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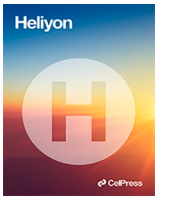

Research article

Statistical bioprocess optimization for enhanced production of a thermo alkalophilic polygalacturonase (PGase) from *Pseudomonas* sp. 13156349 using solid substrate fermentation (SSF)

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ABSTRACT

In this study, a polygalacturonase (PGase) producing bacterial strain was isolated and identified as *Pseudomonas* sp. 13159349 from fruit market soils, and TLC analysis confirmed its pectinolytic activity. Additionally, SSF, Plackett-Burman design (PB), and response surface methodology (RSM) were used to optimize the production of this thermostable and alkalophilic PGase. Wheat bran demonstrated the highest activity (60.13 ± 3.39 U/gm) among the various agricultural wastes used as solid substrates. To further enhance the enzyme production, statistical optimization of media components was investigated using the PB design. Among the 11 variables tested, pH ($p < 0.0001$), inoculum size ($p < 0.0001$), incubation time ($p < 0.0001$), and temperature ($p < 0.0041$) were found to have a positive effect on the production. The interaction and concentration of the selected factors were examined by RSM, which demonstrated the optimal conditions for maximum production (315.65 U/gm) of the enzyme using wheat bran as the solid substrate were pH 10.5, 61–66 h of incubation, and 6–7.5% inoculum size. The model was highly significant, with a p-value of <0.0001, an F-value of 95.33, and a low CV of 2.31. The RSM model was validated by a laboratory-scale experiment showing 30600 ± 400.32 U/100 gm PGase activity. Thus, SSF and the statistical design of media components resulted in a significant 5.2-fold increase in PGase output solely by using agro waste and optimizing the physical parameters, making this a highly cost-effective bioprocess.

1. Introduction

Polygalacturonase (PGase) (EC 3.2.1.15) is a member of the pectinase family of enzymes that has several industrial and commercial applications, especially in sectors that rely on lignocellulosic biomass as raw material. These enzymes aid in the breakdown of α 1,4 D-galacturonic acid linkages in pectin, a structural heteropolysaccharide found in the cell walls of higher plants that accounts for the vast bulk of plant biomass [1]. Numerous sectors rely heavily on the hydrolysis of pectin, including the food and beverage, biofuel, textile, paper, and oil industries, etc. [2], and PGases are essential to these processes. The most sought-after sources of pectinases include microorganisms such as fungi, bacteria, yeast, plants, and so on [3]. The majority of commercial PGases come from fungi like

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Aspergillus, Penicillium, Rhizopus, Trichoderma, and Fusarium, etc, [4] but they are most effective in acidic environments and at low temperatures, making them inefficient in bioprocesses that call for neutral or alkaline pH and high temperatures [5]. However, new research suggests that bacteria such as *Bacillus* sp. produce alkaline PGases [6]. Since alkaline PGases can tolerate high pH and temperature, they are widely utilized in industrial sectors such as biofuels, production of pectin-free starch, coffee, and tea fermentation, oil extraction, bio-scouring of fabrics, polishing of jute fabrics, degumming and retting of natural fibers, paper pulp bleaching, pectic wastewater treatment, etc [7,8]. The application of PGase reported in this study is already established in extraction of olive oil [9]. However, the scarcity of alkaline PGase-producing strains and the optimal culture conditions prevent the large-scale production of this enzyme [10,11].

Increased production cuts not only production costs but also the price of the finished product. Generally, enhanced productivity can be achieved through strain improvement, choice of fermentation, or medium optimization. Advances in fermentation process optimization, particularly in solid substrate fermentation (SSF), have substantially increased enzyme production [12]. SSF typically uses agro-industrial wastes such as fruit peels, paddy straws, bagasse, brans, hull, etc [13]. The selection of an ideal agricultural waste is important for enzyme production. Some of the primary reasons why SSF is superior to other fermentation processes are its simple media composition, less susceptibility to substrate inhibition allowing higher product concentration, higher quality and activity of extracts, minimal effluent generation, reduced downstream processing, cost-effectiveness and reduced ecological footprint, etc [14]. However, despite SSF's success, there are still gaps in the bioprocesses that use this fermentation technique.

In recent decades, great emphasis has been placed on the optimization of media components. Growth parameters, both physical (e.g., pH, temperature, agitation speed, etc.) and nutritional (carbon, nitrogen supply, salts, etc.), appear to have a significant impact on microbial growth and biomolecule production; hence, optimizing these parameters can immensely benefit industrial operations [15]. Previously, researchers used inefficient, time-consuming, and prone-to-error conventional techniques such as one factor at a time (OFAT), where each component was evaluated individually. Nonetheless, the introduction of sophisticated mathematical and statistical methods has led to the widespread usage of media optimization strategies for effective bioprocessing [15,16]. These tools apply models constructed from factorial designs and regression analysis, which yield efficient, accurate, and cost-effective designs. One such approach is the Plackett-Burman (PB) - a multifactorial experimental design used specifically to identify the factors most likely to have an impact on the process output [17]. Once the key components have been determined, RSM may be used to evaluate their measured response at optimum concentration and the influence their interactions have on production [18]. Therefore, integrating PB and RSM in media optimization is an effective strategy, as PB can identify the crucial components and RSM can provide insight into their interactions and the optimal concentrations of the factors for the desired impact.

The objective of the present investigation was the isolation and identification of a thermo-alkalophilic polygalacturonase (PGase) producing bacterial strain. Furthermore, the production parameters were statistically optimized to maximize PGase production by utilizing wheat bran as a solid substrate. Plackett-Burman Design (PBD) was primarily used to screen the factors, followed by Response surface methodology (RSM) to determine optimal conditions for maximum PGase production.

2. Materials and methods

2.1. Isolation and screening of alkalophilic PGase-producing bacterium

Screening for alkalophilic pectinolytic bacteria was carried out with soil samples collected from pectin-rich areas of fruit markets around Bangalore, India. Serially diluted (up to 10^{-5}) samples were spread on pectinase screening agar medium (PSAM) (K_2HPO_4, 3 g/L; KH_2PO_4 2 g/L; $(NH_4)_2HPO_4$ 3 g/L; NaCl 5 g/L; $MgSO_4$ 0.1 g/L; pectin, 5 g/L; agar 25 g/L pH 8) and incubated for 24 h at 37 °C. Following incubation, the plates were stained with a 3% KI solution (1 g iodine, 5 g KI in 330 ml distilled water) to identify the zone of hydrolysis. Colonies showing good zones were further subjected to TLC analysis.

2.2. Thin layer chromatography

The pectinolytic activity of the secreted enzyme was confirmed by thin-layer chromatography (TLC). The enzyme assay was carried out with a 1% pectin substrate and TLC sheets (silica gel 60 F254, Merck) were spotted with 10 μl of the reaction mixture, control (substrate blank), and a galacturonic acid standard (1 mg/ml). Chromatography was conducted with a chloroform-acetic acid-water (3:3.5:0.5 v/v) solvent system, and the separated products were observed by spraying the dried sheets with 1% aniline diphenylamine orthophosphoric acid reagent [19].

2.3. Identification of the bacterial isolate

The isolate was identified based on morphological and biochemical tests following Bergey's manual. Furthermore, the bacterial strain was confirmed using 16S rRNA sequencing (CellKraft Pvt. Ltd). Genomic DNA from the isolate was extracted and used as a template to amplify the 16S rRNA gene sequence. PCR was performed using 27F forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R reverse (5′- GGTTACCTTCTTACGACTT-3′) primers and the obtained 1500 bp PCR product was sequenced with an Applied Biosystems ABI 3130 Genetic Analyzer. The acquired sequence was compared to the NCBI-BLASTn gene database, and the sequences with the highest similarity and percent identity were chosen. These sequences were aligned using Clustal Omega, and a phylogenetic tree was built using the weighbor tree method using the seq scape_v5.2 analysis software, which builds the tree based on the distance matrix generated by the Jukes-Cantor corrected distance model. The nucleotide sequence of the isolate was submitted to GenBank, and

an accession number was assigned.

2.4. Production optimization by solid substrate fermentation (SSF)

2.4.1. Selection and processing of agro-industrial waste substrates

Based on a review of the literature, six agro-industrial wastes (sugarcane bagasse, wheat bran, rice bran, oatmeal, and orange peel) were chosen for PGase enzyme production via solid substrate fermentation (SSF). The agro wastes were cleaned with water, dried overnight in a hot air oven at 80 °C, and coarsely ground to 5–7 mm particle size using a mixer grinder (Godrej Pvt. Ltd). 5 gm of the solid substrate was placed in a 250 ml conical flask and autoclaved. The substrates were inoculated with 0.5 ml of overnight grown pre-inoculum with an $O.D_{600} \approx 1$ and incubated at 37 °C for 48 h [7]. Further optimization was performed on the solid substrate that produced the maximum PGase activity.

2.4.2. Enzyme production

After 48 h of incubation, 50 ml of 0.1 M Tris-HCl pH 9 buffer was added to the flasks and placed in a shaker incubator for 1 h at 100 rpm, followed by 30 min of static incubation at room temperature. The culture was filtered using filter paper before centrifugation at 10,000*g* for 10 min at 4 °C. The supernatant was collected and assayed for pectinase activity.

2.4.3. PGase enzyme assay

PGase enzyme activity was estimated using the DNS technique with citrus pectin as the substrate [9]. The experiment was carried out with 0.2 ml enzyme, 1.3 ml Tris-HCl pH 9 (optimal pH), and 0.5 ml 1% citrus pectin (Sigma-Aldrich). The reaction mixture was incubated at 45 °C (temperature optima) for 2 min before arresting the reaction with 1 ml DNS reagent. The samples were heated for 10 min, cooled, and the optical density was measured at 540 nm using a UV-spectrophotometer (Thermo Fischer). A standard curve of D-galacturonic acid monohydrate (Sigma-Aldrich) was used to quantify enzyme activity, and one unit of enzyme activity (U) was defined as the amount of PGase necessary to release 1 μmol of galacturonic acid per minute per gram of solid substrate utilized. The results were expressed as a relative activity using the following formula.

Relative Activity = Activity of the sample/Sample with maximum activity × 100.

2.5. Statistical optimization of PGase production

2.5.1. Plackett–Burman (PB) experimental design

Plackett-Burmann, a two-factorial experimental design was utilized to evaluate the media components affecting PGase production with wheat bran as the solid substrate. Based on bacterial growth metrics, literature review, and biochemical characteristics [9], eleven independent variables were chosen that included five physical (pH, temperature, moisture, incubation time, and inoculum size) and six nutritional (pectin, starch, $(NH_4)_2SO_4$, yeast extract KH_2PO4, $CoCl_2$) factors (Table 1). Design Expert STAT Ease software, version 11 (Minneapolis, USA), was used to build a random PB matrix with 13 experiments (Table 2), and the impact of each variable on enzyme production was examined at both its upper (+1) and lower limit (−1) by performing the enzyme assay. Significant variables ($p < 0.05$) that influenced PGase production were identified by regression analysis, and the accuracy of the statistical model was measured through the coefficient of determination (R^2).

2.5.2. Central composite rotatable design (CCRD)

According to the PB design, pH (A), incubation time (B), and inoculum size (C) had the utmost effect on PGase production. The CCRD (central composite rotatable design) of response surface methodology (RSM) was used to further analyze the optimal concentrations of the selected variables and the effect of their interactions on enzyme production. A 2^3 full factorial experimental design with the three specified variables at five coded levels (−α, −1, 0, +1, +α) was generated (Table 4). The RSM matrix consisted of twenty experiments with eight factorial, six axial, and six central runs (Table 5). The experiments were conducted and the increase in PGase

Table 1
Factors that influence the PGase production under study.

Codes	Names	Level	
		- 1	+ 1
A	pH	9	11
B	Temperature	30	40
C	Moisture (%)	65	85
D	Incubation Time (hrs)	24	72
E	Inoculum Size (%)	3	10
F	Pectin (%)	1	2
G	Starch (%)	3	5
H	$(NH_4)_2SO_4$ (%)	1	3
I	Yeast Extract (%)	1	2.5
J	KH_2PO_4 (%)	2	5
K	$COCl_2$ (mM)	5	15

Table 2
Placket-Burman Design for PGase media optimization: Plackett-Burman design in coded levels showing actual and predicted response as PGase activity.

Expt	Coded Factors											Actual Response (U/gm)	Predicted Response (U/gm)
	A	B	C	D	E	F	G	H	I	J	K		
1	+1	−1	+1	+1	+1	- 1	- 1	- 1	+1	- 1	+1	276.65	290.21
2	0	0	0	0	0	0	0	0	0	0	0	189.25	178.96
3	+1	+1	- 1	+1	+1	+1	- 1	- 1	- 1	+1	- 1	269.57	268.12
4	- 1	−1	- 1	- 1	- 1	- 1	- 1	- 1	- 1	- 1	- 1	92.92	89.8
5	- 1	−1	+1	- 1	+1	+1	- 1	+1	+1	+1	- 1	156.02	161.86
6	+1	+1	- 1	- 1	- 1	+1	- 1	+1	+1	- 1	+1	150.97	158.03
7	+1	−1	+1	+1	- 1	+1	+1	+1	- 1	- 1	- 1	226.30	218.15
8	- 1	−1	- 1	+1	- 1	+1	+1	- 1	+1	+1	+1	119	127.82
9	+1	+1	+1	- 1	- 1	- 1	+1	- 1	+1	+1	- 1	152.1	158.03
10	- 1	+1	+1	- 1	+1	+1	- 1	- 1	- 1	- 1	+1	138.52	139.76
11	- 1	+1	+1	+1	- 1	- 1	+1	+1	- 1	+1	+1	111.15	105.72
12	- 1	+1	- 1	+1	+1	- 1	+1	+1	+1	- 1	- 1	180.02	177.79
13	+1	+1	- 1	- 1	+1	- 1	+1	+1	- 1	+1	+1	264.02	252.19

production was estimated by enzyme assays. Using the same software, 3D plots of the response were generated, and a quadratic model was deduced.

$$Y = \alpha_0 + \alpha_1 A + \alpha_2 B + \alpha_3 C + \alpha_1 \alpha_1 A^2 + \alpha_2 \alpha_2 B^2 + \alpha_3 \alpha_3 C^2 + \alpha_1 \alpha_2 AB + \alpha_1 \alpha_3 AC + \alpha_2 \alpha_3 BC + \alpha_1 \alpha_2 \alpha_3 ABC$$

where Y is the PGase activity in U/gm of the solid substrate; A, B, and C are the three selected variables; α_0 is the intercept, α_1, α_2, and α3 are linear coefficients; $\alpha_1\alpha_1$, $\alpha_2\alpha_2$, $\alpha_3\alpha_3$ are the squared coefficients; and $\alpha_1\alpha_2$, $\alpha_1\alpha_3$, $\alpha_2\alpha_3$ are the interaction coefficients. Analysis of variance (ANOVA) was used to determine the significance of the coefficients, and the accuracy of the model was determined by comparing the predicted response to the actual response. Lastly, the model was validated by the determination coefficient (R^2) and the adjusted R^2.

2.5.3. RSM design validation

To validate the design, PGase production was attempted in RSM-optimized media (100 gm wheat bran, 65% moisture, and pH 10.5). The flasks were inoculated with 7.5% freshly prepared *Pseudomonas* sp. 13159349 inoculum and grown for 66 h at 37 °C. The crude enzyme was extracted and assayed for PGase activity.

2.6. Statistical analysis

The results of each experiment used in this study were performed in triplicate and reported as mean±standard deviation. The p-value significance ($p < 0.05$) was computed using One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test with IBM SPSS V25 software, and substantially different findings were represented by different alphabets.

3. Results and discussion

3.1. Isolation, screening, and identification of PGase-producing bacteria from fruit market soil samples

More than a hundred isolates were screened for alkalophilic polygalacturonase (PGase) producing bacterial strains from fruit market soil samples collected throughout Bangalore, India. The growth of alkalophilic bacteria was facilitated by culturing the samples on pH 9 media, and five isolates exhibiting a good hydrolytic zone were subsequently screened. The findings of the initial screening

Table 3
ANOVA for PB Design: Statistical significance of the PB design showing the factors influencing the PGase production.

Source	Sum of squares	DF	Mean Squares	F-Value	P-Value	
Model	45858.91	4	11464.73	123.77	**< 0.0001**	Significant
A-pH	24478.53	1	24478.53	264.26	**< 0.0001**	
B-Temperature	1464.79	1	1464.79	15.81	**0.0041**	
C-Incubation time	4336.94	1	4336.94	46.82	0.0001	
D-Inoculum size	15578.65	1	15578.65	168.18	**< 0.0001**	
Residual	741.06	8	92.63			
Core Total	46599.96	12				
Fit Statistics						
Std Deviation	9.62	Mean	178.96	CV%	5.38	
R^2	0.9841	Adjusted R^2	0.9761	Predicted R^2	0.9581	
Adequate precision	33.5763					

revealed P1 isolate showed the largest zone of hydrolysis (Fig. 1A). In addition, thin-layer chromatography confirmed the pectinolytic activity of the extracellularly secreted enzyme since the chromatogram of the reaction mixture indicated the release of galacturonic acid from the pectin substrate following enzyme treatment (Fig. 1B).

Based on morphological features and 16S rRNA molecular sequencing, the P1 strain was identified as *Pseudomonas* sp (Fig. 1C). Nucleotide blast and multiple sequence alignment revealed that the isolate was 99.34% similar to *Pseudomonas* sp. 13159349. The sequence was deposited in the NCBI Gene Bank with accession # OP159848.1. The enzyme secreted by this strain was further purified, and its biochemical characteristics were described in detail [9].

The majority of commercial PGases are derived from *Aspergillus* sp., however, they perform best in acidic environments and at low temperatures. Consequently, they are ineffective in bioprocesses requiring neutral or alkaline pH and high temperatures [5,20]. Studies have demonstrated that bacterial PGases can resist high pH and temperatures and offer advantages such as simple fermentation, low-cost media, and economical production [21,22]. Although *Bacillus* sp. is the most common producer of alkalophilic pectinases [6], several species of *Clostridium*, *Erwinia, Micrococcus, Streptomyces*, and *Pseudomonas* are also capable of making this enzyme [23]. Nonetheless, the only pectinase-producing *Pseudomonas* documented to date are *P. marginalis* CFBP1287 [24], *P.marginalis* MAFF 03-01173 [25], *P.syringeae pv glycinea* [26] and *P. fluorescence* [27]. Since research on extremophilic PGases from bacteria is limited, it is necessary to isolate such strains. *Pseudomonas* sp. 13159349 is a promising pectinolytic strain that produces a thermostable and highly alkalophilic PGase which can retain optimum activity even under harsh industrial conditions that can benefit industrial processes.

3.2. Utilization of agro-wastes as solid substrates for the production of PGase

To optimize PGase production, a two-step strategy was implemented. Initial attempts to improve enzyme production relied on solid substrates (without moisture) as fermentation medium (SSF), including sugarcane bagasse (SCB), wheat bran (WB), rice bran (RB), oatmeal (OM), corn cob (CC), and orange peel (OP). Agro wastes are recognized to have a high nutritious value with a high concentration of carbohydrates, proteins, vitamins, and minerals that promote the growth of microorganisms [28]. Previous studies have mostly reported using SSF for fungal enzyme production since the low water content support fungal growth while being unsuitable for the growth of bacteria. However, enough evidence shows the potential of SSF in bacterial enzyme production since the moisture content and many other parameters can be optimized for the high production of enzymes and other bioactive compounds [29–31].

Wheat bran media resulted in strong PGase activity (60.13 ± 3.39 U/gm) after 48 h incubation at 37 °C. The other substrates were not as effective since SCB yielded (47.25 ± 1.89 U/gm), OP (37.67 ± 0.87 U/gm), OM and RB produced (22.57 ± 0.80 U/gm, 24.43 ± 0.94 U/gm, respectively) (Fig. 2). The results reveal that solid substrate fermentation (SSF) yielded an overall higher PGase activity

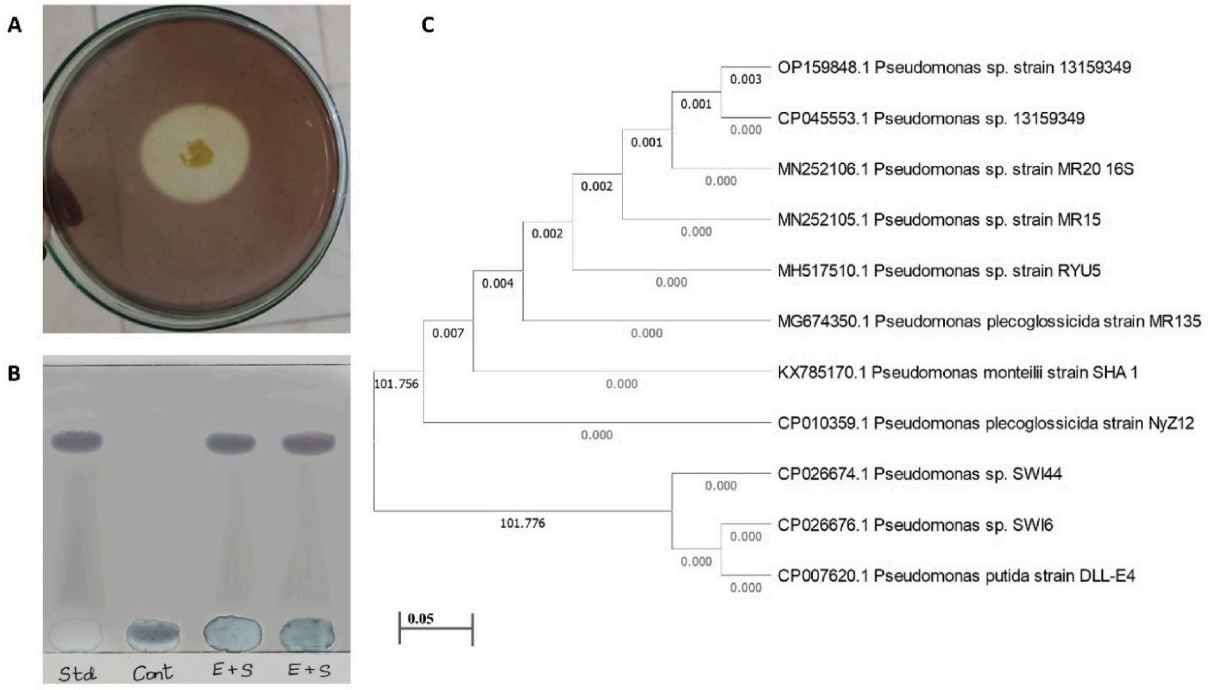

Fig. 1. Isolation and Identification of polygalacturonase-producing bacterial strain. (**A**) Positive Isolate exhibiting zone of hydrolysis (**B**) Thin layer chromatogram (TLC) showing the pectin degradation products confirming the PGase activity. Lane 1: Standard D-galacturonic acid; Lane 2: Control (without Enzyme) Lane 3: 5 μl reaction mixture containing enzyme (cell free supernatant) and pectin substrate, Lane 4: 10 μl reaction mixture. (C) Phylogenetic tree of the 16SrRNA gene sequences showing the relationships between *Pseudomonas* sp. 13159349 and other closely related strains.

compared to submerged fermentation (SMF) with PSAM broth containing 1% apple pectin (8 U/ml). Wheat bran's high lignocellulosic and pectin content makes it an ideal substrate for PGase synthesis without the need for any additional nutrients. Its small size and loose particle binding are known to improve air circulation, which is necessary for the efficient growth of microorganisms [32]. Wheat bran has been described as a promising substrate for the synthesis of PGase in previous researche, but these have all focused on *Bacillus* sp. strains such as; *Bacillus subtilis* strain Btk 27 [7], and *Bacillus megatherium* [33], *Bacillus* sp. MG-cp2 [34]; *Bacillus* sp. DT7 [10]. However, in this study, *Pseudomonas* sp. was successfully cultivated utilizing wheat bran for increased production of PGase.

3.3. Statistical optimization of media components for high PGase production

3.3.1. Screening of critical media components by PB design

Even though wheat bran alone increased enzyme production by 7.5 times, optimizing other media components in conjunction with wheat bran could further improve the enzyme bioprocess. Therefore, a statistical approach was used to screen and identify the media components that could further improve PGase yield.

Eleven variables were tested using 13 PB experiments, and the findings are summarized in Table 2. Plackett-Burman designs can screen numerous variables with a minimal number of experiments, reducing time, expense, and error. The maximum PGase activity (290.21– 218.15 U/gm) was recorded in runs 1, 3, and 11. In addition, the pareto plot and analysis of variance (ANOVA) identified pH ($p < 0.0001$), inoculum size ($p < 0.0001$) incubation time ($p < 0.0001$), and temperature (<0.0041), as the four most influential variables on PGase production (Table 3). Furthermore, the F-value of 123.77 and R^2 of 0.9841 implied that the model was highly significant with a good fit and could be used to navigate the design space.

A central composite rotatable design (CCRD) of RSM was used to further assess the optimum concentration/parameter of the chosen variables and the impact of their interactions on enzyme production.

3.3.2. Statistical optimization by response surface methodology (RSM)

The screening of variables using the PB design predicted that all four physical parameters, as opposed to nutritional factors, contributed considerably to PGase production. The most critical factors pH (A), the incubation period (B), and inoculum size (C) were further selected to investigate the individual influence and their interactions on PGase production through central composite rotatable design (CCRD) of RSM (Table 4). Although moisture and temperature were not significant variables, a steady amount of moisture (65%) in wheat bran and a temperature of 37 °C was necessary for effective enzyme production since moisture supports the growth and metabolism of the microorganisms [35], while the temperature can affect the function of cellular components [36].

The actual and predicted responses of the RSM statistical design are depicted in Table 5. The highest level of enzyme activity was recorded in run number 10, where actual activity (315.65 U/gm) was in close agreement with predicted activity (321.43 U/gm). ANOVA was used to assess the accuracy of the model which showed A, B, C, AB, AC, BC, and C^2 as significant model terms, while A^2 and B^2 as insignificant. Regression analysis revealed that the model is highly significant, with a p-value of <0.0001, an F-value of 95.33, and a low CV (coefficient of variation) of 2.31 (Table 6). Furthermore, the R^2 of 0.9901 suggested that the design could explain 99.01% of the results and was in good agreement with the predicted (0.9032) and adjusted R^2 (0.9803), indicating that the model was adequate for predicting PGase production. Based on the regression model, a second-order polynomial equation was derived to predict enzyme production.

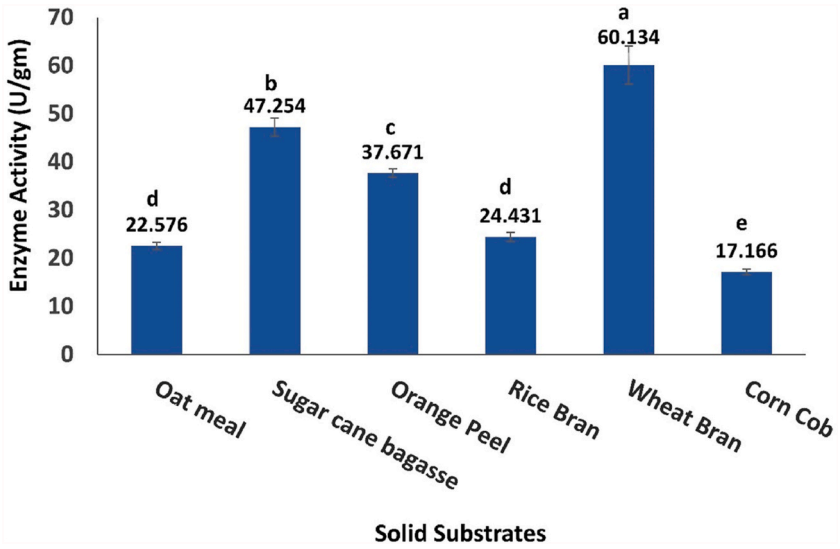

Fig. 2. Effect of solid substrate on PGase activity. The activity of cell free supernatant was assessed after *Pseudomonas* sp. 13159349 was cultured on different Agro wastes with 65% humidity for 48 h at 37 °C. Wheat bran proved to be the ideal substrate, with the good activity of 60.13 U/gm.

Table 4
Influencing factors: Ranges of selected variables for RSM analysis under solid substrate fermentation (SSF).

Factors	Codes				
	-α	**-1**	**0**	**+1**	**+α**
A: pH	10	10.5	11	11.5	11.84
B: Incubation Time (hrs)	61.9	66	72	78	82.09
C: Inoculum size (%)	1.59	5	10	15	18.40

Table 5
CCD – RSM design: Central composite rotatable design in coded levels showing actual and predicted response as PGase activity.

Expt	Codes			Actual Response (U/gm)	Predicted Response (U/gm)
	A	B	C		
1	+α	0	0	193.47	188.79
2	+1	+1	−1	163.25	163.22
3	0	0	0	248.70	252.62
4	−1	+1	+1	266.32	260.78
5	+1	−1	−1	235.45	240.21
6	0	-α	0	294.35	289.20
7	0	0	+α	211.47	213.35
8	0	0	-α	228.15	227.36
9	0	0	0	256.67	252.62
10	-α	0	0	315.65	321.41
11	0	0	0	258.50	252.62
12	0	0	0	251.02	252.62
13	+1	−1	+1	218.55	222.18
14	0	0	0	250	252.62
15	+1	+1	+1	186.62	185.47
16	−1	+1	−1	277.47	273.05
17	0	+α	0	210.42	216.62
18	−1	−1	+1	284.45	283.70
19	0	0	0	251.07	252.62
20	−1	−1	−1	308.60	308.97

Table 6
ANOVA for RSM: Statistical significance of the RSM affecting PGase production.

Source	Sum of squares	DF	Mean Squares	F-Value	P-Value	
Model	30812.5	10	3081.25	95.33	**< 0.0001**	Significant
A-pH	21229.63	1	21229.63	617.61	**< 0.0001**	
B-Incubation time	6352.52	1	6352.52	184.80	**< 0.0001**	
C-Inoculum size	236.92	1	236.92	10.94	**0.0241**	
AB	376.34	1	376.34	6.34	**0.0077**	
AC	218.09	1	218.09	7.20	**0.0288**	
BC	354.71	1	354.71	10.31	**0.0090**	
A^2	11.06	1	11.06	0.0960	0.5729	
B^2	0.1654	1	0.165	0.0354	0.9445	
C^2	1876.13	1	1876.13	58.45	**< 0.0001**	
ABC	92.96	1	92.95	2.70	0.1241	
Residual	290.87	9	32.21			
Lack of Fit	212.71	4	53.17	2.77	0.1059	Not significant
Pure Error	78.16	5	15.63			
Core Total	31103.38	19				
Std. Dev	5.68	**Mean**	245.50	**C.V %**	2.31	
R^2	0.9906	**Adjusted R^2**	0.9803	**Predicted R^2**	0.9032	
Adeq Precision	34.373					

PGase activity (U/gm) $Y = +252.62 - 39.42\,A - 21.56\,B - 4.16C - 6.85\,AB + 5.22\,AC + 6.65\,BC + 0.876\,A^2 - 0.107\,B^2 - 11.40C^2 + 3.40\,ABC$.

Production of the enzyme is affected not only by the individual effects of each variable but also by their interaction and the optimal concentration. 3D surface plots and 2D contour plots are convenient tools to better understand the design space governed by these factors [37]. Two variables were utilized to construct the plots, while the third variable remained constant. The 3D and contour plots in Fig. 3 (A and B) reveal a moderately significant interaction of variables A (pH) and B (incubation time), with modest curvature ($p < 0.0077$), indicating that pH 10.5 and incubation period between 61 and 66 h are favorable for enzyme yield but increasing pH and

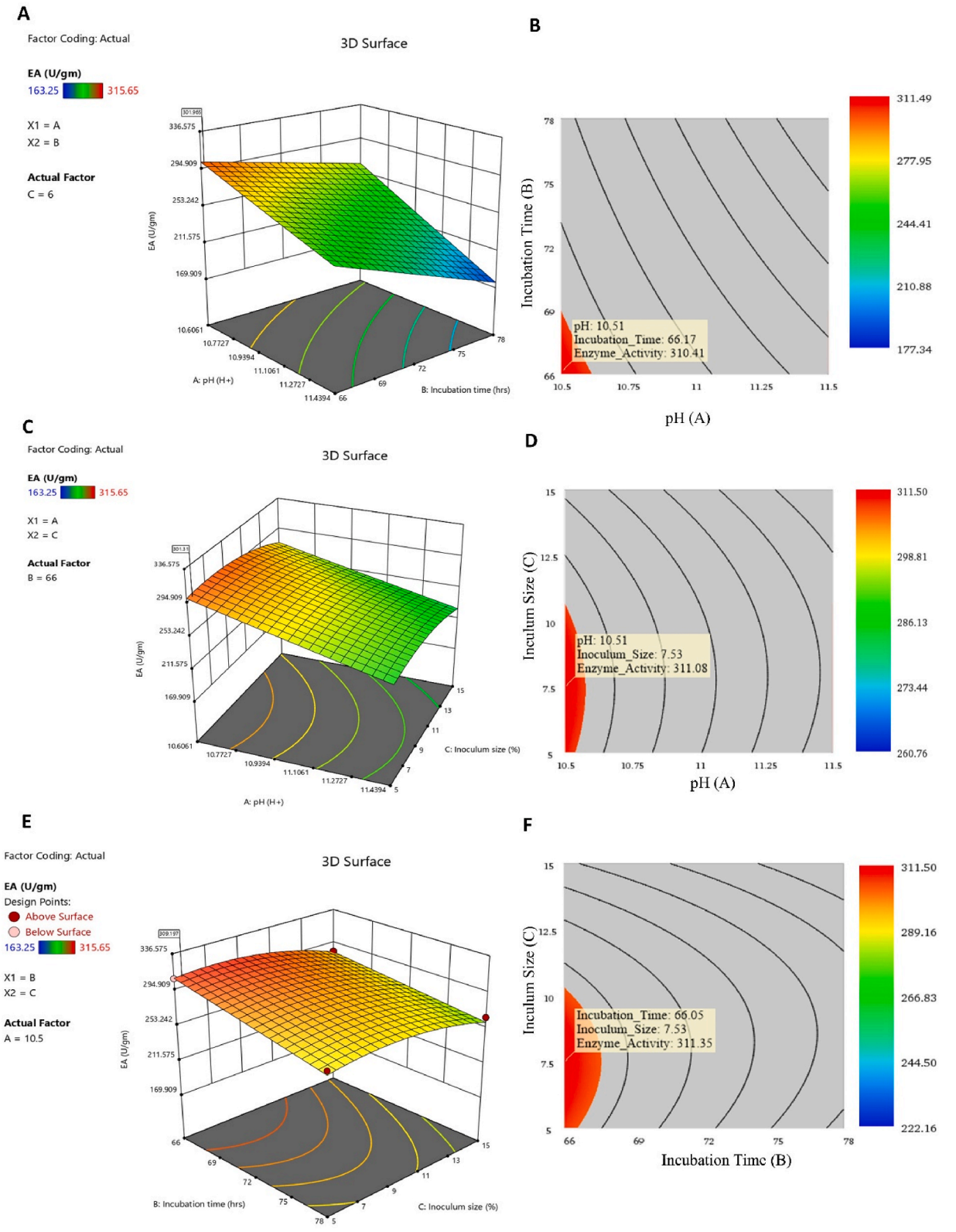

Fig. 3. Response surface plots: 3D surface plots and design space of the critical factors (pH, incubation time, inoculum size) on extracellular production of pectinase by SSF. **A,B** represent plots for pH and incubation time **C,D** show plots for pH and incubation size. **E,F** plots are for incubation time and inoculum size. Design Space shows the maximum enzyme production is at pH 10.5, 66 h incubation time, 6–7.5% inoculum.

incubation time negatively affected the productivity. The interactions between variables A (pH) and C (inoculum size) were likewise significant with good curvature ($p < 0.0241$) (Fig. 3C and D), revealing 6–7.5% of inoculum as a good starter culture for enzyme production at pH 10.5. Interactions between B (incubation time) and C (inoculum size) were found to be most pronounced, as demonstrated by the maximum curvature ($p < 0.0090$) (Fig. 3 E and F). Thus, the study revealed that the optimal design space for maximizing PGase production was pH 10.5, 61–66 h of incubation time, and 6–7.5% inoculum size.

Statistical optimization is a relatively new and effective concept. It is being increasingly applied to maximize enzyme production. There have been many attempts to optimize medium components to boost pectinase enzyme production from various bacterial and fungal strains, with varying degrees of success; however, comparing these studies is difficult because of differences in the growth parameters of the chosen microorganisms, independent factors, and experimental methodology. Nonetheless, few studies statistically optimized pectinase production, such as *Bacillus* sp. Y1 was shown to produce 40 U/ml [38], *Bacillus subtilis PB1* produced 19.50 U/ml [39], *Bacillus pumilis* Dscr1 gave 20,184 U/L [40], and *Bacillus subtilis* exhibited 4.84 U/ml of pectinase production [41]. However, the same design strategy helped in the production of 734.11 U/ml alkaline pectinase from *Bacillus subtilis* ZGL14 [8] in submerged fermentation using starch, peptone, and potassium salts. Similarly, utilizing lactose, tryptone, NH_4SO_4, $MnSO_4$, $MgSO_4$ and NaCl as optimization factors, *Bacillus* ZJ1407 produced 737.61 U/ml pectinase [42]. Recent research by Abdollahzadeh R et al. also enhanced pectinase (14.16 U/mg) production by *Enterobacter* sp. MF84 using NH_4Cl, KH_2PO_4, pectin, and incubation time as the significant factors [43]. Despite the high levels of pectinase production in these studies, this investigation solely employed agricultural waste without additional supplements. The optimization of the physical parameters enhanced enzyme production several folds, making this a highly cost-effective bioprocess.

3.3.3. Validation of the RSM model

The experimental model was validated by evaluating laboratory-scale production of PGase enzyme under RSM-optimized conditions. The enzyme activity ($n = 3$) was found to be (30600 ± 400.32 U/100 gm) which was comparable to the response predicted by the regression model, thus substantiating the model.

4. Conclusion

This study established the isolation and identification of *Pseudomonas* sp. 13156349, a potent alkalophilic and thermostable PGase-producing bacterial strain isolated from soil. Furthermore, the utilization of solid substrate fermentation (SSF) and statistical optimization using PB and RSM design of experiment assisted in significantly improving enzyme production. Wheat bran with a pH of 10.5, 65% moisture, an inoculum size of 6–7.5%, and incubation at 37 °C for 61–66 h resulted in a 5.2-fold increase in enzyme production. These findings highlight the importance of agro wastes and statistical designs in optimizing bioprocesses, which are beneficial to both the biotechnology industry and the environment as a whole due to the time and money saved in the production and reuse of agro wastes respectively.

Author contribution statement

Mary Arpana: Performed the experiments and ; Wrote the paper.
Seema S Rathore: Analyzed and interpreted the data.
Archana S Rao, Ajay Nair: Performed the experiments.
Sunil S More: Contributed reagents, materials, analysis tools or data; Wrote the paper.
Aneesa Fasim: Conceived and designed the experiments, Analyzed and interpreted the data; ; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of competing interest

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

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References

[1] C. Xiao, C.T. Anderson, Roles of pectin in biomass yield and processing for biofuels, Front. Plant Sci. 4 (2013) 67, https://doi.org/10.3389/fpls.2013.00067.
[2] G. Garg, A. Singh, A. Kaur, R. Singh, J. Kaur, R. Mahajan, Microbial pectinases: an ecofriendly tool of nature for industries, 3 Biotech 6 (2016) 47, https://doi.org/10.1007/s13205-016-0371-4.
[3] A. Radha, R. Sneha, R. Kiruthiga, P. Priyadharshini, N. Prabhu, A review on production of polygalacturonase using various organisms and its applications, Asian Journal of Biotechnology and Bioresource Technology (2019) 1–12, https://doi.org/10.9734/ajb2t/2019/v5i330063.
[4] J. Zeni, K. Cence, C.E. Grando, L. Tiggermann, R. Colet, L.A. Lerin, R.L. Cansian, G. Toniazzo, D. de Oliveira, E. Valduga, Screening of pectinase-producing microorganisms with polygalacturonase activity, Appl. Biochem. Biotechnol. 163 (2011) 383–392, https://doi.org/10.1007/s12010-010-9046-5.
[5] M.V.V. de Andrade, A.B. Delatorre, S.A. Ladeira, M.L.L. Martins, Production and partial characterization of alkaline polygalacturonase secreted by thermophilic Bacillus sp. SMIA-2 under submerged culture using pectin and corn steep liquor, Food Sci. Technol. 31 (2011) 204–208, https://doi.org/10.1590/S0101-20612011000100031.
[6] B. Kavuthodi, D. Sebastian, Review on bacterial production of alkaline pectinase with special emphasis on Bacillus species, Biosci. Biotech. Res. Comm. 11 (2018) 18–30, https://doi.org/10.21786/bbrc/11.1/4.
[7] O.J. Oumer, D. Abate, Characterization of pectinase from Bacillus subtilis strain Btk 27 and its potential application in removal of mucilage from coffee beans, Enzym. Res. (2017), e7686904, https://doi.org/10.1155/2017/7686904.
[8] P. Yu, Y. Zhang, D. Gu, Production optimization of a heat-tolerant alkaline pectinase from Bacillus subtilis ZGL14 and its purification and characterization, Bioengineered 8 (2017) 613–623, https://doi.org/10.1080/21655979.2017.1292188.
[9] M. Arpana, A. Fasim, H.K. Manjushree, S.S. More, An alkalophilic and thermostable polygalacturonase (PGase) from Pseudomonas sp. 13159349: purification, biochemical characterization and its efficacy in olive oil extraction, Biologia (2023), https://doi.org/10.1007/s11756-022-01310-5.
[10] D.R. Kashyap, S.K. Soni, R. Tewari, Enhanced production of pectinase by Bacillus sp. DT7 using solid state fermentation, Bioresour. Technol. 88 (2003) 251–254, https://doi.org/10.1016/S0960-8524(02)00206-7.
[11] R.C. Kuhad, M. Kapoor, R. Rustagi, Enhanced production of an alkaline pectinase from Streptomyces sp. RCK-SC by whole-cell immobilization and solid-state cultivation, World J. Microbiol. Biotechnol. 20 (2004) 257–263, https://doi.org/10.1023/B:WIBI.0000023833.15866.45.
[12] M.A. Lizardi-Jiménez, R. Hernández-Martínez, Solid state fermentation (SSF): diversity of applications to valorize waste and biomass, Biotech 7 (2017), https://doi.org/10.1007/s13205-017-0692-y.
[13] N. Martin, S.R. de Souza, R. da Silva, E. Gomes, Pectinase production by fungal strains in solid-state fermentation using agro-industrial bioproduct, Braz. Arch. Biol. Technol. 47 (2004) 813–819, https://doi.org/10.1590/S1516-89132004000500018.
[14] C.R. Soccol, E.S.F. da Costa, L.A.J. Letti, S.G. Karp, A.L. Woiciechowski, L.P. de S. Vandenberghe, Recent developments and innovations in solid state fermentation, Biotechnology Research and Innovation 1 (2017) 52–71, https://doi.org/10.1016/j.biori.2017.01.002.
[15] V. Singh, S. Haque, R. Niwas, A. Srivastava, M. Pasupuleti, C.K.M. Tripathi, Strategies for fermentation medium optimization: an in-depth review, Front. Microbiol. 7 (2016) 2087, https://doi.org/10.3389/fmicb.2016.02087.
[16] A. Fasim, V.S. More, S.S. More, Large-scale production of enzymes for biotechnology uses, Curr. Opin. Biotechnol. 69 (2021) 68–76, https://doi.org/10.1016/j.copbio.2020.12.002.
[17] R.L. Plackett, J.P. Burman, The design of optimum multifactorial experiments, Biometrika 33 (1946) 305–325, https://doi.org/10.1093/biomet/33.4.305.
[18] G.E.P. Box, K.B. Wilson, On the experimental attainment of optimum conditions, J. Roy. Stat. Soc. B 13 (1951) 1–38, https://doi.org/10.1111/j.2517-6161.1951.tb00067.x.
[19] S. Farag, Separation and analysis of some sugars by using thin layer chromatography, JSBR 20 (1979) 251–254, https://doi.org/10.5274/jsbr.20.3.251.
[20] G. Hoondal, R. Tiwari, R. Tewari, N. Dahiya, Q. Beg, Microbial alkaline pectinases and their industrial applications: a review, Appl. Microbiol. Biotechnol. 59 (2002) 409–418, https://doi.org/10.1007/s00253-002-1061-1.
[21] M. Joshi, M. Nerurkar, R. Adivarekar, Characterization, kinetic, and thermodynamic studies of marine pectinase from Bacillus subtilis, Prep. Biochem. Biotechnol. 45 (2015) 205–220, https://doi.org/10.1080/10826068.2014.907181.
[22] R. Singh, M. Kumar, A. Mittal, P.K. Mehta, Microbial enzymes: industrial progress in 21st century, 3 Biotech 6 (2016) 174, https://doi.org/10.1007/s13205-016-0485-8.
[23] O.J. Oumer, D. Abate, Comparative studies of pectinase production by Bacillus subtilis strain Btk 27 in submerged and solid-state fermentations, BioMed Res. Int. (2018), e1514795, https://doi.org/10.1155/2018/1514795.
[24] J.M. Membré, P.M. Burlot, Effects of temperature, pH, and NaCl on growth and pectinolytic activity of Pseudomonas marginalis, Appl. Environ. Microbiol. 60 (1994) 2017–2022, https://doi.org/10.1128/aem.60.6.2017-2022.1994.
[25] K. Hayashi, Y. Inoue, M. Shiga, S. Sato, R. Takano, K. Hirayae, T. Hibi, S. Hara, Pectinolytic enzymes from Pseudomonas marginalis MAFF 03-01173, Phytochemistry 45 (1997) 1359–1363, https://doi.org/10.1016/s0031-9422(97)00191-x.
[26] P. Magro, L. Varvaro, G. Chilosi, C. Avanzo, G.M. Balestra, Pectolytic enzymes produced by Pseudomonas syringae pv. glycinea, FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 117 (1994) 1–5, https://doi.org/10.1111/j.1574-6968.1994.tb06733.x.
[27] C.C. Sakiyama, E.M. Paula, P.C. Pereira, A.C. Borges, D.O. Silva, Characterization of pectin lyase produced by an endophytic strain isolated from coffee cherries, Lett. Appl. Microbiol. 33 (2001) 117–121, https://doi.org/10.1046/j.1472-765x.2001.00961.x.
[28] P.K. Sadh, S. Duhan, J.S. Duhan, Agro-industrial wastes and their utilization using solid state fermentation: a review, Bioresources and Bioprocessing 5 (2018) 1, https://doi.org/10.1186/s40643-017-0187-z.
[29] A. Nighojkar, M.K. Patidar, S. Nighojkar, Pectinases: production and applications for fruit juice beverages, in: Processing and Sustainability of Beverages, Elsevier, 2019, pp. 235–273, https://doi.org/10.1016/B978-0-12-815259-1.00008-2.
[30] L. Yafetto, Application of solid-state fermentation by microbial biotechnology for bioprocessing of agro-industrial wastes from 1970 to 2020: a review and bibliometric analysis, Heliyon 8 (2022), e09173, https://doi.org/10.1016/j.heliyon.2022.e09173.
[31] A. Pandey, C.R. Soccol, D. Mitchell, New developments in solid state fermentation: I-bioprocesses and products, Process Biochemistry 35 (2000) 1153–1169, https://doi.org/10.1016/S0032-9592(00)00152-7.
[32] S. Malathi, R. Chakraborty, Production of alkaline protease by a new Aspergillus flavus isolate under solid-substrate fermentation conditions for use as a depilation agent, Appl. Environ. Microbiol. 57 (1991) 712–716, https://doi.org/10.1128/aem.57.3.712-716.1991.
[33] R.M. El-Shishtawy, S.A. Mohamed, A.M. Asiri, A.M. Gomaa, I.H. Ibrahim, H.A. Al-Talhi, Solid fermentation of wheat bran for hydrolytic enzymes production and saccharification content by a local isolate Bacillus megatherium, BMC Biotechnol. 14 (2014) 29, https://doi.org/10.1186/1472-6750-14-29.
[34] M. Kapoor, R.C. Kuhad, Improved polygalacturonase production from Bacillus sp. MG-cp-2 under submerged (SmF) and solid state (SSF) fermentation, Lett. Appl. Microbiol. 34 (2002) 317–322, https://doi.org/10.1046/j.1472-765x.2002.01107.x.
[35] L. Thomas, C. Larroche, A. Pandey, Current developments in solid-state fermentation, Biochem. Eng. J. 81 (2013) 146–161, https://doi.org/10.1016/j.bej.2013.10.013.
[36] D.B. Nedwell, Effect of low temperature on microbial growth: lowered affinity for substrates limits growth at low temperature, FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 30 (1999) 101–111, https://doi.org/10.1111/j.1574-6941.1999.tb00639.x.
[37] S.B. Jhample, P.K. Bhagwat, P.B. Dandge, Statistical media optimization for enhanced production of fibrinolytic enzyme from newly isolated Proteus penneri SP-20, Biocatal. Agric. Biotechnol. 4 (2015) 370–379, https://doi.org/10.1016/j.bcab.2015.05.006.
[38] F. Guo, X. Li, J. Zhao, G. Li, P. Gao, X. Han, Optimizing culture conditions by statistical approach to enhance production of pectinase from *Bacillus* sp, Y1, BioMed Research International (2019), e8146948, https://doi.org/10.1155/2019/8146948.
[39] M. Zhou, J. Wu, T. Wang, L. Gao, H. Yin, X. Lü, The purification and characterization of a novel alkali-stable pectate lyase produced by Bacillus subtilis PB1, World J. Microbiol. Biotechnol. 33 (2017) 190, https://doi.org/10.1007/s11274-017-2357-8.

[40] D.C. Sharma, T. Satyanarayana, A marked enhancement in the production of a highly alkaline and thermostable pectinase by Bacillus pumilus dcsr1 in submerged fermentation by using statistical methods, Bioresour. Technol. 97 (2006) 727–733, https://doi.org/10.1016/j.biortech.2005.04.012.
[41] S. Uzuner, D. Cekmecelioglu, Enhanced pectinase production by optimizing fermentation conditions of Bacillus subtilis growing on hazelnut shell hydrolyzate, J. Mol. Catal. B Enzym. 113 (2015) 62–67, https://doi.org/10.1016/j.molcatb.2015.01.003.
[42] P. Yu, C. Xu, Production optimization, purification and characterization of a heat-tolerant acidic pectinase from Bacillus sp. ZJ1407, Int. J. Biol. Macromol. 108 (2018) 972–980, https://doi.org/10.1016/j.ijbiomac.2017.11.012.
[43] R. Abdollahzadeh, M. Pazhang, S. Najavand, V. Fallahzadeh-Mamaghani, A.R. Amani-Ghadim, Screening of pectinase-producing bacteria from farmlands and optimization of enzyme production from selected strain by RSM, Folia Microbiol. 65 (2020) 705–719, https://doi.org/10.1007/s12223-020-00776-7.