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Transcription Protease Synthase Discovered by Strain Solibacillus Sylvestris

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ABSTRACT
The goal of this work is to maximise the synthesis of bacterial alkaline protease from a mangrove isolate, Solibacillus silvestris, which has the capacity to create valuable primary and secondary metabolites of biotechnological value. Methodology: Using MALDI-TOF (mass spectrometry), the bacterium (known as Madhwad 103 Summer Zobell) used in the current study was isolated from soil that was obtained from Madhwad, Gir, India, during the summer. It was subsequently examined, utilising various physical and chemical variables, since it exhibited the greatest alkaline protease enzyme activity of the isolates. Selected parameters, i.e., Response Surface Methodology (RSM), were also applied to the optimisation of sucrose, peptone, and salinity. Results: Solibacillus silvestris was identified as the bacterium, which was given the name Madhwad 10-3 Summer Zobell. The results showed that the highest enzyme production can be achieved at pH 8.5, 40 °C of temperature, 48 hours of incubation, and an agitation speed of 120 rpm, while the highest enzyme activity was found to be given by sucrose and peptone with 0% salinity, with values of 221.7 U/ml and 246.7 U/ml, respectively. All the factors were significant in the statistical analysis carried out using RSM, which produced the maximum result of 258.750 U/ml at 2.5% sucrose, 10% peptone, and 0% salinity. Applications and improvements: The enzyme created as a result of its effectiveness at operating at an alkaline pH can be used in bioremediation as well as the detergent and textile industries.

Keywords: alkaline protease, Solibacillus silvestris, Sylvestris, salinity variation, environments

I. INTRODUCTION

The mangrove ecosystem is particularly special because it is subject to regular flooding from both fresh and salt water in estuarine areas, salinity variation, and oxygen depletion from muddy soils. These frequent changes have an impact on how organic matter is converted into nutrients. The biodiversity of the mangrove ecosystem is impacted by changes in organic matter, nutrients, and harsh conditions. All of these factors cause microorganisms to create enzymes and other compounds that have biotechnological and industrial relevance because they can live in highly varied environments.

In the mangrove ecosystem, hydrolases produced by bacteria are crucial in the degradation of different types of biomass, such as wood, branches, fruits, shells, leaves, etc. One of the three main classes of industrial enzymes among the several kinds of enzymes made by bacteria is protease, which can hydrolyze the peptide link in a protein molecule. Detergents, cheese maturation, beer manufacture, meat softening, functional hydrolysate generation, and bioremediation are only a few of the areas where protease enzymes are widely used. A number of physiological and pathological processes, such as protein catabolism, blood coagulation, zymogen activation, and the transport and secretion of proteins across membranes, are also significantly influenced by the enzyme. The detergent business uses proteases primarily to get rid of protein-based stains on clothes. The detergent's enzyme needed to be active in the presence of multiple surfactants, bleaching agents, and other additives, as well as stable across a wide temperature range. Currently, a significant amount of the protease that is sold in stores comes from Bacillus strains, the majority of which are found in coastal areas. According to a review of the prior literature, Gujarat's longest coastline makes it likely that an isolate with excellent alkaline protease production will be found.

Because they grow quickly in small spaces and are simple to genetically modify to produce new modified protease enzymes with new or altered features that are acceptable for various applications, microbes isolated from the mangrove ecosystem are an ideal source of protease enzymes. The optimisation of the parameters is crucial to increasing the protease yield because the production of enzymes from these microbes responds differently to physical (such as pH, temperature, etc.) and chemical (such as carbon source, nitrogen source, etc.) factors. For maximising the synthesis of enzymes from microorganisms, a variety of statistical experimental designs employing response surface methodology (RSM) have been used. Regression analysis and factorial design are two tools that are included in RSM, and they are used

to explore interactions and choose the best circumstances for variables to produce desired responses by assessing key variables and creating models.

Alkaline protease-producing isolates were screened in the current investigation, and the bacteria with the greatest levels of alkaline protease production were then examined with regard to several physical and chemical variables. Selected chemical factors, i.e., the production of alkaline protease, were further optimised using RSM for sucrose as a carbon source, peptone as a nitrogen source, and salinity, which led to a statistically significant improvement in the enzyme activity.

II. RESOURCES AND PROCEDURES

2.1 Sample Gathering

The soil samples were taken from Madhwad, Gir, Gujarat (20.706353, 70.833123), where there were mangroves. These mangroves were immediately identified as belonging to the species Avicennia marina based on their morphology. In the summer, soil samples were collected in triplicate from the rhizospheric zone of mangroves at a depth of 10 cm, and they were preserved in sterile plastic bags. Until the dirt was used in the laboratory, all plastic bags were kept at 4°C.

2.2 Bacterial Isolation from Soil Samples

After being enriched with Zobell marine broth, the soil samples were cultured for 72 hours at 30 °C and 120 rpm. Following enrichment, all samples were serially diluted (from 100 to 104), added to Zobell marine agar, and then incubated for 72 hours at 30 °C. Colonies that were morphologically distinct and distinctive were separated and kept in glycerol stock for later examination.

2.3 Protease-producing Bacteria Screening

Nutrient agar with 10% skimmed milk was used to screen for protease. Due to the colony being surrounded by a clear zone as a result of using milk as a substrate, positive protease producers could be recognised. The isolate with the highest level of protease production was chosen based on the findings, and protease production was optimised.

2.4 Growth Moderate.

The Kathiresan and Manivannan technique was used, with a few modifications, to determine enzyme activity and optimise culture conditions. The growth medium (starch-casein-agar medium) had the following ingredients: 15 g of starch, 5 g of casein, 5 g of peptone, 3 g of beef extract, 5 g of NaCl, 10 l/ml of nalidixic acid, 25 l/ml of nystatin, 10 l/ml of cyclohexamide, 15 g of agar, 500 ml of aged seawater,–500 ml.

2.5 Manufacturing Medium

The modified protease production medium contained 5% starch, 5% yeast extract, 50 ml of a salt solution (KH_2PO_4, $MgSO_4$, K_2HPO_4, and $FeSO_4$), 500 ml of aged seawater, and 500 ml of distilled water, all of which had a pH of 8.5 (25). The 1000 ml of medium was autoclaved for 15 minutes at 121 °C to sterilise it. A loopful culture of the highest alkaline protease producer was added to the sterilised medium before it was cultured for 72 hours at 30 °C and 100 rpm in an orbital shaker. Every 24 hours, 5 ml of the culture filtrate was removed from the culture medium and centrifuged at 7000 rpm for 15 min at 4 °C to test for protease activity in the supernatant.

2.6. Analyse Enzyme

In order to measure the protease activity, 500 ml of 0.5% (w/v) casein solution (pH 9.0, produced in 10 mM Tris-HCl buffer) and 200 ml of supernatant from the centrifuged culture filtrate were combined. The reaction mixture was then incubated at room temperature for 10 minutes. By adding 1 ml of 5% (w/v) trichloroacetic acid, the enzyme reaction was stopped [26]. The reaction mixture was then centrifuged at 7000 rpm for 15 minutes at 4 °C to separate the reaction mixture and leftover substrate. 1 ml of the supernatant from the centrifuged reaction mixture was taken, and it was combined with 5 ml of 0.4 M Na_2CO_3 and 1 ml of Folin-Ciocalteu's reagent that had been diluted by 3. The resultant mixture was incubated in the dark at room temperature for 30 minutes before the absorbance at 660 nm was determined using a UV spectrophotometer. For the enzyme assay utilising the standard procedure, bovine serum albumin (BSA) was used as a standard.

2.7 Alkaline Protease Activity and the Influence

In a production medium, the effects of various physical and chemical factors on the production of alkaline protease were investigated, with each factor being examined separately while the other factors remained constant. When the optimisation experiment for that particular factor was finished, it was added to the optimisation experiment for the next factor. Physical factors like pH, temperature, incubation time, and agitation rate were chosen, while the carbon source, i.e., maltose, sucrose, and glucose; nitrogen from a viz. The production of alkaline protease was optimised using peptone, meat extract, casein, and various salinity concentrations. The Box-Behnken design further improved the chosen carbon and nitrogen source combination.

2.8. Optimising Several Physical Parameters

In modified protease production media, the effects of pH (7 to 11), temperatures (30 to 60 °C), incubation time (24 to 96 h), and agitation rate (0 to 160 rpm) were assessed. The optical density at 600 nm was used to measure the growth.

2.9 Optimising a Range of Chemical Parameters

The result of the carbon source (C-source), i.e., 5% media concentrations of glucose, maltose, and sucrose; and nitrogen source (N-source), i.e., in modified protease production media, the effects of peptone, meat extract, and casein (at 5% concentration), as well as salinity (0–40% of seawater), were assessed. The optical density at 600 nm was used to measure the growth.

2.10 Production of Alkaline Protease

The chosen C and N source combo, i.e., the Box-Behnken design, was used to further optimise sucrose and peptone with % salinity for the production of alkaline protease. Minitab 16, Minitab Ltd., and the UK produced a design with 15 experiments and three centre points. Runs 4, 8, and 9 served as the centre points and were replicated three times to estimate design error. Each variable was examined at low, medium, and high concentration levels and assigned the codes 1, 0, and +1 (Table 1). The same software was used to predict parameters and produce a response counter plot for the model. To determine the importance of the model parameters, ANOVA was used.

Table 1: Experimental range and levels of the independent variables

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

III. OBSERVATIONS

3.1 Screening and Isolation

From the soil samples taken from Madhwad, Gir, a total of 17 morphologically distinct bacterial colonies were extracted and evaluated for their protease activity. The enzyme activity of these isolates was recorded as the Index of Relative Enzyme Activity and calculated using the below-described formula:

Table 2: The results of the extracellular enzyme activity of each isolated sample

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 strongest alkaline protease-generating isolate, dubbed Madhwad 103, Summer Zobell was discovered as Solibacillus silvestris using MALDI-TOF mass spectrometry (Bruker Corporation, Billerica, MA) at Neuberg Supratech, Ahmedabad, and Gujarat, India.

3.3 Optimisation of Various Physical and Chemical Parameters

Among the varied physical parameters, maximal alkaline protease production by Solibacillus silvestris was obtained at pH 8.5, 40°C of temperature, 48 h of incubation period, and an agitation speed of 120 rpm (Figures 1–2). Among the chemical parameters, sucrose (5%) as a C-source and peptone (5%) as an N-source, along with 0% salinity, provided the best enzyme activity, with 221.7 U/ml, 246.7 U/ml, and 246.7 U/ml, respectively.

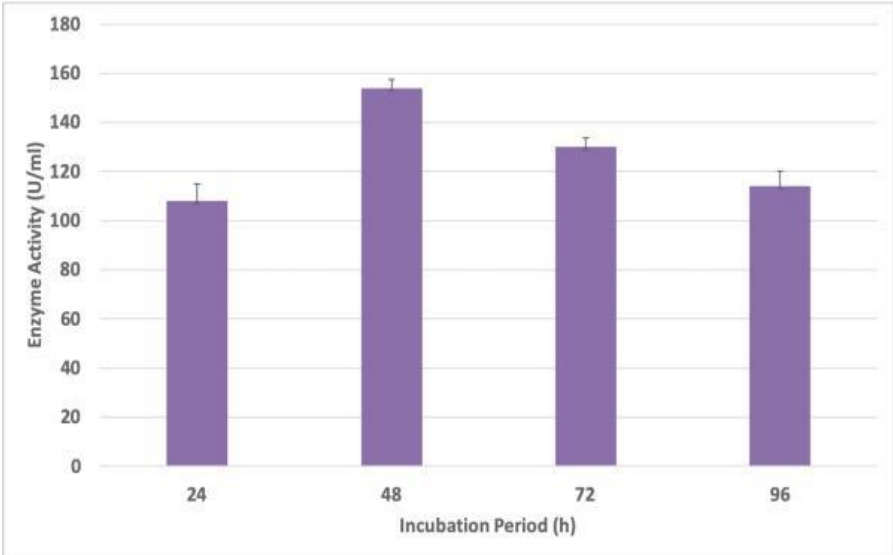

Figure 1: Effect of Incubation period on the alkaline protease production by Solibacillus silvestris

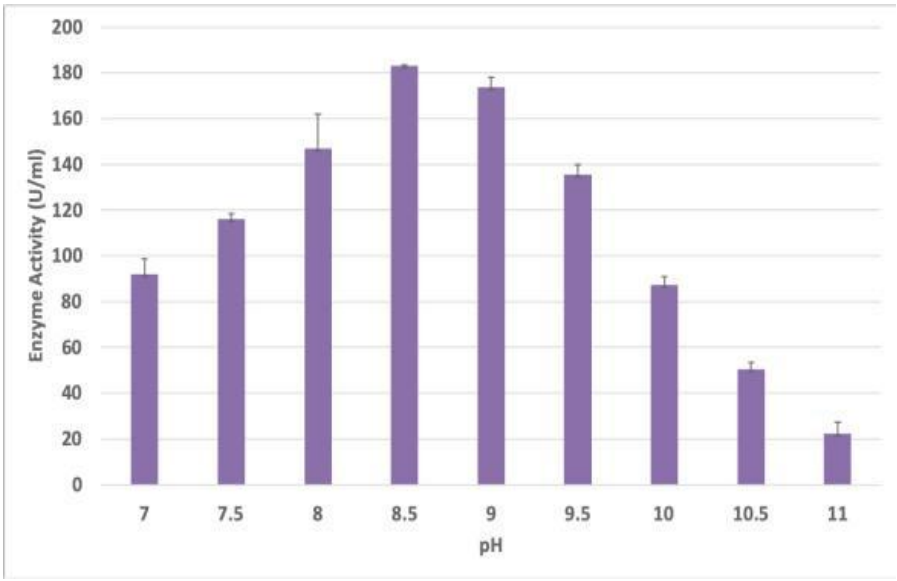

Figure 2: Effect of pH on the alkaline protease production by Solibacillus silvestris

3.4 Production of Alkaline Protease

Sucrose, peptone, and salinity were the variables selected for optimisation by RSM while fixing the optimum physical parameters. The experimental design with related protease activity is provided in Table 3. ANOVA of the quadratic regression model implies that it was a significant model as determined by Fisher's F test with the allowed probability value. The model F value of 78.76 shows that the model is significant. There is just a 0.04% probability that a model F value this large may emerge due to noise. Values of p less than 0.05 imply that model terms are significant. The coefficients and p values of all the variables in the linear (Sucrose, Peptone, Salinity), quadratic (Sucrose*Sucrose, Peptone*Peptone, Salinity*Salinity), and interaction (Sucrose*Peptone, Sucrose*Salinity, Peptone*Salinity) terms were found and reported in Table 4. Among the linear coefficients, sucrose, peptone, and salinity had a substantial effect on protease production. While all the quadratic coefficients were significant, only the interaction had a meaningful effect on protease production. The following regression equation was established:

Table 3: Results of Box–Behnken using three independent variables

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

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

The behaviour of alkaline protease production and interaction effect of three factors—sucrose, peptone, and salinity—at varied values were illustrated in contour plots. The optimum production of the enzyme was found to be 258.75 U/ml with the sucrose, peptone, and percent salinity at concentrations of 2.5%, 10%, and 0%, respectively, in the expected value. Then, based on those values, additional experiments were carried out in triplicate to validate the results, which produced an optimal enzyme production of 259 U/ml, which was higher than the activity obtained in a one-variable-at-a-time approach in physical parameters without any statistical approach, i.e., 218 U/ml. The modified R2 (98.04%) value, which is closer to 100% than the posited R2 (88.1%) value for protease production, showed better fit to the experimental data than the predicated R2 (88.1%) value.

IV. CONCLUSION

Alkaline proteases are used extensively in a variety of fields, including environmental bioremediation. The current study demonstrates that cultural contexts and media elements have a significant impact on protease production. Solibacillus silvestris, an isolate from the rhizospheric soil of the Madhwad, Gir, mangrove region, produced alkaline proteases most effectively at 40 °C, pH 8.5, 48 hours of incubation, sucrose as a C-source, and peptone as an N-source. The two stages of optimisation used for alkaline protease production included a statistical method with MINITAB 16 and a one variable at a time approach. After optimisation, the synthesis of alkaline protease rose from 218 U/ml using the one variable at a time method to 259 U/ml using the statistical method. The findings show that the bacterial isolate is valuable for industry, and more research is being done to characterise and purify the enzyme for usage in industry.

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