Research Article

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Screening and optimization of extracellular pectinase produced by *Bacillus thuringiensis* SH7

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Abstract: The objective of the current research was to identify and evaluate the possibility of production of pectinase, also known as pectin degrading enzymes, from indigenous bacterial strains. Qualitative screening of isolated bacterial strains showed that among 29 bacterial strains, 5 have maximum enzymatic activity. The highest pectinase producing strains were quantitatively analyzed for enzyme production. SH7 strain was found as highest pectinase producer (0.77 IU/mL) that was further analyzed to molecular level by amplification of 16s rRNA. It was found 100% similar with other reported strains of *Bacillus thuringiensis*. Medium optimization was performed to optimize fermentation conditions for maximum enzyme yield. An experimental design containing 12 experimental runs was designed by Plackett–Burman design (PBD). Maximum pectinase activity was obtained at 45°C after 24 h when the growth medium was supplemented with 2.5% nitrogen, 5.0% substrate, $MgSO_4$ as metal ion, 1% inoculum size, and pH was adjusted to 6. Factorial regression analysis of the PBD design was performed and the overall design was also found significant in terms of *R* square value. In PBD, the most significant factors for production were temperature, pH, metal ion concentration, and nitrogen source. Central composite design (CCD) design consisting of 26 experimental runs was employed to optimize these four significant factors. The overall model summary showed maximum pectinase activity (19.2 IU/mL) at 37°C temperature, 0.08 NaCl, 1.7% nitrogen source, and pH 8.4. In CCD, NaCl, nitrogen source, and pH were also reported as significant factors by the Pareto chart, probability plots, and 3D interactions.

Keywords: Plackett–Burman design, central composite design, pectinases, optimization, fermentation

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1 Introduction

Enzymes are biomolecules that speed up reactions in any metabolic pathway. Many benefits can be acquired by using enzymes that cannot be obtained by traditional ways of treating chemical. They have been more valuable in industrial operations due to their capacity to accomplish very specific chemical transformation. With the passage of time, as the energy consumptions increases, natural resources will be depleted at an alarming rate. Therefore, the need for additional energy resources is critical. There are numerous advantages of applying enzymes over traditional methods [1].

Pectin is a polymeric substance containing group of carbohydrates that esterifies with methyl alcohol. It is a crucial part of the plants' cell wall. This substance is mostly found in the terrestrial plants and middle plate, where it serves as a binding agent between neighboring cells as well as it also has a role in cell wall hydration. Pectic polymers have been suggested to play a variety of activities, such as cell growth, controlling cell–cell adhesion, wall mechanical characteristics, mediating cell porosity, acting as a source of signaling molecules (oligosaccharides), and taking part in cell organogenesis and differentiation [2]. Pectinases belong to class of enzymes which carried out the cleavage of pectin substances found in the cell walls of plants. Pectic enzymes primarily fall into two categories: those that catalyze the de-esterification of pectins (de-esterifying enzymes) and those that depolymerize glycosidic-(1–4) links within

GalA residues (depolymerizing enzymes), through hydrolysis (hydrolases).

There are many sources by which enzymes are synthesized such as plants, animals, and microbes. However, it is well recognized that microorganisms are a key origin of enzymes since they enable inexpensive technique with low waste production and less resource consumption that does not raise any environmental difficulties, as is the case with sources from animals and plants [3]. According to reports, fungi, yeast, and bacteria are responsible for producing 50% of all known enzymes, followed by 35% by bacteria and the remaining 15% by either plants or animals [4]. Many microbial species have been reported for production of extracellular and intracellular pectinases, these include *Bacillus subtilis*, *Bacillus pumilis*, *Aspergillus niger*, *Geotrichumklebahnii*, *Cystofilobasidium capitatum*, and *Geotrichum lactis* [5].

Pectinases can be used in various industrial sectors. The microbial pectinases are being used in the fruit juice industries for the enhancement of clarification and manufacturing of juices. They also minimize the density of fluid and enhance the recovery process [6]. Pectinolytic enzymes also have significant role in wine industry. Wine which is processed enzymatically has better stability and less filtration time as compared to other methods [7]. Many fiber crops are retted and degummed using pectinases. Microorganisms use this process to break down the pectin in bark, releasing fibers in the process [8]. By adding particular enzymes to fibers, bioscouring is used to remove noncellulosic contaminants. The enzymatic approach has replaced the harmful chemicals that were previously used in conventional scouring [9]. Other carbohydrases are added to pectinolytic enzymes to remove the sizing agent from cotton fibers without generating any negative side effects [10]. Similarly, pectinases are added to the process of tea and coffee fermentation in order to hydrolyze and break the cell wall of tea leaves and to remove the mucilage layer around the coffee beans, which reduces the ability of instant tea powder to froth. In contrast to the conventional technique, pectinase, xylanase, and cellulose from yeasts and bacteria improved the various black tea components [11].

For maximum yield of enzyme various fermentation factors are optimized. Previously one factor/variable at time technique was used for optimizing process parameters but it has many limitations like time consumption and laborious, superficial optimization, no factor interaction, and large number of experimental runs. Nowadays many statistical approaches are being practiced for optimization like Box–Behnken design, Plackett–Burman design (PBD), central composite design (CCD), and Graeco–Latin squares [12]. Among the statistical approaches, PBD method can quickly and efficiently filter out the most crucial parameters from a large number of factors using a single strategy. As a combination of different mathematical and statistical approaches or modeling, response surface methodology (RSM) is typically used to analyze the performance of complex systems and optimize the multiple process conditions [13]. This work aims to isolate and identify pectinase producing bacterial strains from indigenous soil samples and optimize fermentation conditions (both physical and chemical) to increase enzyme production initially by PBD and then followed by CCD.

2 Materials and methods

2.1 Isolation of pectinolytic bacteria

Soil samples were collected in winters from vegetable and fruit dump areas of district Haripur, Abbottabad and Mansehra. Isolation of collected strains was done by tenfold serial dilution in Luria Basal (LB) media to reduce microbial load. From the last three dilutions, a small measured volume (100 µL) of each dilution was used to spread on LB plates containing yeast extract (10 g), sodium chloride (10 g), tryptone (10 g), agar (2 g), and 1% substrate. Initial pH of the medium was adjusted to 9 before sterilization at 121°C for 20 min. After inoculation with selected strain, the plates were incubated at 37°C for 24 h. After 24 h of incubation, separate distinct colonies were picked with sterilize wire loop and streaked on LB agar media plate and labeled accordingly. Then, the plates were again subjected to incubation at 37°C for 24 h for further growth. The process was repeated for a number of times to get the individual cell. Gram staining and biochemical assay were performed for initial identification of isolated species [14].

2.2 Qualitative screening

Bacterial strains were initially screened by inoculating in fermentation media containing glucose (2%), $MgSO_4$ (0.05%), ammonium sulphate (0.3%), NaCl (0.1%), K_2HpO_4 (0.1%), and pectin substrate (1%). Selected colonies were inoculated on the media plates followed by incubation for 24 h at 37°C. After the complete incubation colonial plates were stained with 2% iodine dye, appearance of clear zone around colonies showed pectinolytic bacteria. The pectinolytic bacterial colonies were further qualitatively screened for optimum pectinase production on the basis of zone diameter around the colonies.

Zone of hydrolysis was analyzed and enzyme index was calculated using the formula [15] as follows:

Enzyme index = Diameter of hydrolysis zone/Diameter of a colony.

2.3 Quantitative screening of selected bacterial strains

Submerged fermentation was used to evaluate the strains that showed pectinolytic activity in the qualitative assay. Fermentation media (100 mL) with 1% (v/v) inoculum was incubated in shaking incubator at 37°C for 24 h (agitation 150 rpm). After incubation period, the broth culture was centrifuged for 15 min at 8,000 rpm. The supernatant was used as crude enzyme for the enzyme assay. Then, 1 mL of substrate solution (1 g substrate in 100 mL distilled water) was mixed with 1 mL of cell free filtrate and incubated for 30 min in a water bath at 37°C. The reaction was then stopped with 3 mL of dinitrosalicylic acid reagent followed by boiling for 5 min. After cooling for 5 min in an ice bath, 720 µL distilled water was added to the reaction mixture and enzyme production was analyzed at 540 nm. Along with the test, a control was prepared by using heat-killed enzyme. All the experiments were performed in triplicate and the results are the mean values. According to standard assay, one unit of enzyme is defined as the quantity of enzyme required to release 1 mg of reducing sugar from galacturonic acid [16].

2.4 Phenotypic and biochemical characterizations

The highest pectinolytic isolates were characterized on the basis of various morphological, microscopic, and biochemical characteristics to identify the isolated strains to genus level as per results of Bergey's Manual of Determinative Bacteriology. The test parameters were gram stain, catalase test, citrate, pectinase, lactose, oxidase, etc. Microscopic characteristics and colonial feature were also studied [17].

2.5 Molecular identification

Genomic DNA from maximum pectin producing strain was extracted according to the methods for bacterial DNA extraction by using Promega DNA extraction kit.

2.5.1 PCR of 16s rRNA gene of pectinolytic bacterial isolate

16s rRNA gene of highest pectinase producing strain was amplified by C-1000 Touch thermocycler using universal primer sequences. The PCR mixture was prepared by adding Hot start PCR master mix, forward primers (5′-AGA GTT TGA TCI TGG CTC AG-3′), reverse primers (5′-ACG GIT ACC TTG TTA CGA CTT-3′), and DNA template. The reaction was carried out at the conditions of denaturation at 94°C for 1 min followed by 35 cycles, 55°C annealing for 30 s, 72°C extension for 1 min and final elongation at 72°C for 10 min [18].

2.5.2 Gene sequencing and analysis of 16s rRNA

For gene sequencing, 16s rRNA sequence was analyzed by BioEdit Sequence Alignment Editor Software and compared to GenBank nucleotide data library. BLAST program of NCBI was used for the nucleotide sequence alignment and to determine its closest phylogenetic relatives. The 16S rRNA sequence was analyzed by MEGA11 software and compared to the sequences in GenBank nucleotide data library [19].

2.6 Statistical optimization of pectinase production by multi factorial experiments

The overall optimization of fermentative enzyme production was completed in three major steps. At first, various nutritional and physical parameters were evaluated for pectinase production by using PBD. In the next step, most significant variables were further optimized to their optimum level by using CCD. At last, the quality of model fitness was verified by computational analysis expressed by R^2, the coefficient of determination.

2.6.1 PBD

PBD was generated to assess the effect of the seven factors influencing pectinase enzyme production. Temperature, inoculum size, pH, incubation period, substrate concentration, metal ion, and nitrogen ion concentration were chosen as independent factors. All the selected variables were selected at low (−1) and high (+1) level (Table 1).

Table 1: Variable levels screened in the PBD

Factor	Low level (−1)	High level (+1)	Factor	Low level (−1)	High level (+1)
Temperature (°C)	25	45	Nitrogen source (%)	0.5	2.5
pH	6	10	Substrate (%)	1	5
Inoculum size (%)	1	5	Metal ion (%)	Magnesium sulphate	Sodium chloride
Incubation period (h)	24	96			

Minitab software was used to create a design matrix of 12 experiments (Table 2). All experiments were performed in triplicate in 250 mL Erlenmeyer flasks containing 50 ml fermentation medium. PBD screening design depends on the first order model.

$$A = \alpha_0 + \sum \alpha_i B_i.$$

Here A represents the response, i.e., pectinase activity, α_0 is the model intercept, α_i is the variable estimate, and B_i shows the independent variable levels. Significance of each variable was best demonstrated by Pareto Plot. All the variables with p-values less than 0.05 were noted as significant. Statistical significance of the model was also determined by T and R^2 values. All factors that were found statistically significant were chosen for further steps [20].

2.6.2 Optimization of significant variables using CCD

CCD of RSM was employed to optimize significant factors for pectinase activity. Number of experimental combinations in CCD is $2h + 2h +$ no, where h represents the number of independent variables and no represents the repetition of experiments at center point. Four factors were identified as significant during the initial screening of factors (in PBD), including initial temperature, pH, nitrogen source, and NaCl were further optimized using CCD in RSM. Three levels for each variable are low (−),central (0), and high (+) (Table 3). A second-order polynomial equation was used to analyze the effect of these variables on response.

$$A = \alpha 0 + \Sigma \alpha_i B_i + \Sigma \alpha_{ii} B_i 2 + \Sigma \alpha_{ij} B_i B_j,$$

where A = predicted response, $\alpha 0$ = intercept term, α_i = linear effect, α_{ii} = squared effect, α_{ij} = interaction effect, and B_i and B_j = independent variables. An experimental model of 26 runs was designed (Table 4). Pectinase activity was determined and used as a response, while the rest of the optimized factors were kept constant for subsequent designed experimental runs [21].

Table 3: Factors with their levels in CCD

Factors	Levels		
	−1	0	+1
NaCl concentration (%)	0.05	0.075	0.08
pH	6	7	8
Nitrogen source concentration (%)	0.5	1.5	1.7
Temperature	33°C	37°C	45°C

Table 2: PBD for screening significant factors affecting pectinase production

Run	B1: temp (°C)	B2: pH	B3: incubation period (h)	B4: inoculum size (%)	B5: metal ions	B6: nitrogen source (%)	B7: substrate (%)	A: pectinase (IU/mL)
1	45	6	96	1	$MgSO_4$	0.5	5	0.43
2	45	10	24	5	$MgSO_4$	0.5	1	0.21
3	25	10	96	1	NaCl	0.5	1	0.43
4	45	6	96	5	$MgSO_4$	2.5	1	0.59
5	45	10	24	5	NaCl	0.5	5	0.43
6	45	10	96	1	NaCl	2.5	1	0.59
7	25	10	96	5	$MgSO_4$	2.5	5	0.3
8	25	6	96	5	$MgSO_4$	0.5	5	0.42
9	25	6	24	5	$MgSO_4$	2.5	1	0.59
10	45	6	24	1	$MgSO_4$	2.5	5	0.83
11	25	10	24	1	$MgSO_4$	2.5	5	0.3
12	25	6	24	1	$MgSO_4$	0.5	1	0.35

2.7 Model validation

Designed model in the RSM was validated by different point studies. Obtained data were compared to predicted values and prediction errors.

2.8 Statistical analysis of data

All the designed run orders were carried out in triplicate and response was expressed as mean of observed values. ANOVA analysis was applied to the model data and the quality of polynomial equation in term of fit was accessed by evaluating the R^2 coefficient to Adj R^2 coefficient; model significance was checked by F test and P value. 3D surface and 2D contour plots were plotted to express the significance of independent variables on the results.

Table 4: CCD Optimization of pectinase production (IU/mL)

Run order	NaCl	pH	Nitrogen source	Temperature °C	IU/mL
1	0.07	7.6	1.3	33	1.95
2	0.08	7.6	1.3	33	5.4
3	0.07	8.4	1.3	33	4.8
4	0.08	8.4	1.3	33	9
5	0.07	7.6	1.7	33	9
6	0.08	7.6	1.7	33	9.6
7	0.07	8.4	1.7	33	11.7
8	0.08	8.4	1.7	33	8.7
9	0.07	7.6	1.3	37	9
10	0.08	7.6	1.3	37	10.2
11	0.07	8.4	1.3	37	9.6
12	0.08	8.4	1.3	37	5.7
13	0.07	7.6	1.7	37	15.3
14	0.08	7.6	1.7	37	1.2
15	0.07	8.4	1.7	37	19.2
16	0.08	8.4	1.7	37	3.3
17	0.075	8	1.5	35	10.8
18	0.075	8	1.5	35	4.95
19	0.05	8	1.5	35	14.4
20	0.1	8	1.5	35	4.5
21	0.075	6	1.5	35	6.75
22	0.075	10	1.5	35	13.6
23	0.075	8	0.5	35	2.1
24	0.075	8	2.5	35	14.4
25	0.075	8	1.5	25	6.6
26	0.075	8	1.5	45	9.6

3 Results

3.1 Screening for maximum pectinase producing bacteria

Strains with particularly considering their conditions were isolated from soil samples and were qualitatively analyzed for pectinase production by dropping iodine dye on pectinase agar plate. Eight strains (SH1, SH3, SH5, SH7, SH16, SH17, SH18, and SH25) showed positive results with the formation of clear zone surrounding the colonies due to pectin hydrolysis at dark purple background. The largest zone was observed around strain SH7 (Figure 1). Quantitative screening of the potent pectinolytic isolates was conducted using the enzyme activity assay. The highest pectinolytic activity was shown by isolate SH7 (0.77 IU/mL) which was consistent with the results obtained from the

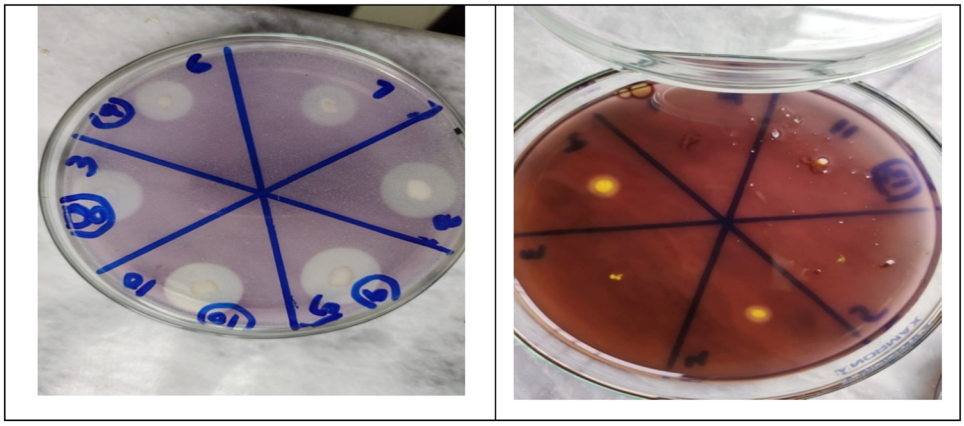

Figure 1: (a) Pectinase producers and (b) non-producers.

previous qualitative assessment. Due to its potent activity, SH7 strain was chosen for further investigation (Figure 2).

3.2 Characterization of pectinolytic bacterial isolate

Highest pectinase producing isolate, i.e., SH7 strain was subjected to morphological, microscopic, and biochemical characterization according to methods of Bergey's Manual of Determinative Bacteriology. The results showed that isolate SH7 belongs to the genus Bacillus, gram (+), citrate (+), catalase (+), oxidase (−), lactose (+), glucose (+), urease (+), pectinase (+), short rod shaped in chains, aerobic, and spore forming organism.

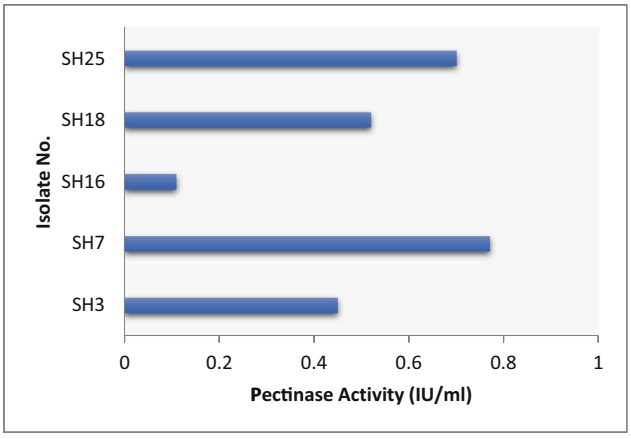

Figure 2: Qualitative screening of pectinase enzyme.

3.3 Molecular identification of pectinolytic bacterial isolate (SH7)

SH7 strain was subjected to molecular identification for further confirmation and identification. The PCR was used to amplify 16s rRNA of Bacillus SH7. Through agarose gel electrophoresis band of 1.5 kb was obtained. After purification and sequencing of reamplified 16s rRNA, 1,400 nucleotide sequence was obtained that contained both variable and conserved sections. The sequenced data were run to the BLAST study for identification of the bacterial strain (SH7). Phylogenetic tree was constructed on the basis of 16s rRNA sequences of pectinase producing SH7 strain and close species sequences were downloaded from NCBI. The sequences were aligned by CLUSTAL W2 and neighbor-joining tree was constructed in MEGA11 by Kimura-2-parameter [22]. Phylogenetic tree from *Bacillus thuringiensis* showed 100% similarity with their closest phylogenetic relatives *Bacillus thuringiensis* LAA3 in the NCBI (Figure 3).

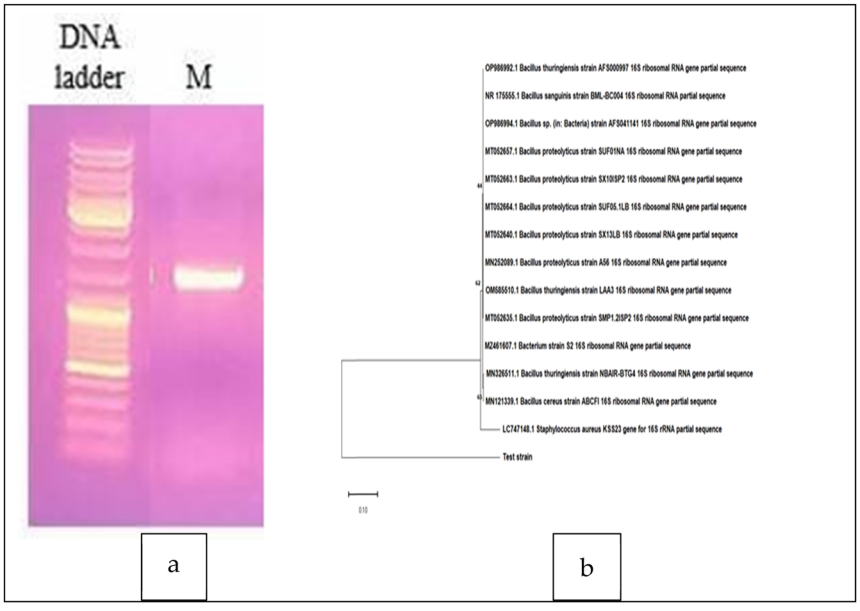

Figure 3: Electrophoresis shows PCR products of 16s rRNA of *Bacillus thuringiensis* SH7 (M) (a) and phylogenetic tree using multiple CLUSTAL W2 alignment software of MEGA 11 (b).

3.4 Multi factorial optimization designs for pectinase production

Current study is focused on statistical approach to gradually enhance pectinase production by using different as well as minimize cost.

3.4.1 PBD

PBD was conducted to find out the significant variables that affect the production of pectinase. Totally 12 experiments were designed at 2 levels i.e., high and low. Temperature, incubation period, pH, incubation temperature, nitrogen source, metal ions, and substrate concentration were primarily selected as optimization parameters. Table 2 demonstrates the average pectinase activity (IU/mL). The lowest pectinase activity (0.2 IU/mL) was observed in run order 2 whereas highest pectinase activity (0.8 IU/mL) was obtained in run over 10 having pH 6, sodium chloride as metal ion, incubation period of 24 h, inoculum size of 1%, nitrogen source of 2.5%, substrate concentration of 5%, and temperature of 450°C. Multi regression analysis on the experimental model established the first order polynomial equation to show the production of pectinase as follows:

$$A\ (\text{IU/mL}) = 0.503 + 0.00575\,B1 - 0.03958\,B2 + 0.000116B3 - 0.01625\,B4 + 0.0925B5 + 0.0775\,B6 - 0.00208\,B7,$$

where A is the pectinase activity; B1 is the temperature; B2 is the pH; B3 is the incubation period; B4 is the inoculum size; B5 is the metal ions; B6 is the nitrogen source; and B7 is the substrate concentration. Regression analysis in the form of Pareto chart showed that four factors including temperature (P = 0.031), pH (P = 0.011), nitrogen source (P = 0.012), and metal ions (P = 0.006) have significant effects on pectinase production because these factors clearly crossed the reference line (Figure 4).

3.4.2 CCD

Second order model (CCD) was performed to analyze the four factors (pH, temperature, nitrogen source, and metal ions) that were found as significant factors in PBD while making non-significant factors constant as per highest run over values. The overall model summary showed that the highest pectinase activity (19.2 IU/mL) was observed in run over 15 whereas lowest pectinase activity of 1.2 IU/mL was observed in run over 14. Regression analysis for the experimental data showed that predicted response can be obtained by using second-order polynomial equation as follows:

$$A\ (\text{IU/mL}) = -1{,}141 + 1{,}3181\,B1 + 18.5\,B2 + 215\,B3 + 23.5\,B4 + 5{,}511\,B1{*}B1 + 1.04\,B2{*}B2 + 2.2\,B3{*}B3 + 0.021\,B4{*}B4 - 305\,B1{*}B2 - 2{,}334\,B1{*}B3 - 237.2\,B1{*}B4 + 4.10\,B2{*}B3 - 0.480\,B2{*}B4 - 2.09\,B3{*}B4,$$

where A is the pectinase activity; B1 is sodium chloride; B2 is the pH; B3 is the nitrogen source; and B4 is the temperature. ANOVA analysis for pectinase production was employed on CCD design (Table 5). High precision of model was showed by

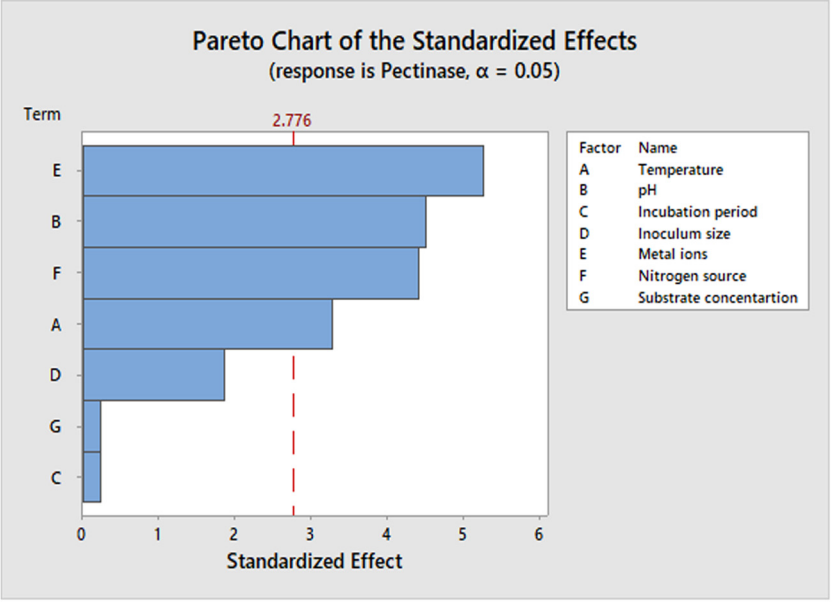

Figure 4: Pareto chart (PBD) showing effect of different factors on pectinase production.

Table 5: Variance analysis of CCD for pectinase production

Source	DF	Seq SS	Contribution (%)	Adj SS	Adj MS	*F*-Value	*P* Value
Model	15	445.912	87.47	445.912	29.727	4.65	
Blocks	1	2.666	0.52	0.214	0.214	0.03	
Linear	4	238.561	46.79	238.561	59.640	9.33	
B1	1	89.717	17.60	89.717	89.717	14.04	0.004
B2	1	30.139	5.91	30.139	30.139	4.72	0.055
B3	1	106.528	20.90	106.528	106.528	16.67	0.002
B4	1	12.178	2.39	12.178	12.178	1.91	0.197
Square	4	6.317	1.24	6.317	1.579	0.25	
B1*B1	1	0.596	0.12	0.531	0.531	0.08	0.779
B2*B2	1	5.486	1.08	0.778	0.778	0.12	0.734
B3*B3	1	0.039	0.01	0.225	0.225	0.04	0.855
B4*B4	1	0.196	0.196	0.196	0.196	0.03	0.864
Two-way interaction	6	198.368	38.91	198.368	33.061	5.17	
B1*B2	1	5.941	1.17	5.941	5.941	0.93	0.358
B1*B3	1	87.189	17.10	87.189	87.189	13.65	0.004
B1*B4	1	90.013	17.66	90.013	90.013	14.09	0.004
B2*B3	1	1.723	0.34	1.723	1.723	0.27	0.615
B2*B4	1	2.364	0.46	2.364	2.364	0.37	0.557
B3*B4	1	11.139	2.18	11.139	11.139	1.74	0.216
Error	10	63.891	12.53	63.891	6.389		
Lack-of-fit	9	46.779	9.18	46.779	5.198	0.30	
Pure error	1	17.111	3.36	17.111	17.111		
Total	25	509.803	100.00				

B_1 = Sodium chloride, B_2 = pH, B_3 = Nitrogen source, B_4 = Temperature.

low magnitude of P value (at 1% level of significance). Significance of each coefficient was indicated as lower *P* and higher *T* values. Sodium chloride ($P = 0.004$), pH (0.055), and nitrogen source (0.002) were seen as significant linear term whereas no square term was found significant throughout the design. Two-way interaction showed significant value for sodium chloride and nitrogen (0.004), and sodium chloride and temperature (0.004) interactions. Significance of model was also depicted by values of R^2 (coefficient of determination). In the current experimental model, value of R^2 (87.47%) was found high as compare to adjusted R^2 (68.67%) that also confirms the accuracy of designed experiments.

The response analysis was obtained after running the CCD design. Pareto charts were plotted to determine the significant effects for pectinase enzyme. In the Pareto chart, three factors including metal ion (NaCl) (*P* value 0.004), pH (*P* value 0.05), and nitrogen source (*P* value 0.002) were found statistically significant, as these factors clearly crossed the reference line (Figure 5).

Expression of model regression equation in the form of surface plots (3D) and contour plots (2D) graphs provides a rapid and structured way to visualize the best values of significant variable. Elliptical contours show maximum interaction among the selected variables whereas circular shaped contour explains no interaction among variables. 3D surface plots further explain the relationship between dependent and independent variables. As depicted by these graphical representations, nitrogen concentration of 0.657386, sodium chloride of 0.075, and pH 8 resulted in a remarkable increase in the enzyme yield (22.5 IU/mL) as compared to the results of PBD (0.8 IU/mL) (Figure 6).

4 Discussion

Nowadays chemicals have been replaced with biomolecules for accelerating various reactions. Among these biomolecules, enzymes are most commonly used in many industrial sectors. It is important to synthesize these biocatalysts with enhanced and desired characteristics that can meet the industrial demands. Pectinases are among industrially important enzymes that have numerous applications in various fields. Although, pectinases are broadly synthesized by higher plants but advances in microbial biotechnology have revealed the enormous pectinolytic potential of microbes, particularly bacteria and filamentous fungi. In general, filamentous fungi are the basis of acidic pectinases, whereas bacteria are the source of alkaline pectinases [23]. Bacteria have various advantages over

filamentous fungi as they do not experience rheological issues in fermentation broth and do not produce a mixture of pectinases [24]. Locally isolated bacteria are yet to be investigated for biotechnological applications [25].

The current study was conducted with the isolation of 50 microbial isolates from soil of fruit and vegetable dumps areas. Indeed soil dumped with rotten vegetables and fruits is a well-known productive habitat in order to isolate pectinolytic bacteria because pectin promotes their growth by supplying nutritious requirements [26]. The qualitative screening of bacterial isolates was carried out by growing them on pectin fermentation media and treating with potassium iodide solution. Potassium iodide is useful because when it is oxidized, a color change is produced which shows the presence of pectin polymer that gives color change [27]. Following the initial screening, 5 out of 50 (10%) strains were obtained as potential pectinase producer. Our study is in correspondence with the findings of Merín et al. [19]. Quantitative enzyme production showed that SH7 has maximum enzyme production ability as compared to other strains. SH7 strain was chosen for further study and subjected to colonial, microscopic, biochemical characterization, and molecular identification. The results showed that selected bacterial isolate displayed 100% similarity with other reported strains of *Bacillus thuringiensis*. Liu and coworkers also reported identification of pectinase producing strain as *Bacillus thuringiensis* [28]. Similarly, Namasivayam isolated bacterial strain from soil sample and identified it as *Bacillus thuringiensis* [29].

Generally, the production of pectinase is majorly affected by biological, physical, and chemical parameters. The production of enzymes from microbes can be improved by analyzing the influence of nutritional and ecological factors mostly known as optimization methods which ultimately results in decrease in production cost. Conventionally, factors that affect the production process were studied one after another by keeping the other factors constant. In this strategy, the influence of values and factors on the analysis can be obtained separately. However, this technique is found as laborious and does not explain the interactions between variables [30]. Later, different statistical techniques have been developed for the optimization of biological and chemical factors. In the current research, two levels of statistical designs were used in order to perform optimization, the first one is PBD and the second is CCD.

PBD is an effective method for screening the influence of several factors on the response [31]. 12 runs of the experiment were performed as obtained by PBD. Maximum pectinase units 0.83 IU/mL was produced in PBD when fermentation was carried for 24 h at 450°C by maintaining the medium at pH 10, inoculum size of 5%, substrate concentration of 1%, nitrogen source of 0.5%, and $MnSO_4$ as metal ion. The results of recent work are in accordance with the findings of Ahmed et al. [5]. Factorial regression analysis of PBD design was performed that showed significance of multiple factors. Among all test variables, temperature, pH, nitrogen source, and metal ion concentration were statistically significant for pectinase production as these factors have positive *R*-coefficient and *P* value less than 0.05. In the current model, value of R^2 (87.47%) was found high as compared to adjusted R^2 (68.67%) that also confirms the accuracy of designed

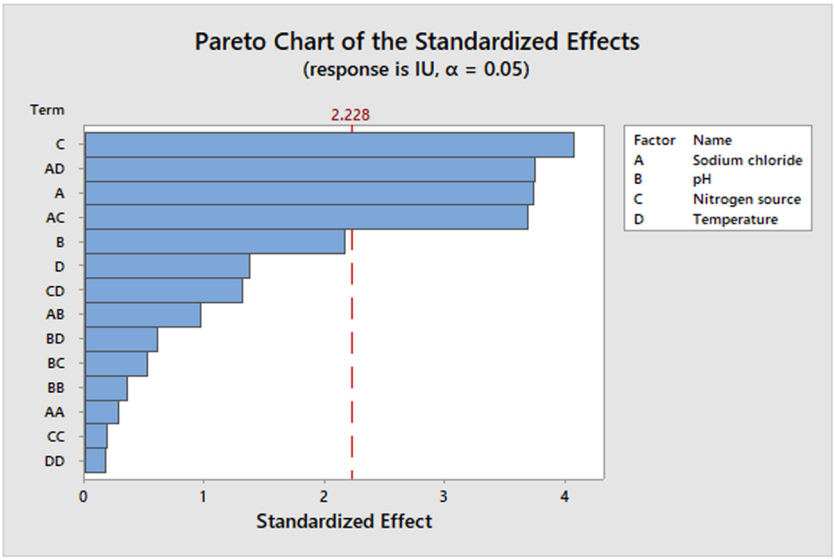

Figure 5: Pareto chart showing the effect of NaCl, pH, nitrogen source, and temperature on pectinase by *B. thuringiensis*strain-SH7.

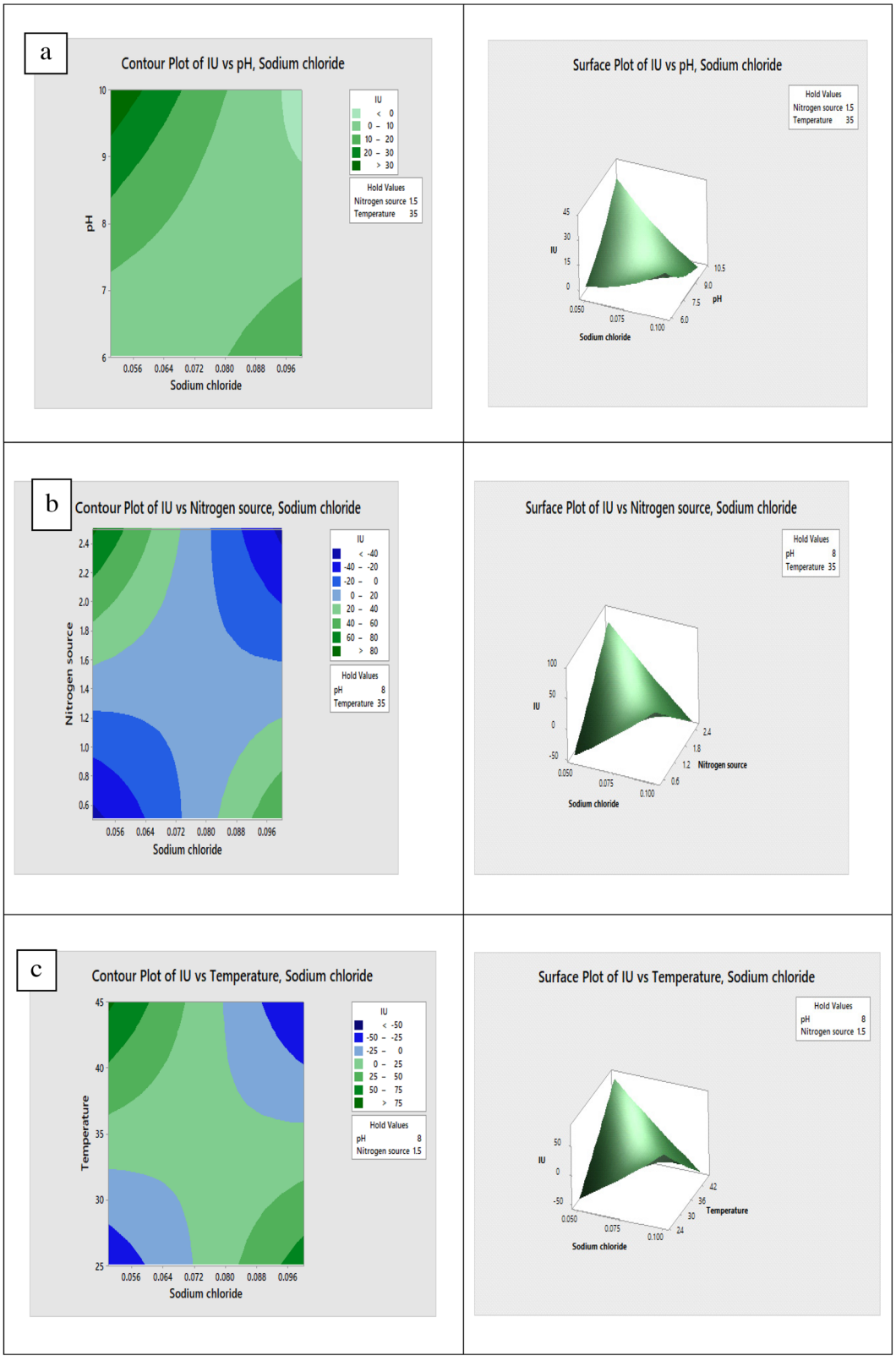

Figure 6: Response surface 2D and 3D plots representing interaction between variables affecting pectinase production: (a) pH and Nacl, (b) Nacl and nitrogen source, (c) temperature and Nacl, (d) nitrogen and pH, (e) temperature and pH, and (f) temperature and nitrogen source.

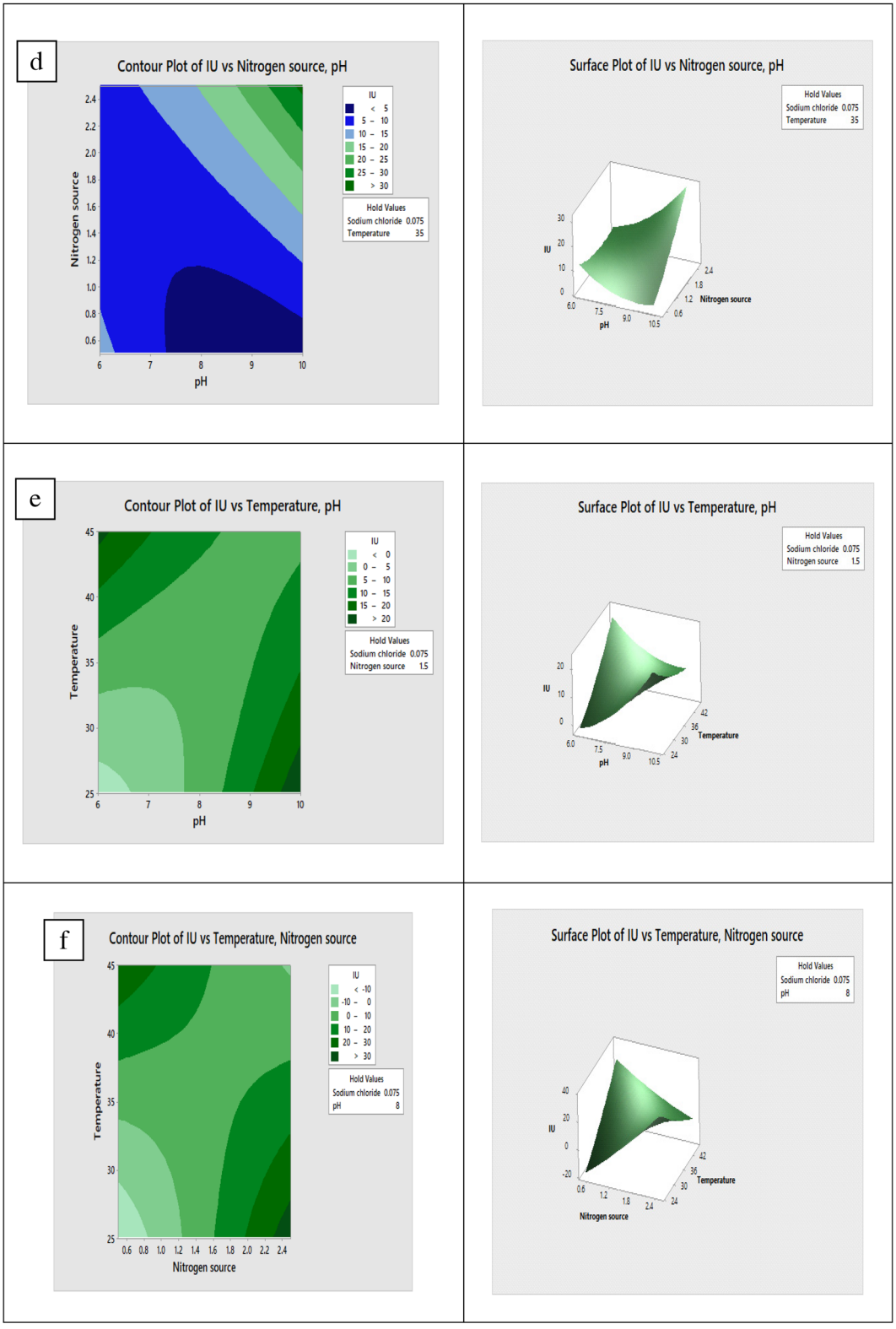

Figure 6: (*Continued*)

experiments. Accuracy of optimization design for pectinase production in terms of R^2 value was also reported in an earlier study (99.93%) [32].

Pareto chart clearly shows significant effects for enzyme by a reference line. Exceeding bars from reference line shows significant factors for response analysis. In a recent study, response analysis was obtained after running the PBD design. The most significant factors for production of pectinase were sodium chloride, temperature, pH, and nitrogen source. Our results are found in accordance with the findings of Venil et al., who reported pH and nitrogen source as significant factor by response analysis in terms of Pareto [33].

During statistical analysis, PBD was used to screen significant factors that have profound effect on enzyme production. In the current study, the significant factors by PBD design were further optimized by using central composite. In CCD, the overall model summary showed that the significant factors are pH, nitrogen source, and metal ion concentrations that showed the maximum pectinase yield of (22.5 IU/mL) at 0.657% nitrogen source, 0.075% NaCl, and pH 8. Accuracy of CCD design was also checked by using ANOVA analysis. Each significant coefficient was analyzed by lower P and higher T values. All factors showing p values less than 0.05 was a significant variable. Whereas no square term was found significant throughout the design. A two-way interaction showed significant value for sodium chloride and nitrogen (0.004) and sodium chloride, and temperature (0.004) interactions. Our results are in contrast with earlier findings that showed maximum pectinase production (10 IU/mL) increase in the pectinase activity at 40°C and 24 h by *Aspergillus niger* [34]. In the current experimental model, the value of R^2 (87.47%) was found high as compared to adjusted R^2 (68.67%) that also confirms the accuracy of designed experiments [35].

Representation of the model regression equation in the form of surface plots (3D) and contour plots (2D) graphs provides a rapid and structured way to visualize the best values of significant variable. Multiple combinations among two variables (keeping all the other selected parameters at their respective zero level) are graphically presented in the form of contour curves. Elliptical contours show maximum interaction among selected variables whereas circular shaped contour explains no interaction among variables [36]. 3D surface plots further explain the relationship between independent and dependent variables. As depicted by these graphical representations, nitrogen concentration of 0.657386, sodium chloride of 0.075, and pH 8 resulted in a significant increase in the enzyme yield (22.5 IU/mL) as compared to the results of PBD (0.8 IU/mL). Significant interaction of fermentation variables such as $CaCl_2$ (0.8%), temperature (40°C), inoculum size (1.5%), and pectin concentration (2.5 g/L) for optimum pectinase yield by *B. subtilis* BKDS1 was also reported in previous findings [37].

The current study resulted in isolation of pectinolytic bacterial strain from indigenous soil samples. Moreover, optimization studies showed that significant titer of enzyme could be used for different industrial applications.

5 Conclusion

Members of genus bacillus have been reported for production of biotechnologically important products. Among these commercially important products, enzymes are well documented by *Bacillus thuringiensis*. The current study was focused on isolation of pectinolytic bacillus strain from soil sample. Optimum pectinase producing strain was identified as *Bacillus thuringiensis* SH7 on the basis of microscopic, morphological, biochemical, and molecular characteristics. Pectinase production was optimized in two-step process, PBD followed by CCD. Optimization of fermentation variable by PBD showed that among seven selected variables, temperature, sodium chloride, nitrogen source, and pH have a remarkable effect on extracellular pectinase production. The significant factors by PBD were optimized to their optimal level by CCD of RSM. Temperature, NaCl, and pH were also found as optimum variables after CCD analysis. The overall optimization model was found significant in terms of R, P, and F values. The present study resulted in a significant statistical optimization of enzyme that can be used for different biotechnologically important processes.

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Ethical approval: The conducted research is not related to either human or animal use.

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