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Effect of Fermentation Response on Biosynthesis of Endopolygalacturonase from a Potent Strain of *Bacillus* by Utilizing Polymeric Substrates of Agricultural Origin

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Abstract: Endopolygalacturonase (EndoPGase), EC: 3.2.1.15. is one of the crucial pectinases belonging to the class of carbohydrase. The catalytic action of EndoPGase captivates the attention of the production of this extremely valuable catalyst in the industrial sector. The main focus was to ascertain a potential bacterial candidate for endoPGase production. The isolated bacterial strain was further identified by 16S rRNA gene sequencing. The parameters for enzyme biosynthesis were optimized by a single and multiple factor approach at a time. The results of our investigation led to the identification of a potent strain of *Bacillus subtilis* NR2 [strain 168]. The sequence of 16S rRNA amplified from the isolated bacterium has been submitted to GenBank under accession number ON738697. The strain was found active for pectic enzyme activity under shaking- flask fermentation at pH 5.0 and 50 °C temperature of incubation. Among all monomeric and polymeric substrates (inducer-substrates), citrus pectin, followed by potato starch and pectin (Sigma) were considered the best enzyme inducers at 1% concentration. In comparison, an increased wheat bran concentration at 5% was proved to be ideal for EndoPGase biosynthesis Moreover, an increasing trend in enzyme activity was observed with the increasing concentration of inducer. The combined effect of three variables (pH, inducer-substrates, and inducer-substrate concentration) was explored by response surface methodology (RSM) involving a Box–Behnken design (BBD). Based on the results, we concluded that the soil-isolated *B. subtilis* can be effectively utilized for commercial-scale pectinase enzyme biosynthesis.

Keywords: endopolygalacturonase; biocatalysts; biosynthesis; heteropolysaccharide; inducer; submerged fermentation

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1. Introduction

The word pectinase refers to various heterotypic enzymes of the related group comprising pectin depolymerases or polygalacturonases (PG), polymethylgalacturonases (PMG), transeliminases or lyases, and pectin esterases (PE) also known as pectin methyl esterases (PME). These enzymes can cleave the glycosidic bonds and transform or debranch pectin, the most abundant constituent of fruits [1]. Pectin is an acidic heteropolysaccharide containing galacturonic acid units in which carboxyl groups are esterified with methanol. The heteropolysaccharide-based pectin is the most important component in fruits, vegetables,

and cereals. Pectic substances are high molecular weight, non-toxic anionic natural polysaccharides, biocompatible, and principal constituents found in the middle lamella of the cell wall of plants [2].

Pectinolytic enzymes being heterogeneous in nature are used to hydrolyze pectic substrates and therefore have gained high interest in various industrial sectors such as the food, textile, and paper industries [3]. In food industries, pectinases have decisive roles in juice extraction from fruits and wine clarification; cocoa, tea, and coffee fermentation and concentration; vegetable oil extraction; pickling, and preparation of jams and jellies [4,5]. Additionally, these enzymes are used in; pulp and paper industries for oil extraction, bleaching of paper, retting, and degumming of plant fibers, bio-scouring of cotton, treatment of pectic wastewater from juice industries, poultry feed additive, protoplast fusion technology, and production of bioenergy [5,6].

The primary production sources of pectinase enzymes are microbes and plants. However, because of commercial and technical practicability, the production of pectic enzymes from microbial sources is becoming central in research considerations [7,8]. The extensive utilization and efficiency of microbial pectic biocatalysts in various production industries has significantly increased its global demand. Pectinases from microbial sources are frequently used because of their convenient production procedure and distinctive physicochemical properties [9]. Previously, researchers have reported that pectinic enzymes from the microbial source account for about 25% of the global sale of food and industrial enzymes, and their market is constantly increasing [10]. Predominantly, enzymes embrace an engrained global market which was predicted to reach USD 6.3 billion in 2021 [11].

It has been publicized that the fermentation conditions and inducer-substrate composition significantly impact the biosynthesis of pectinase enzymes using microbes [12,13]. Hence, it is a well-established fact now that pectinolytic enzyme production is affected by the fermentation environment and the carbon, nitrogen, and mineral salts. Therefore, the nutritional composition of the fermentation medium is a critical aspect of industrial-scale enzyme production since one-third of the enzyme production cost has primarily been found associated with the expenditure of culture medium [14]. Therefore, choosing appropriate medium components and understanding components' interaction is critical. Moreover, compositional optimization of the medium is also an essential factor, which supports minimalizing production costs and enhancing the actual yield [15].

The shake flask cultivation scheme has been extensively employed mainly in enzyme production; however, the statistical design of experiments [16] may vary to analyze the biotechnological parameters to calibrate the production efficiency.

During the past decades, industrial process optimization was achieved by applying statistical tools to reduce production costs. The process optimization usually begins with identifying the variables which significantly affect enzyme production, followed by optimization of their concentration. However, the methodology of one-factor-at-a-time for culture medium optimization has some limitations in accuracy because of interaction among the variables [17]. Therefore, statistical strategies of the multiple-factor-at-a-time approach provide economically efficient alternatives for studying the interaction between variables or factors.

Therefore, in the present investigation, a potent bacterial strain of *Bacillus subtilis* was isolated from the soil for endopolygalacturonase production. The isolated strain was further characterized to explore its status as a potential candidate for pectinolytic enzyme production on a commercial scale. The submerged-fermentation medium conditions were optimized using available natural and synthesized polymeric inducer-substrate to release the endoPGase by employing the multiple-factors-at-a-time approach for the first time.

2. Results

2.1. Isolation, Identification and Screening of Bacillus subtilis

Initially, three bacterial strains (named: NR1, NR2, and NR3) isolated on pectin-containing-medium were screened using pectinase screening agar plates, followed by the

appearance of a yellow-colored halo of pectin depolymerization. The pure culture of NR2 shown in Figure 1a–c exhibited a large yellow zone around the colony after staining with iodine and was selected for further experimental work. The culture was identified as *B. subtilis* based on morpho-molecular characterization. Potential pectinolytic bacterial, actinomycete, and fungal species have been screened and identified molecularly by sequencing 16Sr RNA gene amplification. Seventy percent of isolates were confirmed to be *Bacillus* strains, and among all bacterial species, two isolates were identified as *B. subtilis* and previous findings coincided with our results [11]. Our current results of isolation and screening agree with previous reports on bacterial strains' isolation for pectinolytic activity [18–22].

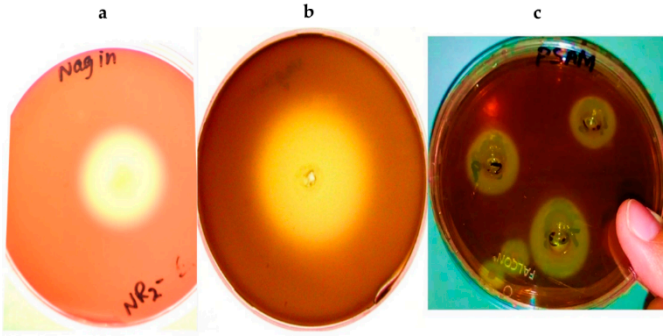

Figure 1. (**a**) Screening of pectinolytic *B. subtilis* isolate using PSAM (pectinase screening agar medium) containing 0.5% citrus pectin from Sigma. After inoculation, the plate was incubated overnight at 37 °C, flooded with 0.5 mM iodine solution for 20 min, and rinsed with water; (**b**) Selected colony based on the large halo of pectin depolymerization; (**c**) well plate assay of endoPGase enzyme on PSAM.

2.1.1. Scanning Electron Microscopy and Transmission Electron Microscopy of Isolated Bacillus Strain

Analysis by SEM exposed that the bacterium was rod-shaped bacillus having a size of ~2.2 µm as shown in Figure 2a. Moreover, transmission electron microscopy revealed that the bacterium was rod-shaped with a size of 1.4 µm which revealed that the culture might contain both short and long rods. The ultra-thin section, about 1.4 µm in size, of positively screened *Bacillus* isolate is shown in Figure 2b. Our results concord with a previously reported study by Kumari and co-authors [23], who showed the size of *B. subtilis* around 2–3 µm. Microbial and biochemical analysis [24] showed that the microbe could produce pectinolytic enzymes and was thus referred to be *Bacillus*. Previously, El-Sayed [18] reported the isolation and screening of pectinase-producing *Bacillus* sp. The microbial strain they isolated was identified as *B. subtilis* because of physiological, morphological, biochemical, and molecular characterization which supported our current identification.

All these reporters isolated *Bacillus* (most commonly *B. subtilis*) and screened for pectinolytic enzyme activity using pectinase screening agar medium. Another previous study revealed that *B. subtilis* was isolated from soil and screened for polygalacturonase production using a plate assay [25]. The strain was then subjected to colony PCR for 16S rRNA analysis and further confirmation was performed by sequencing. Our results agree with the study of Nawawi et al. [26], in which a potential xylanopectinolytic *B. subtilis* strain was reported. The strain isolated from compost based on zone formation on pectin-agar-medium was screened for enzyme production. The 16S rRNA gene sequencing data revealed that the strain culture belonged to the genus *Bacillus* and was finally identified as *B. subtilis*. Kaur and Gupta [27] also reported similar results for isolation and 16S rRNA identification of bacterial strain *B. subtilis* for pectinase production coinciding with our present observations. Similarly, Adeyefa and Ebuehi [28] also reported the production of pectinase enzyme by *B. subtilis*, strongly supporting our findings. Some novel pectinolytic bacteria were also screened and identified by gene sequencing by Shrestha et al. [29] previously.

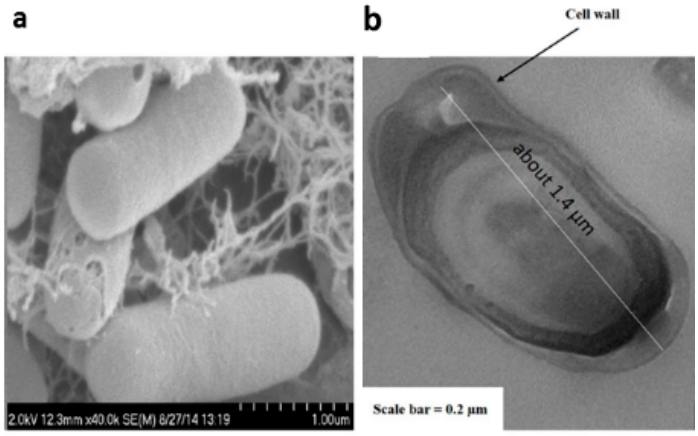

Figure 2. (**a**) Scanning electron micrograph of the *Bacillus* isolate. (**b**) Transmission electron micrograph of positively screened *Bacillus* isolate.

2.1.2. Molecular Identification of the isolated strains

The molecular identification of bacterial isolate from soil samples was carried out based on 16S rRNA sequencing data analysis. The products of PCR were first purified and then sequenced. The acquired sequence data were analyzed using NCBI BLAST, and the likely microorganisms were identified following Oumer and Abate [11]. The 16S rRNA sequence of the amplified PCR product was about 1438 bp long. Comparative analysis of the 16S rRNA sequence from the newly isolated strain with the GenBank database sequences showed 99% similarity with the equivalent sequences from the *B. subtilis* species. According to Bergey's Bacterial Identification Manual and 16S rRNA results [30], the bacterium is *B. Subtilis* strain 168. The 16S rRNA sequence of the isolated bacterium was submitted to GenBank under accession number ON738697.

2.1.3. Evolutionary Relationships of Taxa

The evolutionary history was inferred using the neighbor-joining method reported by Saito and Nei [31]. The bootstrap consensus tree inferred from 500 replicates [32] was taken to represent the evolutionary history of the taxa analyzed [32]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) were shown next to the branches [32]. The evolutionary distances were computed using the maximum composite likelihood method [33] and were in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. All ambiguous positions were removed for each sequence pair using the pairwise deletion option. There were a total of 1552 positions in the final dataset. Evolutionary analyses were conducted in MEGA 11 following Tamura [34]. The phylogenetic tree based on the 16S rRNA sequences alignment among the isolated *Bacillus* and the related *Bacillus* strains is presented in Figure 3.

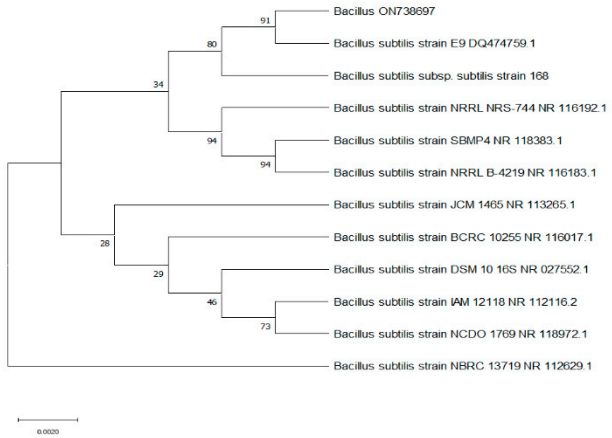

Figure 3. Phylogenetic tree showing evolutionary relationship based on the 16S rRNA sequences alignment among the isolated *Bacillus* strain GenBank accession ON738697 with the other related *Bacillus subtilis* strains: *Bacillus subtilis* subsp. *subtilis* strain 168 16S (NR_102783.2); *Bacillus subtilis* strain DSM 10 (NR_027552.1); *Bacillus subtilis* strain JCM 1465 (NR_113265.1); *Bacillus subtilis* strain NBRC 13719 (NR_112629.1); *Bacillus subtilis* strain IAM 12118 (NR_112116.2); *Bacillus subtilis* strain BCRC 10255 (NR_116017.1); *Bacillus subtilis* strain SBMP4 (NR_118383.1); *Bacillus subtilis* strain NCDO 1769 (NR_118972.1); *Bacillus subtilis* strain NRRL NRS-744 (NR_116192.1); *Bacillus subtilis* strain NRRL B-4219 (NR_116183.1); *Bacillus subtilis* strain E9 (DQ474759.1).

2.1.4. Partial Purification and SDS-PAGE Analysis

The molecular weight of purified endoPGase from the isolated *Bacillus* revealed by SDS-PAGE is shown in Figure 4. It showed a molecular weight of 47 kDa in the gel, which has been well reported earlier and thus confirmed the expressional product of the EndoPGase genes. Fratebianchi et al. [35] have also presented a molecular weight of 47 kDa for an endoPGase isolated from a fungal strain of *Aspergillus sojae.* However, endoPGase from *B. tequilensis* CAS-MEI-2-33 was 45.4 kDa, reported by Zhang et al. [36], whereas it was 43.0 kDa, as observed by Anand et al. [37]. The molecular mass of PGase enzymes ranged between 37 kDa and 66 kDa from *Bacillus* strains [38]. Similar findings were reported by Nazir et al. [39] who investigated a protein of 47 kDa by SDS-PAGE analysis while screening grapes for polygalacturonases. Another study reported a molecular mass of 48 kDa of pectinase enzyme from *A. niger*, revealing a near consensus with our findings [40].

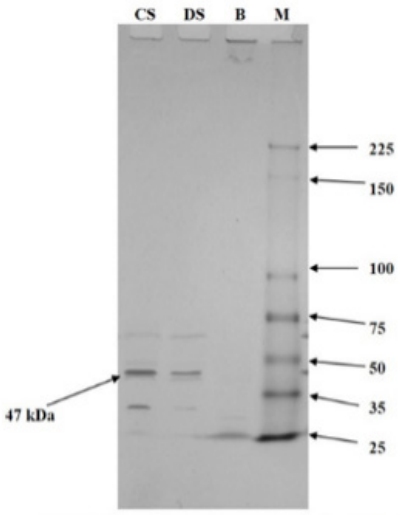

Figure 4. Molecular identification of partially purified endoPGase by SDS-PAGE. Where: M (marker), B (blank) DS (diluted enzyme sample) and CS (concentrated enzyme sample), respectively.

2.2. Standardization of Submerged Bioprocess Parameters

2.2.1. Optimization of Initial pH of the Fermentation Medium

The synthesis of enzyme metabolites is affected by the initial pH of the fermentation medium, which affects the growth of microorganisms. The effect of pH was studied by culturing *B. subtilis* in growth medium having a variable pH range (4.0–10.0), and it was found that *B. subtilis* could produce PGase on a wide range of pH. The maximum endoPGase activity (68.22 IU/mL) was observed at pH 5.0, shown in Figure 5a,b, and no significant decrease in enzyme units was found with variation in pH from the optimal level. In contrast, any variation in pH towards extreme acidic or alkaline resulted in a 50% decrease in endo-PGase activity. pH is crucial to regulate the synthesis of extracellular enzymes by microorganisms and the growth of microbes. An acidophilic *B. subtilis* SAV-21 was reported by Kaur and Gupta [27]. They described that the decrease in enzyme units at pH values above the optimal might be because of a reduction in the growth of the bacillus strain. Though the maximal enzyme production was experiential at pH 4.0, the isolated PGase enzyme was found active over a varied pH range. Our study also presented a similar pattern of activity decline. Our results also concord with the finding of Adeyefa and Ebuehi [28], who reported maximal pectinase enzyme production at pH 5.00 by using *B. subtilis* and *Aspergillus niger* separately as culture microbes. Previously, Munir and co-authors [41] reported a study on the optimization of polygalacturonase production and showed maximum enzyme units at pH 5.0. A similar outcome reported by Aminzadeh et al. [42] showed a higher polygalacturonase yield at pH 5.0 by using *Tetracoccosporium sp.* as a host organism. Vasanthi [43] has also reported maximal polygalacturonase activity at pH 5.0. Hence, such a coincidence with the previous reports confirmed that the enzyme is pH sensitive, and the optimum pH must be set at 5.00 for maximum enzymatic production and activity.

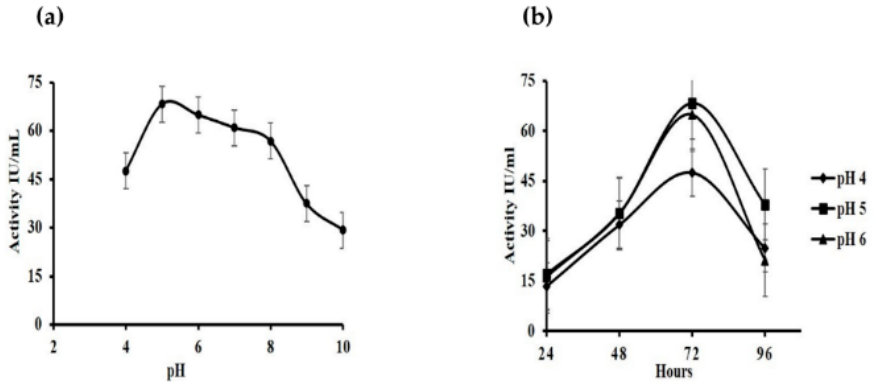

Figure 5. (**a**) Effect of initial medium pH on endoPGase activity; (**b**) effect of pH on enzyme production with relation to the fermentation time.

2.2.2. Temperature Influence on EndoPGase Production Efficiency

The incubation temperature of the production medium has a critical impact on the yield of the enzyme. Since temperature is among the most significant factors for the successful fermentation of biocatalysts, it directly influences the growth of enzyme-producing microbes [27]. The resultant values of our investigation showed that the endoPGase production was increasing with the temperature rise, and 50 °C was found to be the optimum for maximum enzyme production. A further decline in activity showed that the increase in temperature above 50 negatively influenced microbial growth, which ultimately inhibited enzyme production. An apparent decline in activity from 98.87 to 87.86 u/mL was observed, as presented in Figure 6. The increase or decrease in temperature from the optimum affects the endoPGase production level. However, Raju and Divakar [7] investigated the influence of different temperatures on pectinase enzyme production by *Bacillus circulans* and found maximum yield at 40 °C. Another report revealed 35 °C as the optimal temperature for pectinase production by *B. subtilis;* at 50 °C, the enzyme production was 63.54% of the

maximum production under solid state fermentation [27]. This dissimilarity in both studies might be due to the difference in fermentation conditions and the potential of strains for pectinase enzyme synthesis. Our finding is significantly in line with that reported previously by Demir et al. [44] in which the ideal temperature for pectin lyase production by *B. borstelensis* (P35) was recorded at 50 °C.

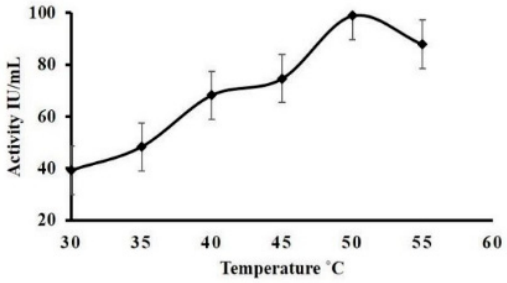

Figure 6. Effect of fermentation temperature on endopolygalacturonase production.

2.2.3. Influence of Inducer-Substrate's Induction on EndoPGase Activity

The induction of inducer-substrates significantly influences endoPGase production by *B. subtilis*. Various agricultural wastes and synthetic starches/sugars were supplemented in a production medium with 1% concentration as a carbon source to induce the production of endoPGase, and fermentation conditions were adjusted at pH 6.00 and a temperature of 37 °C. Among all inducer-substrates, citrus peel, as shown in Figure 7, was found to be a more appropriate and economical substrate for commercial endoPGase production. Following the citrus peel, potato starch and pectin (Sigma) were also found to be good inducers-substrates for endopolygalacturonase activity, as shown in Figure 7. However, the minimum units were observed when the medium was supplemented with glucose (42.85 IU/mL), sucrose (29.17 IU/mL), and CMC (22.34 IU/mL) shown in Figure 7. Aminzadeh et al. [42] reported the repressed biosynthesis of polygalacturonase from *Tetracocosporium* sp. by using monosaccharides (glucose and fructose) and procured the same results. Such an inhibition might be due to the influence of catabolite repression in the presence of simple sugars. However, some agro-residual inducer-substrates such as gram bran, wheat bran, rice starch, and corn starch were also found to produce a low yield of endoPGase at a 1% concentration. Munir et al. [41] have also recorded similar results. The increased concentration of reported agro-based inducers results in significant progress in the expression of the EndoPGase enzyme. Pectinolytic enzyme production has been reported previously by many researchers by using various wastes of agricultural origin. However, there is no report on the comparative effect of synthetic monomers and polymeric substrates with agro-industrial wastes as inducers of endo-pectinase enzyme production. A previous study [27] suggested orange peel with 5% concentration as the best inducer-substrate for pectinase production by *B. subtilis* under solid state fermentation with 450.50 units/g, which is far lower than our finding of 1066 units/mL with 5% inducer concentration of citrus peel (data not shown). The comparative analysis revealed that the difference in results was possible because of submerged fermentation since the shake flask fermentation provides better distribution of mineral nutrients for the growth of microbes.

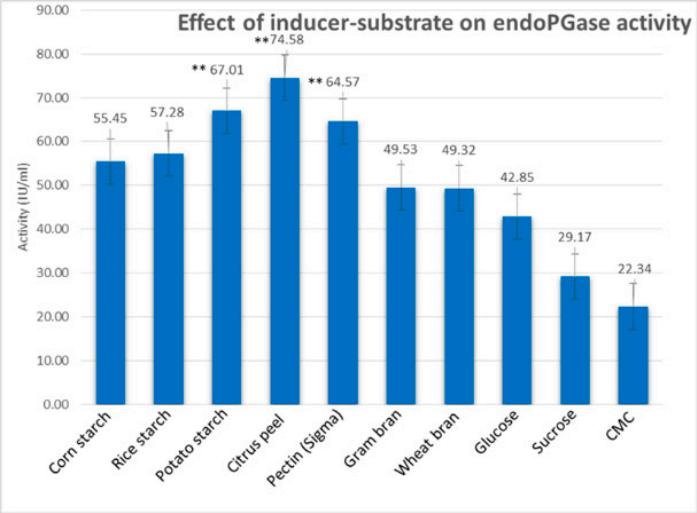

Figure 7. Effect of agro-based monomeric and dimeric substrates on endopolygalacturonase activity. ** The mean values are highly significant at 1% level of significance with a *p*-value of 0.05 representing the highest significant effect of citrus peel in comparison with potato starch and pectin (Sigma) on EndoPGase activity.

In comparison with our study, Jahan et al. [45] also reported the utilization of agro-industrial wastes for polygalacturonase production by a mesophilic strain of *Bacillus licheniformis*. They evaluated wheat bran at 1% concentration as the most suitable source for PGase enzyme production, while citrus peel was reported for its minimal enzyme production. The minimal enzyme activity on citrus peel may be because of the inability of mesophilic bacillus to hydrolyze the substrate. In the case of monomeric substrates, the enzyme production may undergo catabolite repression [46] because glucose is known for its catabolic repression of transcription of the gene encoding enzymes, which are the prime requirement for utilization of alternative inducer-substrates [47]. However, as for as the use of corn as an inducer-substrates is concerned, we used it since corn is an abundant crop in our region. Moreover, we explored different monomeric, dimeric, and polymeric substrates for enzyme production; therefore, it was also important to test corn starch. However, we found that the corn was not among the best inducer-substrates.

2.3. Fermentation Medium Optimization by Response Surface Methodology (RSM)

Process parameter optimization is the most significant factor in reducing the cost of production. Response surface methodology is a composition of statistical and numerical techniques for simultaneously investigating the effect of numerous independent variables [48]. The optimal level of independent variables is later useful for analyzing the best combination response of different variables. This approach can evaluate the relationship between a set of variables and experimental results. The interaction of variables can be assessed with a fewer number of experiments. Hence this methodology has been verified as an important tool to investigate the effect of multiple process parameters with limited experimental trials.

The preliminary study by one factor at a time approach evaluated the effect of substrates' induction, temperature, and pH on enzyme activity to determine the significant effective parameters. The optimization of EndoPGase production was then completed using response surface methodology in which three process variables, such as inducer-substrates, pH, and inducer-substrate's concentration with three levels [49,50], were selected to determine the combined effect of these variables at 50 °C. The initial results revealed that the induction of carbon source, pH, and inducer-substrate's concentration was the most effective as compared to other parameters. Based on these results, the important parame-

ters were also studied by RSM after designing Box–Behnken experimental design, and the results are shown in Figure 8.

The effect of three polymeric inducer-substrates was also observed along with the interaction of the initial pH of the production medium and the concentration of three inducing substrates by employing RSM. The simple Box–Behnken experiment was designed for three independent variables via Design-Expert software (version 8.0.7.1). The independent variables were coded as; A. ×1 for initial medium pH (4, 5, 6), B. ×2 for inducer-substrate concentration (1%, 4%, 6%) while C. ×3 represented three different organic and synthetic inducers (wheat bran, corn starch) as a minimal and maximal level (citrus peel) of induction efficiency. The three inducer-substrates were selected on the basis of initial one-factor experimental results. The following quadratic equation in terms of actual factors was obtained from experimental data through Design-Expert software version 8.0.7.1.

$$\text{Activity} = 48.16 - 0.33 \times A + 0.23 \times B + 0.63 \times C + 0.63 \times A \times B + 0.83 \times A \times C + 0.017 \times B \times C + 0.28 \times A2 + 0.56 \times B2 + 0.097 \times C2$$

Analysis of variance [ANOVA] table [Partial sum of squares—Type III] showing highly significant, significant, and non-significant results for response surface quadratic model is presented in Table 1.

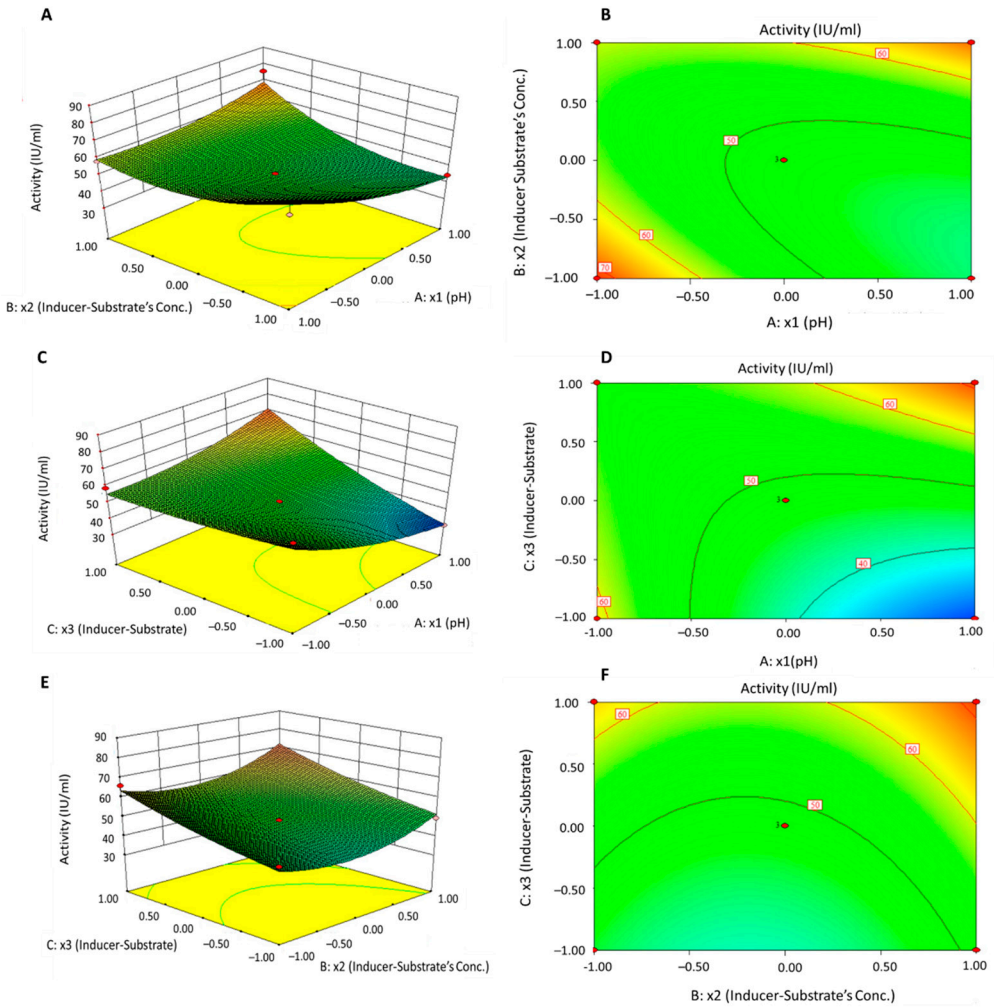

Figure 8. Response surface graph of EndoPGase activity of the isolated bacterial strain *B. subtilis* with respect to different experimental factors. (**A**,**B**) pH (4, 5, 6) vs. inducer-substrate's concentration (1%, 4%, 6%); (**C**,**D**) pH vs. inducer-substrate (wheat bran, corn starch, and citrus peel); (**E**,**F**) inducer-substrate's concentration vs. inducer-substrate, respectively.

Table 1. Analysis of variance [ANOVA] table [Partial sum of squares—Type III] for response surface quadratic model.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Model	10.22	9	1.14	9.50	0.0116 *
A-×1	0.87	1	0.87	7.28	0.0429 *
B-×2	0.43	1	0.43	3.62	0.1154
C-×3	3.19	1	3.19	26.68	0.0036 **
AB	1.59	1	1.59	13.32	0.0148 *
AC	2.76	1	2.76	23.14	0.0048 **
BC	1.182×10^{-3}	1	1.182×10^{-3}	9.891×10^{-3}	0.9246
A2	0.29	1	0.29	2.44	0.1789
B2	1.16	1	1.16	9.68	0.0265
C2	0.035	1	0.035	0.29	0.6130
Residual	0.60	5	0.12		
Lack of Fit	0.60	3	0.20		
Pure Error	0.000	2	0.000		
Cor Total	10.82	14			

* Denotes a significant effect. ** Denotes a highly significant effect; no asterisk denotes a non-significant effect at 95% confidence level.

The prediction profile of EndoPGase activity response of three factors (pH, inducer-substrate's concentration, and inducer-substrates) is shown in Figure 9.

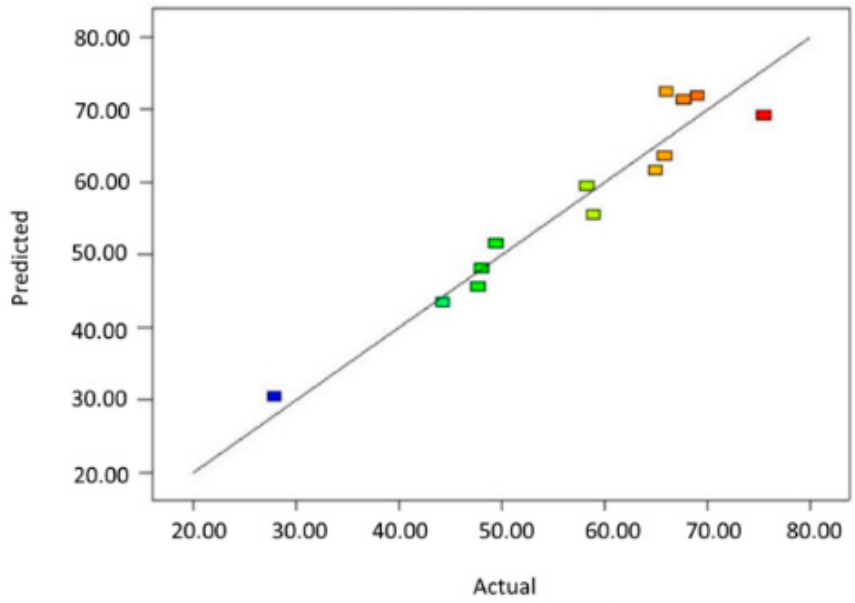

Figure 9. Residual plots calculated from RSM graphs for the EndoPGase activity exhibited by the isolated bacterial strain of *B. subtilis* in repose to pH, inducer-substrates, and inducer-substrates' concentration where; blue, green, light red, and red color squares show minimum, medium, and the maximum enzyme activity response respectively.

3. Materials and Methods

3.1. Screening of Pure Culture Strain

A 100 mL of soil compost was serially diluted in sterile 0.9% normal saline solution. A mineral salt solution containing 0.2 g/100 mL K_2HPO_4, 0.2 g/100 mL $MgSO_4 \cdot 7H_2O$, 0.004 g/100 mL $CuSO_4 \cdot 5H_2O$, 0.008 g/100 mL $FeSO_4$, 0.008 g/100 mL $ZnSO_4$, and 0.008 g/100 mL $MnSO_4$ [51] supplemented with 0.2 g/100 mL of pectin as the sole carbon source was prepared and added to the growth medium. An agar of 2% concentration was added to the medium, and the pH was adjusted to 6.0. Then, the medium was first autoclaved at 121°C for 20 min, and each diluted

sample was spread in triplicates on the agar medium in the plates. Subsequently, the plates were incubated at 37 °C for three days for the growth of the inoculums [52].

3.2. Qualitative Assay for Pectinolytic Activity

Colonies with different morphology were subcultured on pectin-supplemented-agar-media (PSAM) plates. PSAM plates were inoculated with each pure colony. After 24 h of incubation at 37 °C, the PSAM plates were stained with 0.5 mM iodine solution (iodine 1 g, potassium iodide 5 g, dissolved in 330 mL of H_2O) and incubated for 20 min. Then, the PSAM plates were slowly washed with de-ionized water. A clear yellow zone around the colony was picked positive for polygalacturonase (PG) activity [53].

3.3. Scanning and Transmission Electron Microscopy (SEM)

A pure colony of selected culture was placed overnight in 0.1 M sodium cacodylate buffer (pH 6.9) containing glutaraldehyde (2.5%) and formaldehyde (2%). The cells' aliquots were washed in the buffer solution three times to fix and then rinsed three times with 1% osmium tetraoxide mixed in 1% phosphate buffer for 60 min. The cell aliquots were centrifuged (in a micro-centrifuge machine, Fisher Scientific (Hampton, NH, USA)) for 10 s to make a pellet. The pellet was resuspended and rinsed with 1% phosphate-buffered osmium tetraoxide. It was then filtered through a 0.1 µm membrane filter made of poly-l-lysine (Nuclepore track-etch membrane filter, Whatman, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The filters were dehydrated with a critical point dryer. Each filter containing culture cell was fixed onto aluminum stubs (Ted Pella Inc., Redding, CA, USA), in a Denton Desk II, covered by a gold-palladium coating unit (Denton Vacuum, Inc., Moorestown, NJ, USA). The fixed culture colonies were visualized at 2 kV resolution power in a Hitachi S4700 field emission scanning electron microscope (Hitachi, Tokyo, Japan) [54].

From post-fixed samples prepared for SEM, 70 nm thick portions were cut and kept on 400-mesh copper grids and subsequently stained for 10 min with 1% aqueous uranyl acetate solution. After three times rinsing with deionized water, the grid samples were dried using filter paper slices. The grid sections were visualized at 80 kV for photograph resolution in an FEI Technai 12 transmission electron microscope (FEI, Hillsboro, OR, USA).

3.4. Genetic Identification of the Isolate

The morphology of the selected colony based on color, shape, and texture was observed on fresh PSAM and nutrient agar plates. Gram staining was performed, and each colony was checked under a microscope, and based on SEM and TEM observations, the NR2 strain was selected for genetic identification by 16S RNA sequencing.

3.4.1. Extraction and Purification of Genomic DNA

For the extraction and purification of genomic DNA, a fresh sterile 100 mL PSM broth media was prepared and the selected colony was inoculated and incubated at 37 °C for 24 h growth. Five milliliters of culture growth from PSM broth was centrifuged at 8000 rpm for 10 min. The cell pellet was separated, and the supernatant of PSM media was discarded. A sterile 500 µL TE Buffer composed of 1 mM EDTA, 10 mM Tris HCl, and 1 M NaCl, pH 8.0, was added to the cell pellet. A 200 µL 10% SDS was added, and the pellet was heated at 80 °C for 60 min. Two hundred microliters of 1 molar Tris-HCl containing proteinase K buffer was added and the test tubes were optimally placed at 50 °C for 60 min in a water bath. After that, 10 µL of 40 g RNase was added at room temperature for 30 min. The test tubes were centrifuged at 8000 rpm for 15 min after the addition of 100 µL of 5 M NaCl solution and chilled with absolute ethanol. The cell supernatant was added to fresh eppendorfs. A chilled 1 mL of phenol-chloroform-iso amylalcohol was added. The eppendorfs were centrifuged again at 8000 rpm for 15 min. The uppermost layer of the eppendorfs was picked and transferred to fresh eppendorfs. This layer was washed and centrifuged three times with 70% chilled ethanol. The cell pellet was dissolved in 200 µL of TE buffer. A 0.5× solution of TBE buffer was used to prepare 0.8% agarose gel. This

TBE-agarose solution was heated in a microwave oven for 30 s twice and cooled to 45 °C. For DNA visualization, 0.5 µg/mL ethidium bromide (Roche, Mannheim, Germany) was added. Two microliters of the extracted DNA sample was loaded to the well next to the well loaded with a standard DNA marker. Electrophoresis was carried out for 30 min at 100 V [53].

3.4.2. PCR Amplification

The 16S rRNA sequence of colony DNA was amplified with universal primers; p16S-8 forward primer FD1 (5-AGAGTTTGATCCTGGCTCAG-3) and p16S-1541 reverse primer RD1 (5-AAGGAGGTGATCCAGCC-3) respectively. A PCR master mix solution was prepared with a composition of 1 µL of Taq DNA polymerase (Fermentas, Waltham, MA, USA), 10× PCR supermix (15 µL), 1.5 µL of (25 ng/µL) forward and reverse primer, 1 µL of DNA (30 ng), and 10 µL of PCR buffer water. PCR conditions were optimized at the following conditions: one cycle at 94°C for 120 sec for initial denaturation, 60 s at 94°C, 60 s at 52°C for annealing, and 60 s at 72°C for extension. The cycle was repeated for 30 rounds. The PCR amplified product was cleaned with the PCR Purification Kit (Qiagen, Germantown, MD, USA). A 2µL of PCR product was checked on electrophoresis gel and a digital photograph was taken under UV Transilluminator (UVItec, EEC). The amplified PCR 16S rRNA was sequenced from ELIM BIO San Francisco, California USA. The raw sequence of PCR was filtered through the sequence analysis package (DNA-Star). Fasta sequence of 16S rRNA was searched through NCBI Basic Local Alignment Search Tool (BLAST) for genetically similar species strains. Eleven sequences of most similar species strains were retrieved, and a phylogenetic tree was built using MEGA 11 software as described earlier [55,56].

3.5. Submerged Fermentation Medium (SMF)

A liquid mineral salt medium PSM supplemented with 0.6 g/100 mL K_2HPO_4, 0.1 g/100 mL $MgSO_4 \cdot 7H_2O$, 0.004 g/100 mL $CuSO_4 \cdot 5H_2O$, 0.008 g/100 mL $FeSO_4$, 0.008 g/100 mL $ZnSO_4$, 0.008 g/100 mL $MnSO_4$, and 1% citrus pectin as the sole carbon source was mixed well. The pH of the media was adjusted to 6.0. A pure culture with 0.6 OD/600 nm was inoculated into 50 mL PSM in 250 mL capacity Erlenmeyer flasks. The flasks were kept at 45 °C on a rotary shaker with a shaking speed of 150 rpm. After two days' growth time, the 10 mL of culture cells were centrifuged at 4 °C, @10,000 rpm for 10 min. Then, enzyme supernatant was tested for the estimation of polygalacturonase (PG) activity [57].

Enzyme Extraction and Endo-Polygalacturonase Enzyme Assay

A fresh sterile liquid mineral salt medium (PSM), supplemented with 1% citrus pectin, was inoculated with pure culture. The starting inoculum was 0.2 OD/600 nm into 100 mL PSM/ 250 mL Erlenmeyer flasks. After every 12 h, 5 mL of culture cells were centrifuged at 4 °C, @13,000 rpm for 20 min. The crude supernatant was assayed for pectinase enzyme as described earlier by Maciel et al. [58]. The polygalacturonase assay was carried out using 1000 µL of 0.2 M sodium acetate buffer pH 5.5, 500 µL of 1% pectin substrate, and 500 µL of crude enzyme supernatant. The reaction was carried out for 30 min at 37 °C in a boiling water bath. Dinitrosalisylic salt (DNS) (3000 µL) was added to stop the enzyme reaction. To find out the amount of releasing sugars from pectin, the absorbance was read at 575 nm [59].

3.6. Partial Purification and SDS-PAGE Analysis

The cell-free enzyme supernatant was filtered through a Nalgene filtration assembly, treated with 70% ammonium sulphate with constant stirring, and centrifuged for 20 min at 13,000 rpm. The enzyme supernatant was separated from precipitated impurities and loaded on an anion exchange Q-sepharose 112,014 column for further purification. Enzyme fractions of 3 mL were collected by using a fraction collector up to 130 fractions. Fractions containing the highest EndoPGase activity were pooled desalted, concentrated by a tan-

gential flow filtration system, and reloaded on a Q-sepharose 112,014 column that had been equilibrated by 20 mM Tris-HCl buffer (pH 7.5). The fractions having the highest EndoPGase activity were pooled and concentrated again for conducting sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular weight determination using a mini gel system of Novex (Invitrogen) through Nu-PAGE 10% Bis-Tris gels in the presence of MES-SDS running buffer followed by electrophoresis for 40 min. A protein standard marker of known molecular weight (kDa) was loaded in the first well to enumerate the unknown weight of the diluted and concentrated enzyme samples. The gel was cyber stained and photographed soon after the bands appeared clearly [60].

3.7. Optimization Parameters

The purified culture was tested to find out the optimum activity conditions of pH ranging from 3 to 8, temperature ranging from 30 to 60 °C, and carbon source (pectin, galactose, glucose, fructose, sucrose, citrus peel, wheat bran, and starch following the method of Doan et al. [38].

3.8. Statistical Method

Data for the effect of agro-based, monomeric and dimeric inducer-substrates on endoPGase activity were analyzed by analysis of variance (ANOVA) using SPSS (20.0) statistical software (IBM). Initially, a one-factor-at-a-time approach was applied to optimize medium conditions for enzyme production. Then, three parameters, viz., pH, inducer-substrate, and inducer-substrate's concentration, were further studied via a multiple-factor-at-a-time approach by applying a Box–Behnken design for enzyme production and to produce graphs of response surface using Design Expert Software trial version 8.0.7.1.

4. Conclusions

In conclusion, *Bacillus subtilis* was recognized as a promising candidate for endopolygalacturonase production under a submerged fermentation system. The enzyme activity was maximal at 50 °C fermentation temperature and pH 5 in the presence of citrus peel as an inducer-substrate. In order to obtain maximum enzyme activity, Box–Behnken design, response surface methodology (RSM) use was found to be a suitable method for the enhancement of endoPGase production and the optimization of fermentation parameters. The enzyme was stable under a variable range of pH, and inducer-substrates which also indicated that the *Bacillus subtilis* strain 168 could be a potential microbe for its utilization in various biotechnology industries. The multiple-factors-at-a-time approach indicated both citrus peel and wheat bran as ideal substrates for enzyme production at pH 5 and 6, respectively. The observed temperature for endoPGase production supports that the enzyme is quite suitable for industrial-scale applications. The genetic study of the strain assured its commercial-scale application for pectinase production and utilization in food formulations and processing since *B. subtilis* is a proven GRAS (generally recognized as safe) bacterial strain; thus, offering potential future use as a promising cell factory in the food, beverages, paper, and textile industries.

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