1.4
6	Madhwad 10-1 Summer Zobell	1.4
7	Madhwad 10-1 Summer Zobell	1.3
8	Madhwad 10-1 Summer Zobell	1.4
9	Madhwad 10-3 Summer Zobell	1.6
10	Madhwad 10-3 Summer Zobell	0.0

11	Madhwad 10-3 Summer Zobell	1.4
12	Madhwad 10-3 Summer Zobell	1.1
13	Madhwad 10-3 Summer Zobell	0.0
14	Madhwad R2A 1	1.7
15	Madhwad R2A 2	1.5
16	Madhwad R2A 3	1.9
17	Madhwad 10-2 R2A	1.8

3.2. Identification of the Selected Bacterium

The highest alkaline protease producing isolate named Madhwad 10^{-3} Summer Zobell was identified as *Solibacillus silvestris* using the MALDI-TOF mass spectrometry (Bruker Corporation, Billerica, MA) at Neuberg Supratech, Ahmedabad, and Gujarat, India.

3.3. Optimization of Various Physical and Chemical Parameters for Protease Production

Among the different physical parameters, highest alkaline protease production by *Solibacillus silvestris* was obtained at pH 8.5, 40°C of temperature, 48h of incubation period, and agitation speed of 120 rpm (Figures 1–4). Among the chemical parameters, Sucrose (5%) as a C-source and Peptone (5%) as N-source along with 0% salinity were given best enzyme activity 221.7 U/ml, 246.7 U/ml, and 246.7 U/ml, respectively (Figures 5–7).

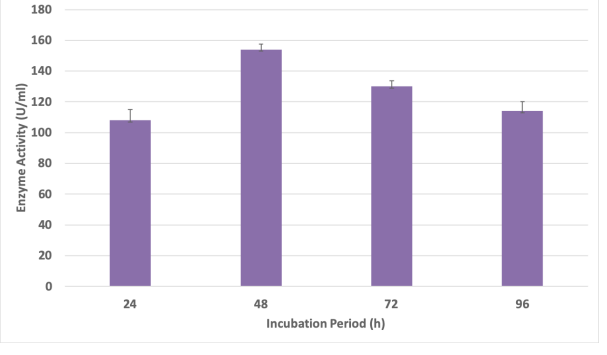

FIGURE 1. Effect of Incubation period on the alkaline protease production by Solibacillus silvestris.

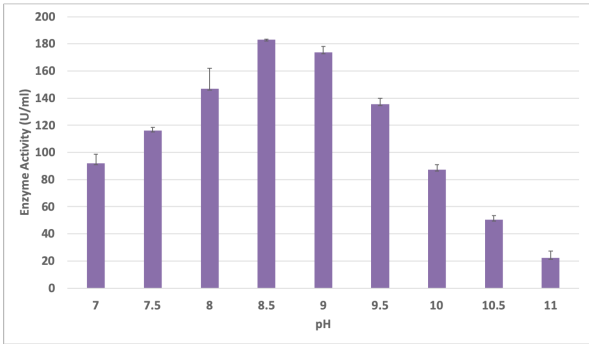

FIGURE 2. Effect of pH on the alkaline protease production by Solibacillus silvestris.

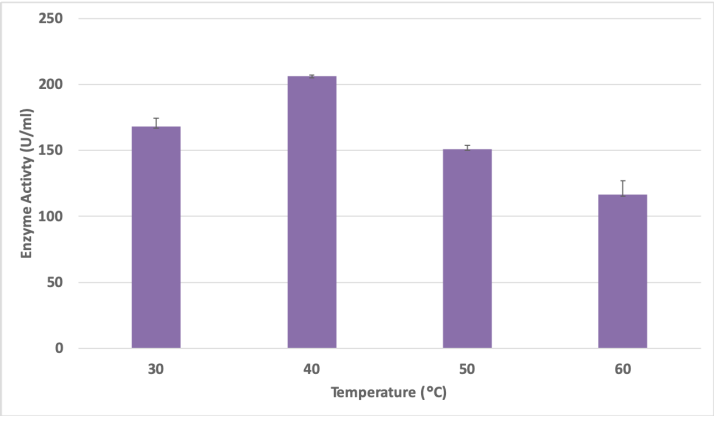

FIGURE 3. Effect of temperature on the alkaline protease production by Solibacillus silvestris.

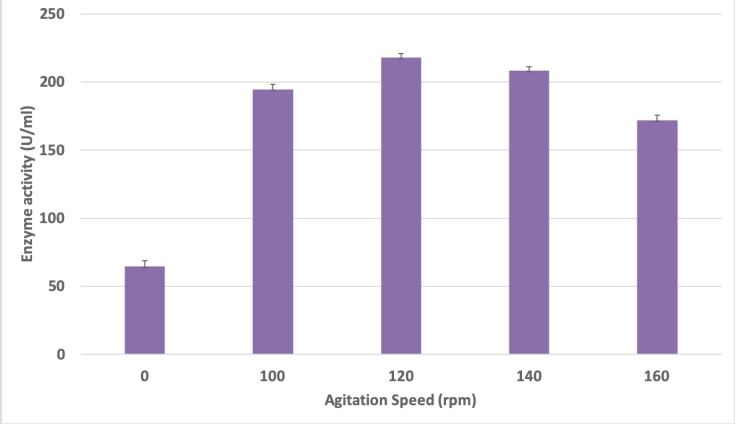

FIGURE 4. Effect of agitation speed on the alkaline protease production by Solibacillus silvestris.

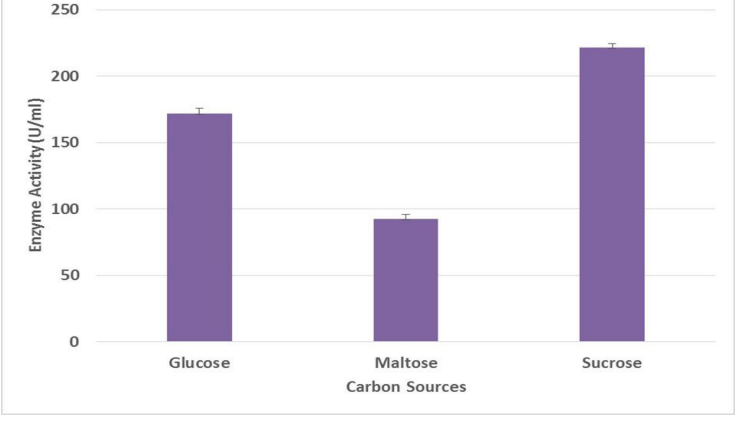

FIGURE 5. Effect of carbon source on the alkaline protease production by Solibacillus silvestris.

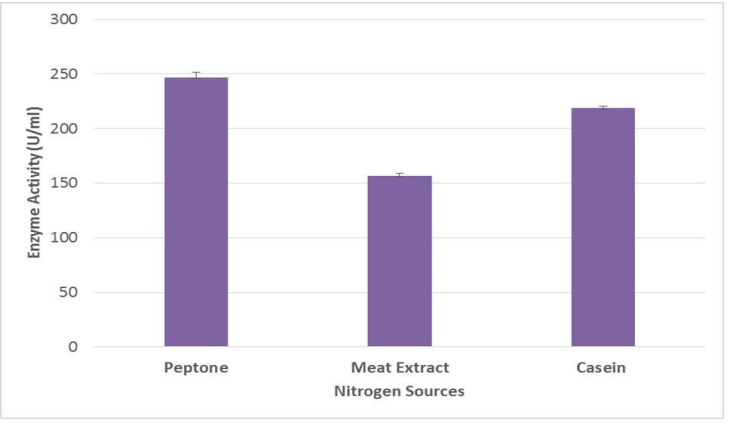

FIGURE 6. Effect of nitrogen source on the alkaline protease production by *Solibacillus silvestris*.

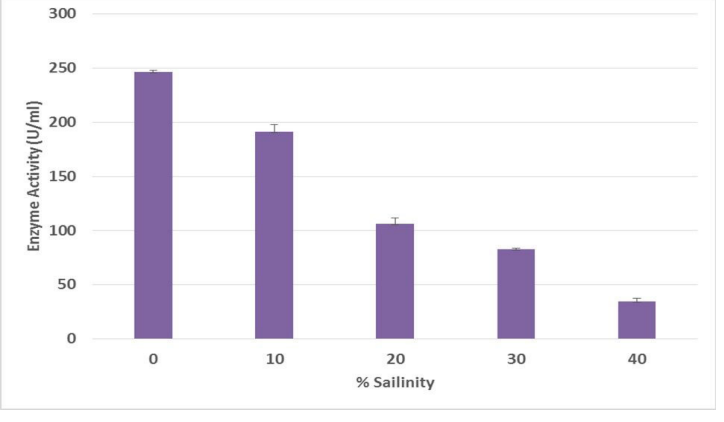

FIGURE 7. Effect of % salinity on the alkaline protease production by Solibacillus silvestris.

3.4. Statistical Optimization of Alkaline Protease Production

Sucrose, Peptone, and Salinity were the variables selected for optimization by RSM while fixing the optimized physical parameters. The experimental design with corresponding Protease activity is shown in Table 3. ANOVA of the quadratic regression model implies that it was a significant model as the Fisher's *F* test with allowed probability value. The model *F* value of 78.76 implies that the model is significant (Table 4). There is only a 0.04% chance that a model *F* value this large could occur due to noise. Values of *p* less than 0.05 indicate that model terms are significant. The coefficients and *p* values of all the variables of linear (Sucrose, Peptone, Salinity), quadratic (Sucrose*Sucrose, Peptone*Peptone, Salinity*Salinity), and interaction (Sucrose*Peptone, Sucrose*Salinity, Peptone*Salinity) terms were determined and presented in Table 4. Among the linear coefficients, Sucrose, Peptone, and Salinity had a significant effect on protease production. While all the quadratic coefficients were significant, only the interaction had a significant effect on the protease production. The following regression equation was established:

Protease (U/ml) = 114.7 + 31.73 Sucrose + 9.39 Peptone – 13.48 Salinity – 3.847 Sucrose X Sucrose + 0.028 Peptone × Peptone + 0.3571 Salinity × Salinity – 0.320 Sucrose × Peptone – 0.520 Sucrose × Salinity + 0.0750 Peptone × Salinity

TABLE 3. Results of Box–Behnken using three independent variables and three centre points showing predicted and observed response of protease production by Solibacillus silvestris

Run no.	Sucrose (%)	Peptone (%)	Salinity	Experimental value (U/ml)	Predicted value (U/ml)
1	0.0	5	0	169	162.375
2	0.0	0	10	7	15.625
3	2.5	0	20	17	17.250
4	2.5	5	10	106	105.333
5	2.5	0	0	172	170.000
6	0.0	10	10	113	119.875
7	5.0	0	10	59	52.125
8	2.5	5	10	105	105.333
9	2.5	5	10	105	105.333
10	5.0	5	20	39	45.625
11	2.5	10	20	119	121.000
12	5.0	10	10	149	140.375
13	5.0	5	0	208	216.875
14	2.5	10	0	259	258.750
15	0.0	5	20	52	43.125

TABLE 4. Analysis of variance (ANOVA) of response surface quadratic model

Source	DF	Adj SS	Adj MS	*F*-value	*p* Value
Model	9	70517.5	7835.3	78.76	0
Linear	3	62347.8	20782.6	208.91	0
Sucrose	1	1624.5	1624.5	16.33	0.01
Peptone	1	18528.1	18528.1	186.24	0
Salinity	1	42195.1	42195.1	424.14	0
Square	3	7373.5	2457.8	24.71	0.002
Sucrose*sucrose	1	2134.2	2134.2	21.45	0.006
Peptone*peptone	1	1.9	1.9	0.02	0.897
Salinity*salinity	1	4708	4708	47.32	0.001
2-way interaction	3	796.2	265.4	2.67	0.159
Sucrose*PEPTONE	1	64	64	0.64	0.459
Sucrose*Salinity	1	676	676	6.8	0.048
Peptone*salinity	1	56.2	56.2	0.57	0.486
Error	5	497.4	99.5		
Lack-of-fit	3	496.7	165.6	496.75	0.002
Pure error	2	0.7	0.3		
Total	14	71014.9			

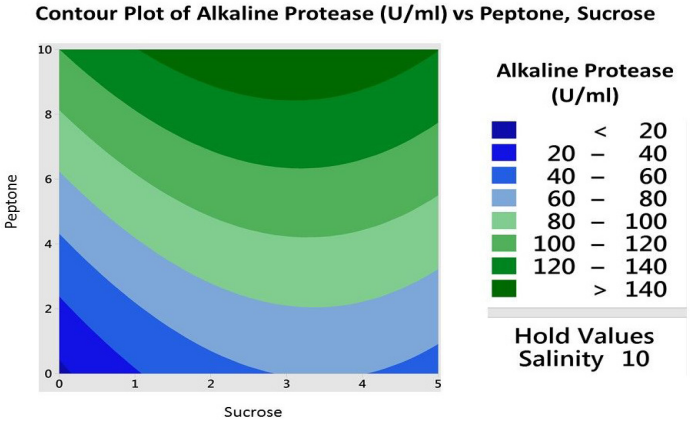

FIGURE 8. Contour plot of alkaline protease (U/ml) vs peptone, sucrose by Solibacillus silvestris.

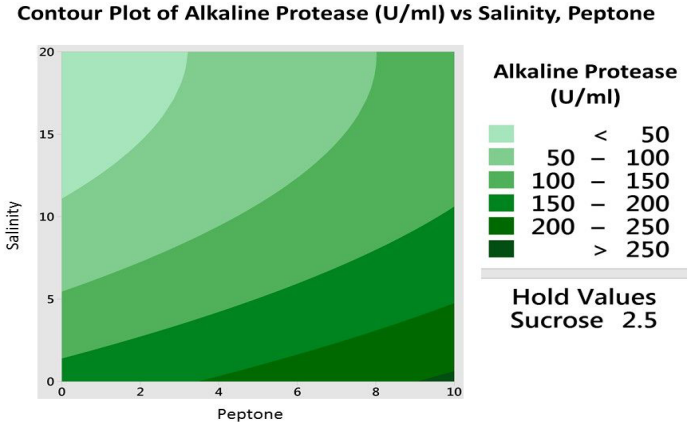

FIGURE 9. Contour plot of alkaline protease (U/ml) vs salinity, peptone by *Solibacillus silvestris*.

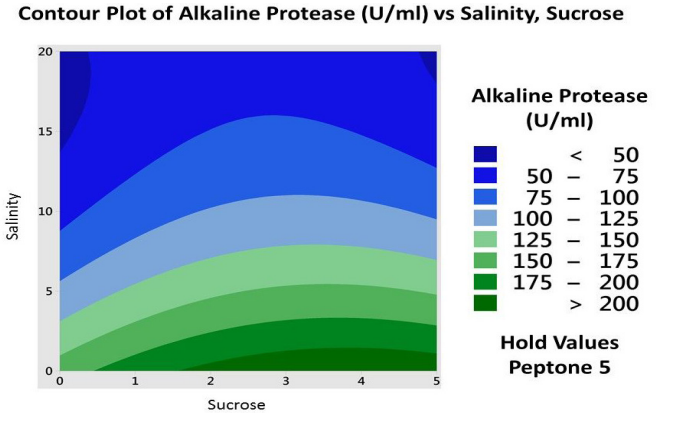

FIGURE 10. Contour plot of alkaline protease (U/ml) vs salinity, sucrose by *Solibacillus silvestris*.

The behaviour of alkaline protease production and interaction effect of three factors: Sucrose, Peptone, and % Salinity at different values were represented in contour plots (Figures 8–10). The optimum production of the enzyme was found to be 258.75 U/ml with the sucrose, peptone, and % salinity at the concentration of 2.5%, 10%, and 0%, respectively in the predicted value. Then based on that values, additional experiments in triplicate were performed to validate the values which yielded optimum enzyme production of 259 U/ml which was higher than the activity obtained in one-variable-at-a-time approach in physical parameters without any statistical approach i.e. 218 U/ml. The predicated R^2 (88.1%) and adjusted R^2 (98.04%) values for the Protease production were in reasonable agreement with the value of R^2 (99.30%), which is closer to 100%, indicating the better fitness of the model in the experimental data.

4. Conclusion

Alkaline proteases have many applications in different industries and environmental bioremediations. The present study shows that protease production is greatly influenced by cultural conditions and media components. The optimum alkaline protease production by *Solibacillus silvestris*, an isolate from rhizospheric soil of Mangrove region of the Madhwad, Gir, was observed at 40 °C, pH 8.5, 48 h of incubation period, Sucrose as a C-source, Peptone as a N-source. Alkaline protease production was also optimized at two levels i.e. one-variable-at-a-time and statistical approach using MINITAB 16. After optimization, alkaline protease production was increased from 218 U/ml in one-variable-at-a-time to 259 U/ml in statistical approach. The results indicate the industrial usefulness of the bacterial isolate and further studies are in progress in order to purify and characterize the enzyme for commercial applications.

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