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

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**Abstract:**

Endopolygalacturonase (EndoPGase) is one of the crucial pectinases belonging to the class of carbohydrase. The catalytic action of EndoPGase captivate the attention for production of this extremely valuable catalyst of industrial sector. The main focus was to ascertain a potential bacterial candidate for endoPGase production. The isolated bacterial strain was further identified by 16S rDNA gene sequencing. A genomic library was constructed by using Lambda ZAP II vector system to investigate the pectinolytic potential of the expressed genes. The parameters for enzyme biosynthesis were optimized by single as well as multiple factor approach at a time. The results of our investigation led to the identification of a potent strain of *Bacillus subtilis* NR2. 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, citrus pectin followed by wheat bran was considered the best enzyme inducers at 1 % concentration. Moreover, an increasing trend in enzyme activity was observed with the increasing inducer concentration. The combined effect of three variables (pH, substrates, and substrate concentration) was explored by response surface methodology involving Box Benken Design (BBD). The study concluded that the soil isolated *B. subtilis* can be utilized for commercial scale pectinase enzyme production.

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**Key Words:** Endopolygalacturonase, biocatalysts, biosynthesis, heteropolysaccharide, inducer, submerged fermentation.

## Introduction

The word pectinase refers to various heterotypic enzymes of related group comprising of pectin depolymerases or polygalacturonases (PG), polymethylgalacturonases (PMG), transeliminases or lyases, and pectin esterases (PE) also known as pectin methyl esterases (PME). These enzymes have capability of both cleaving the glycosidic bonds and transforming or debranching pectin, the most abundant constituent of fruits [1]. Pectin is an acidic heteropolysaccharide containing galacturonic acid units in which carboxyl groups are esterified with the 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 middle lamella of 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; 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, as a poultry feed additive, protoplast fusion technology, and production of bioenergy [5, 6].

The major production sources of pectinase enzyme are microbes and plants. However, because of commercial and technical practicability, production of pectic enzyme from microbial sources is becoming the foremost area in research considerations [7, 8]. The extensive utilization and efficiency of microbial pectic biocatalysts in various production industries have 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 of microbial source accounts for about 25% of global sale of food and industrial enzymes, and their market is increasing constantly [10]. Principally, enzymes embrace an engrained global market which was predicted to reach 6.3 billion US dollar in 2021 [11].

It has been publicized that the fermentation-conditions and substrate-composition have a significant impact on biosynthesis of pectinase enzymes using microbes [12, 13]. Hence, it is well-established fact now that the pectinolytic enzyme production is affected by the condition of fermentation environment and the carbon, nitrogen and mineral salts sources. Therefore, the nutritional composition of fermentation medium is critical aspect for the industrial scale enzyme production since, one-third of the enzyme production cost has primarily been found associated to the expenditure of the culture medium [14]. For that reason, the choice of appropriate medium

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components and understanding of components interaction are critical. Moreover, compositional optimization of medium is also an important factor, which supports to minimalize costs of production and to enhance the actual-yield [15].

Shake flask fermentation scheme has been extensively employed especially, in enzymes production however, statistical design of experiments [16] may vary for analysis of the biotechnological parameters to calibrate the production efficiency.

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

For present investigation a potent bacterial strain was isolated from soil for endopolygalacturonase production. The strain was further characterized to explore its status to be a potential candidate for pectinolytic enzyme production on commercial scale and three important pectinase genes have been isolated from its genomic library. The submerged fermentation conditions were optimized using available natural and synthesized polymeric substrate to release the PGase by employing multiple factors at a time approach.

## Materials and Methods

### Screening of Pure Culture Strain

A 100 mL of soil compost was serially diluted in sterile 0.9% normal saline solution. Mineral salt solution containing 0.2g/100mL $K_2HPO_4$, 0.2 g/100mL $MgSO_4.7H_2O$, 0.004 g/100mL $CuSO_4.5H_2O$, 0.008 g/100mL $FeSO_4$, 0.008 g/100mL $ZnSO_4$, and 0.008 g/100mL $MnSO_4$, supplemented with 0.2g/100mL of pectin as sole carbon source were prepared and added to the growth medium. An agar of 2% was added to the medium and the pH was adjusted at 6.0. Then, medium was first autoclaved at 121°C for 20 minutes and each of the 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 growth of the inoculums [18].

### 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 hours of incubation at 37 °C, the PSAM plates were stained with 0.5mM iodine solution (iodine 1 g, potassium iodide 5 g, dissolved in 330 mL of $H_2O$) and incubated for 20 minutes. 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 [19].

### Genetic Identification of the Isolate

Morphology of the selected colony viz., color, shape, and texture was observed on fresh PSAM and nutrient agar plates. Gram staining was done and each colony was checked under microscope.

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**Extraction and Purification of Genomic DNA**

Fresh sterile 100 mL PSM broth media was prepared. The selected colony was inoculated and incubated at 37 °C for 24 hours growth. 5 mL of culture growth from PSM broth was centrifuged at 8000rpm for 10 minutes. Cells pellet was separated and the supernatant of PSM media was discarded. A sterile 500 µL TE Buffer composed of 1mM EDTA, 10 mM Tris HCl and 1 M NaCl, pH 8.0 was added to cells pellet. A 200 µL 10% SDS was added and the pellet was heated at 80°C for 60 minutes. 200 µL of 1 molar Tris HCl containing Proteinase K buffer was added. The test tubes were optimally placed at 50°C for 60 minutes in water bath. 10µL of 40g RNase was added at room temperature for 30 minutes. The test tubes were centrifuged at 8000rpm for 15 minutes after addition of 100 µL of 5M-NaCl solution and chilled absolute ethanol. The cells supernatant was added to fresh eppendorfs. A chilled 1mL of phenol-chloroform-iso amylalcohol was added. The eppendorfs were centrifuged again at 8000rpm for 15 minutes. The upper most layer of the eppendorfs was picked and transferred to fresh eppendorfs. This layer was washed and centrifuged three times with 70% chilled ethanol. The cells pellet was dissolved in 200 µL of TE buffer. A 0.5x solution of TBE was used to prepare 0.8% agarose gel. This TBE-Agarose solution was heated in microwave oven for 30 second twice and cooled to 45°C. For DNA visualization, a 0.5µg/mL ethidium bromide (Roche, Germany) was added. A 2µL of the extracted DNA sample was loaded to the well next to the well loaded with a standard DNA marker. The electrophoresis was carried out for 30 minutes at 100 V [19].

**PCR Amplification**

The 16S rRNA sequence of colony DNA was amplified with forward primer FD1 (5- AGAGTTTGATCCTGGCTCAG-3) and reverse primer RD1 primer (5- AAGGAGGTGATCCAGCC-3). A PCR master mix solution was prepared with composition of 1 µL of Taq DNA polymerase (Fermentas, USA), 10x PCR Super mix (15µL), 1.5µL of (25ng/µL) forward and reverse primer, 1 µL of DNA (30ng) and 10 µL of PCR buffer water. PCR conditions were optimized at the following conditions: one cycle at 94°C for 120 sec initial denaturation, 60 seconds at 94°C, 60 seconds at 52°C for annealing and 60 seconds at 72°C for extension. The cycle was repeated for 30 rounds. The PCR amplified product was cleaned with PCR Purification Kit (Qiagen, MD, USA). A 2µL of PCR product was checked on electrophoresis gel and 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. A 20 sequences of most similar species strains were retrieved and phylogenetic tree was Build using MEGA7 software as described earlier [20, 21].

**Scanning and Transmission Electron Microscopy (SEM)**

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 three time in the buffer solution to fix and then rinsed three times with 1% osmium tetraoxide mixed in 1 % phosphate buffer for 60 minutes. The cells aliquots were centrifuged (in a Micro-Centrifuge, Fisher Scientific machine) for 10 seconds to make a pellet. The cells pellet was resuspended again and rinsed with 1 % phosphate buffered osmium tetraoxide. It was then filtered through 0.1 µm membrane filter made of poly-l-lysine (Nuclepore track-etch membrane filter, Whatman, GE Healthcare Bio-Sciences, Pittsburgh, PA). The filters were dehydrated with

critical point dryer. Each filter containing culture cell was fixed onto aluminum stubs (Ted Pella Inc., Redding, CA), in a Denton Desk II, covered by gold-palladium coating unit (Denton Vacuum, Inc., Moorestown, NJ). The fixed culture colonies were visualized at 2 kV resolution power in a Hitachi S4700 field emission scanning electron microscope (Hitachi, Japan) [22].
From post-fixed samples prepared for SEM, 70nm thick portions were cut and kept on 400-mesh copper grids. It was stained for 10 minutes with 1% aqueous uranyl acetate solution. After three time 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).

**Submerged Fermentation Medium (SMF)**

A liquid mineral salt medium PSM supplemented with 0.6g/100mL $K_2HPO_4$, 0.1g/100mL $MgSO_4.7H_2O$, 0.004 g/100mL $CuSO_4.5H_2O$, 0.008 g/100mL $FeSO_4$, 0.008 g/100mL $ZnSO_4$, 0.008 g/100mL $MnSO_4$, and 1 % citrus pectin as sole carbon source were mixed-well. The pH of media was adjusted at 6.0. A pure culture with 0.6 OD/600nm was inoculated into 50mL PSM in 250mL capacity Erlenmeyers flasks. The flasks were kept at 45°C on rotary shaker with a shaking speed of 150 rpm. After two days' growth time the 10mL of culture cells were centrifugation at 4°C, @10000 rpm for 10 min. Then, enzyme supernatant was tested for estimation of polygalacturonase (PG) activity [23].

**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/600nm into 100mL PSM/ 250mL Erlenmeyers flasks. After every 12 hours, 5 mL of culture cells were centrifuged at 4 °C, @13000 rpm for 20 minutes. The crude supernatant was assayed for pectinase enzyme as described earlier (Maciel *et al.,* 2011). The assay of polygalacturonase was carried out using 1000uL of 0.2 M sodium acetate buffer pH 5.5, 500uL of 1% pectin substrate, and, 500uL of crude enzyme supernatant. The reaction was carried out for 30 minutes at 37 °C in a boiling water bath. Dinitrosalisylic salt (DNS) of 3000 uL was added to stop the enzyme reaction. To find out the amount of releasing sugars from pectin, the absorbance was read at 575 nm [24].

**Optimization Parameters**

The purified culture was tested to find out the optimum condition 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.

**Statistical Method**

Initially one factor at a time approach was applied for optimizing medium conditions for enzyme production and then three parameters were further studied via multiple factor at a time approach by applying Box Benken Design for enzyme production and to produce graphs of response surface using Design Expert Software trial version 8.0.7.1.

## Results and Discussion

The recent biotechnological implications of pectinase enzyme in industries demand the development of economically efficient bioprocess for biosynthesis of pectin degrading enzyme. The projected study was aimed to investigate a prospective microbe for biosynthetic production of endoPGase enzyme.

### 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 by appearance of yellow colored halo of pectin depolymerization. The pure culture NR2 (**Fig. 1A and 1B**) exhibited a large yellow zone around the colony after staining with iodine was selected for further experimental work. The culture was identified as *Bacillus subtilis* based on morpho-molecular characterization. Potential pectinolytic bacterial, actinomycete and fungal species have been screened and identified molecularly by sequencing of 16Sr DNA gene amplification. Among these 70% isolates were confirmed to be *Bacillus* strains. Among all bacterial species, two isolates were reported as *Bacillus subtilis* and previous findings were in coincidence with our results [11] The results of isolation and screening are in good agreement with previous reports related to bacterial strains isolation having pectinolytic activity [25, 26, 27, 28, 29]. All these previous reports isolated *Bacillus* (most commonly *Bacillus subtilis*) and screened for pectinolytic enzyme activity by using pectinase screening agar medium. Another previous study revealed that *Bacillus subtilis* was isolated from soil and screened for polygalacturonase production using plate assay [30]. The strain was then subjected to colony PCR for 16S rDNA analysis and further confirmation was done by sequencing. Our results are in agreement with the study of Muhammad Hariadi Nawawi [31] in which a potential xylanopectinolytic *Bacillus subtilis* strain was reported. The strain isolated from compost and on the basis of zone formation on pectin agar medium was screened for enzyme production. The 16SrDNA gene sequencing data revealed that the strain culture belonged to the genus Bacillus and finally identified as *B. subtilis*. Similar results for isolation and 16SrDNA identification of bacterial strain *Bacillus subtilis* for pectinase production was also reported by Kaur & Gupta [32] coinciding with our present observations. Similarly, Adeyefa and Ebuehi [33] also reported the production of pectinase enzyme by *Bacillus subtilis* strongly supporting our findings. Some novel pectinolytic bacteria were screened and identified by gene sequencing by Shrestha [34].

### Molecular Identification of the isolated strains

The products of PCR were first purified and then sequenced. The acquired sequence data were analyzed using NCBI BLAST for the likely microorganisms were identified following Oumer and Abate [11] and are presented in **Table 1.** Molecular weight of purified endoPGase revealed by SDS-PAGE is shown in **Figure 6.** It showed a molecular weight of 47kda in the gel which has been well reported earlier and thus confirmed the expressioal product of the EndoPGase genes.

**Table 1**

Identification of the isolated strains using BLAST (Basic Local Alignment Search Tool) .

| Isolate | Molecular identification | GenBank accession number | %similarity | Taxonomy | Habitat |
|---|---|---|---|---|---|
| **NR1** | Bacillus subtilis Strain ATCCC 19217 | CP009749.1 | 97.24 | Bacillus | Soil |
| **NR2** | Bacillus subtilis Strain Bs-916 | CP009611.1 | 98.37 | Bacillus | Soil/Mud |
| **NR3** | Bacillus subtilis Strain Bs-916 | CP009611.1 | 98.37 | Bacillus | Field Mud |

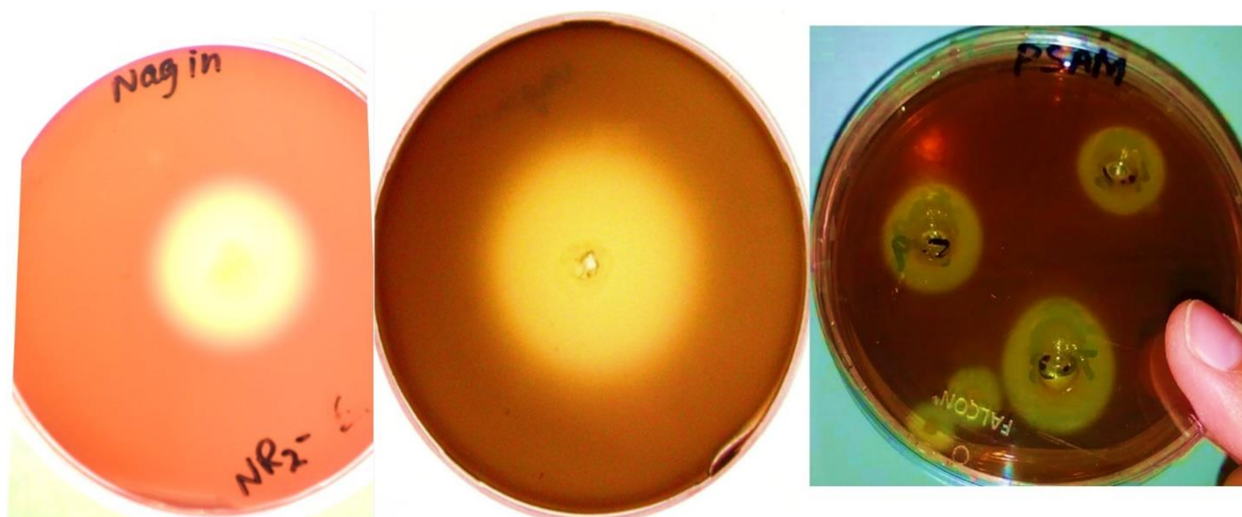


**Figure 1. A. Screening of pectinolytic Bacillus subtilis isolate using PSAM (pectinase agar screening medium) containing 0.5 % citrus pectin from Sigma. After inoculation plate was incubated overnight at 37 °C and flooded with 0.5 mM iodine solution for 20 min and rinse with water. B. selected colony on the basis of large halo of pectin depolymerizarion. C. Well plate assay of Endo-polygalacturonase enzyme on PSAM.**

**Figure 1. A.** Screening of pectinolytic *Bacillus subtilis* isolate using PSAM (pectinase agar screening medium) containing 0.5 % citrus pectin from Sigma. After inoculation plate was incubated overnight at 37 ºC and flooded with 0.5 mM iodine solution for 20 min and rinse with

water. **B.** selected colony on the basis of large halo of pectin depolymerizarion. C. Well plate assay of Endo-polygalacturonase enzyme on PSAM.

**Transmission Electron Microscopy and Scanning Electron Microscopy of Isolated Bacillus Strain**

Analysis by SEM exposed that bacterium was rod shaped bacillus having a size of ~ 2.2 µm as shown in Fig. 2B. Also, transmission electron microscopy revealed that bacterium was rod shaped with a size of 1.4 µm (**Fig. 2A**) which revealed that the culture might contain both short and long rods shown in **Fig. 2A** Ultra-thin section about 1.4 µm in size of positively screened bacillus isolate, (**Fig. 2B**). Scanning electron microscopy of bacillus isolate (scale bar=1.00 µm). Our results are in concordance with a previously reported study by Kumari and co-authors [35] which showed the size of *Bacillus subtilis* around 2-3 µm. Microbial and biochemical analysis [36] showed that the microbe could produce pectinolytic enzymes and was referred to as *Bacillus*. Previously, El-Sayed [25] reported the isolation and screening of pectinase producing bacteria *Bacillus* sp. The microbial strain was identified as *Bacillus subtilis* because of physiological, morphological, biochemical as well as molecular characterization which supported our outcomes.

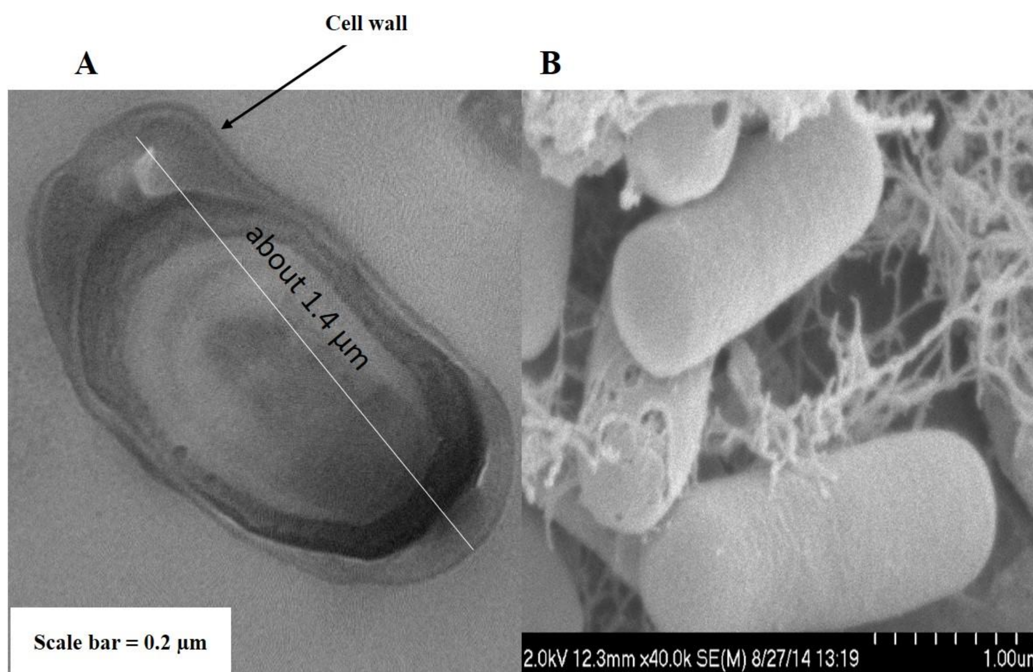


**Fig. 2A. Transmission Electron Microscopy of positively screened Bacillus isolate, 2B. Scanning Electron Microscopy of Bacillus isolate**

**Standardization of submerged bioprocess parameters**

**Optimization of Initial pH of the medium**

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The synthesis of enzyme metabolites is affected by initial pH of the fermentation medium which affects the growth of microorganism. The effect of pH was studied by culturing *Bacillus subtilis* in growth medium having 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 endo PGase activity (68.22 IU/mL) was observed at pH 5.0 shown in **Fig. 3A and B,** and no significant decrease in enzyme units was found with variation in pH from the optimal level. While any variation in pH towards extreme acidic or alkaline resulted in 50 % decrease in endo-PGase activity. pH is crucial to regulate the synthesis of extracellular enzymes by microorganisms and growth of microbes as well. An acidophilic *B. subtilis* SAV- 21 was reported by Kaur and Gupta [32] who described that the decrease in enzyme unit at pH values above the optimal might be because of reduction in 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 range of pH and our study also presented the similar pattern of activity-decline. Our results are also in concordance with the finding of Adeyefa and Ebuehi [33] who reported maximal pectinase enzyme production at pH 5.00 by using *Bacillus subtilis* and *Aspergillus niger* separately as culture microbe. Previously, Munir and co-authors [37] reported a study on optimization of polygalacturonase production and showed the maximum enzyme units at pH 5.0. A similar outcome reported by Aminzadeh et al [38] showed the higher polygalacturonase yield at pH 5.0 by using *Tetracoccosporium sp*. as a host organism. Vasanthi [39] has also reported the maximal polygalacturonase activity at pH 5.0. Hence, such coincidence with the previous reports confirmed that the enzyme is pH sensitive and optimum pH must be set at 5.00 for the maximum enzymatic production and activity.

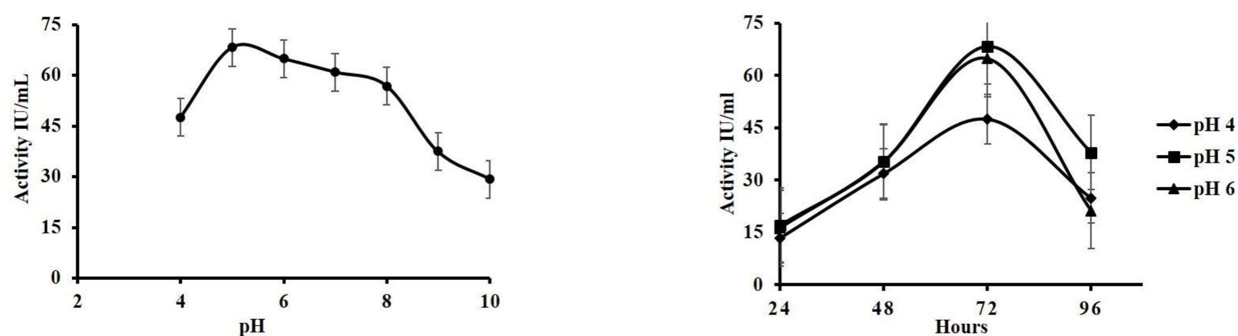


**Figure 3. A.** Effect of initial-medium-pH on endo-PGase activity, **B.** Effect of pH on enzyme production with relation to the fermentation time.

**Temperature influence on EndoPGase Production Efficiency**

Incubation temperature of production medium has a great impact on the yield of the enzyme. Since temperature is among one of the most significant factors for the successful fermentation of biocatalysts as it directly influences the growth of enzyme producing

microbes [32]. The resultant values of our investigation showed that the endo PGase production was increasing with the rise in temperature, and 50 ºC was found to be the optimum for utmost enzyme production and further decline in activity showed that the increase in temperature above 50 have negative influence on microbial growth which ultimately inhibited the enzyme production and a clear decline in activity from 98.87 to 87.86 u/ml was observed as presented in **Figure 4**. The increase or decrease in temperature from the optimum affect the endo PGase production level. However, Raju and Divaker [7] investigated the influence of different temperatures on pectinase enzyme production by *Bacillus circulans* and found maximum yield at 40 ºC. Another report revealed 35 ºC as the optimal temperature for pectinase production by *B. subtilis* but at 50 ºC the enzyme production was 63.54 % of the maximum production under solid state fermentation [32]. This dissimilarity in both studies might be due to the difference in fermentation conditions and potential of strains for pectinase enzyme synthesis. Our finding is significantly in line with that reported previously by Demir et al [40] in which the ideal temperature for pectin lyase production by *B. borstelensis* (P35) was recorded at 50 °C.

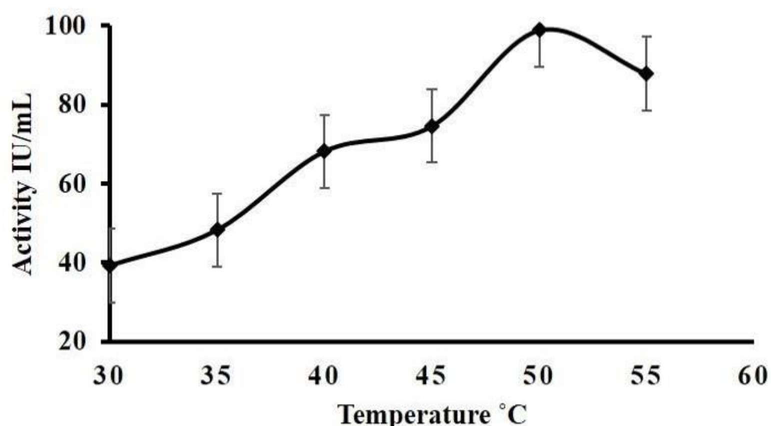


**Figure 4.** Effect of fermentation temperature on Endopolygalacturonase production.

### Influence of Substrate Induction on EndoPGase activity

The induction of substrates and carbon sources has significant influence on endoPGase production by *B. subtilis*. Various agricultural wastes, and synthetic starches/sugars were supplemented in production medium with 1 % concentration as a carbon source to induce the production of endo-PGase and fermentation conditions were adjusted at pH 6.00 and temperature 37 °C. Among all substrates citrus peel, as shown in **Fig. 5** was found more appropriate and economical substrate for commercial endo-PGase production. Following citrus peel, potato starch, and pectin (Sigma), were also found to be good carbon sources for

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endopolygalacturonase activity as shown in **Fig.5**. However, the minimum units were observed when medium was supplemented with glucose (42.85 IU/mL), sucrose (29.17 IU/mL), and CMC (22.34 IU/mL) shown in Fig. 14. Aminzadha [38] reported the repressed biosynthesis of polygalacturonase from *Tetracocosporium* sp. by using monosaccharides (glucose and fructose) and procured the same results. The reason behind this inhibition might be due to the influence of catabolite repression in the presence of simple sugars. Although some agro- residual substrates such as gram bran, wheat bran, rice starch, and corn starch were also found to produce a low yield of endo-PGase at 1 % concentration. Munir et al. [37] have also recorded the similar results. The increase in concentration of reported agro-based inducers results in significant progresses in the expression of the EndoPGase enzyme. Pectinolytic enzyme production has been reported previously by many researchers by using various wastes of agricultural origin and to date there is no report on comparative effect of synthetic monomers and polymeric substrates with agro-industrial wastes as inducers of Endo-pectinase enzyme production. A previous study [32] suggested orange peel with 5 % concentration as the best substrate for pectinase production by *Bacillus subtilis* under solid state fermentation with 450.50 units/g which is far lower than our finding which is 1066 units/ml with 5 % inducer concentration of citrus peel (data not shown). The comparative analysis revealed that difference in results was possibly because of submerged fermentation as the shake flask fermentation provides better distribution of mineral nutrients for the growth of microbes. In comparison with our study, Nayyar Jahan [41] also reported the utilization of agro industrial wastes for polygalacturonase production by a mesophilic strain of *Bacillus licheniformis* and 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. As far as monomeric substrates are concerned, the enzyme production may undergo catabolite repression [42] because glucose is known for its catabolic repression of transcription of the gene encoding enzymes which are the prime requirement for utilization of alternative carbon substrates [43].

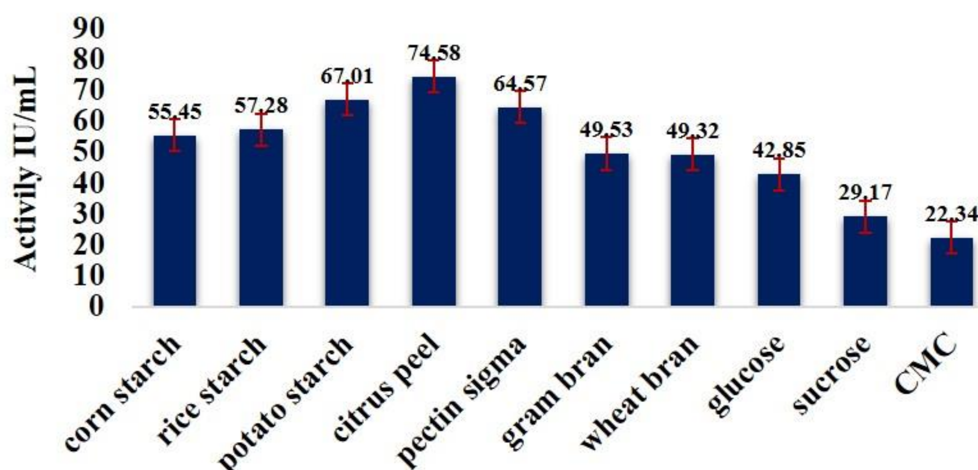

**Figure 5.** Effect of agro based, monomeric and dimeric substrates on endopolygalacturonase production.

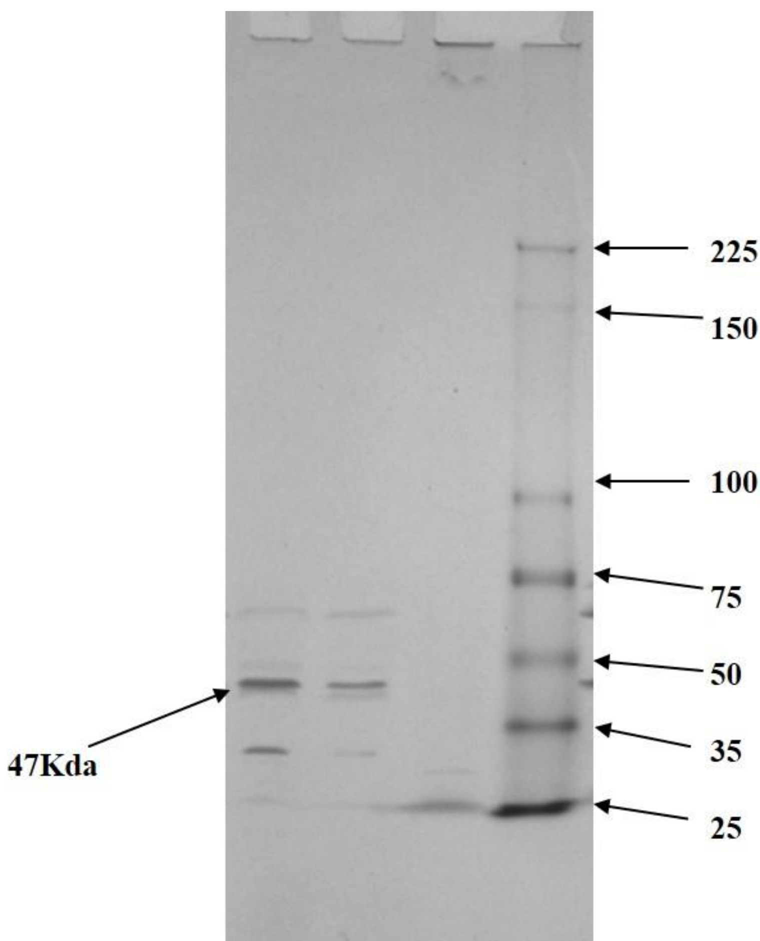


**Figure 6. Molecular weight of purified endoPGase by SDS-PAGE.**

**Fermentation medium optimization by 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 investigating the effect of numerous independent variables at the same time [44]. The optimal level of independent variables is later useful for analyzing the best combination response of different variables. This type of approach can be utilized for evaluation of relationship among a set of variables and experimental results. The interaction of variables can be assessed with fewer number of experiments. Hence this methodology has

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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 major effective parameters. The optimization of EndoPGase production was also completed using response surface methodology (RSM) in which three process variables such as; substrates, pH and substrate concentration with three levels, were selected to determine the combine effect of these variables at 50 °C. The initial results revealed that induction of carbon source, pH, and substrate concentration were the most effective as compared to other parameters. Based on these results, the important parameters were also studied by RSM after designing Box Benken experimental design and results showed in Figure 7. The effect of three polymeric substrates was also observed along with the interaction of initial pH of production medium and concentration of three inducing substrates by employing RSM. The simple Box Benken experiment was designed for three independent variables via Design Expert software (version 8.0.7.1). The independent variables were coded as; A. x1 for initial medium pH (4,5,6) , B. x2 for substrate concentration (1%, 4%, 6%) while C. x3 represented three different organic and synthetic inducers as a minimal (Corn starch) medium (Wheat Bran) and maximal level (Citrus peel) of induction efficiency. The three 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.

$$\text{Activity} = 48.16-0.33 \times A + 0.23 \times B + 0.63 \times C + 0.63 \times A \times B + 0.83 \times A \times C + 0.017 \times B \times C + 0.28 \times A^2 + 0.56 \times B^2 + 0.097 \times C^2$$

The prediction profile of EndoPGase activity response of three factors (pH, substrate concentration and substrates) is shown in **Figure 8.**

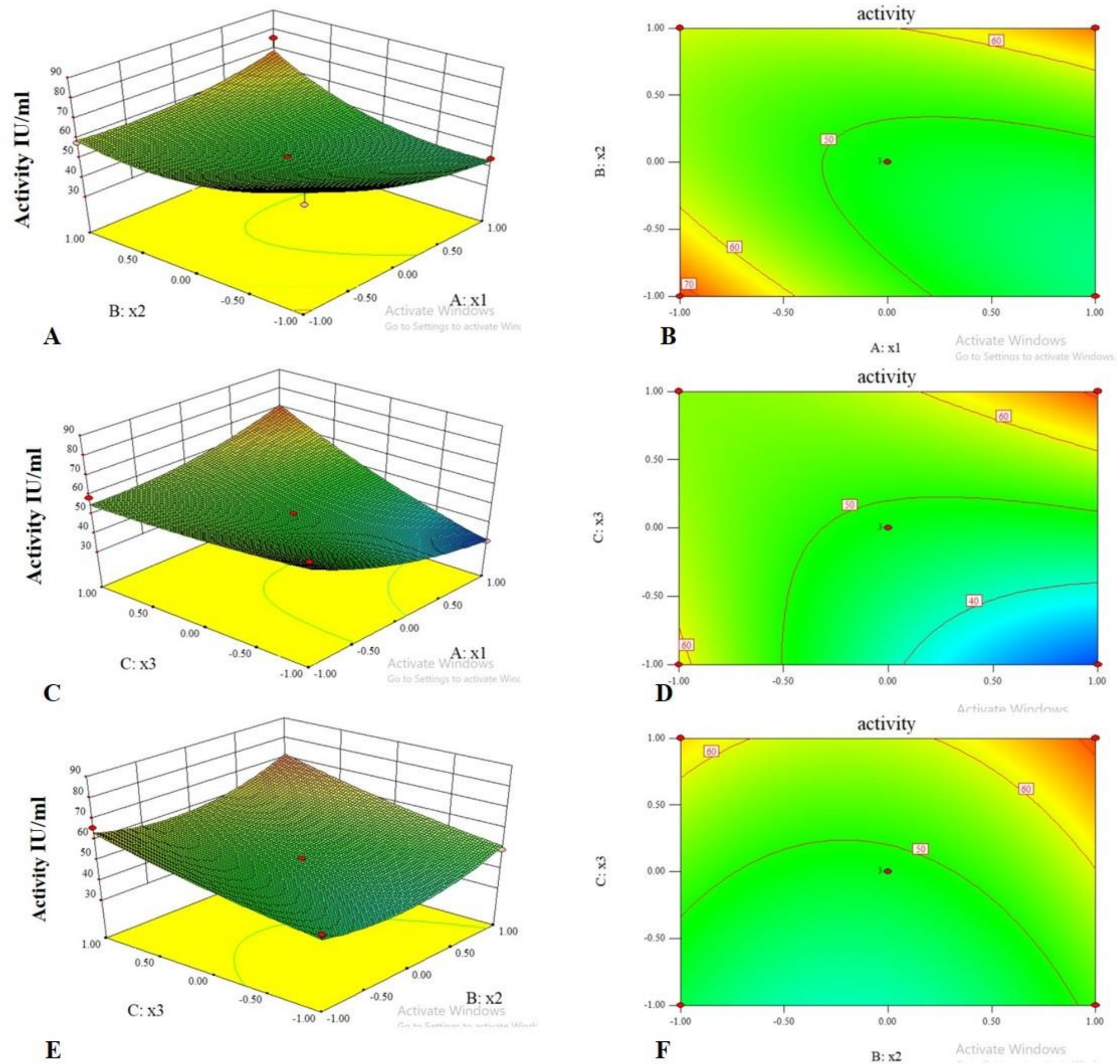


**Figure 7.** Response Graphs and Contour Graphs of three variables viz., substrates, pH and substrate concentration

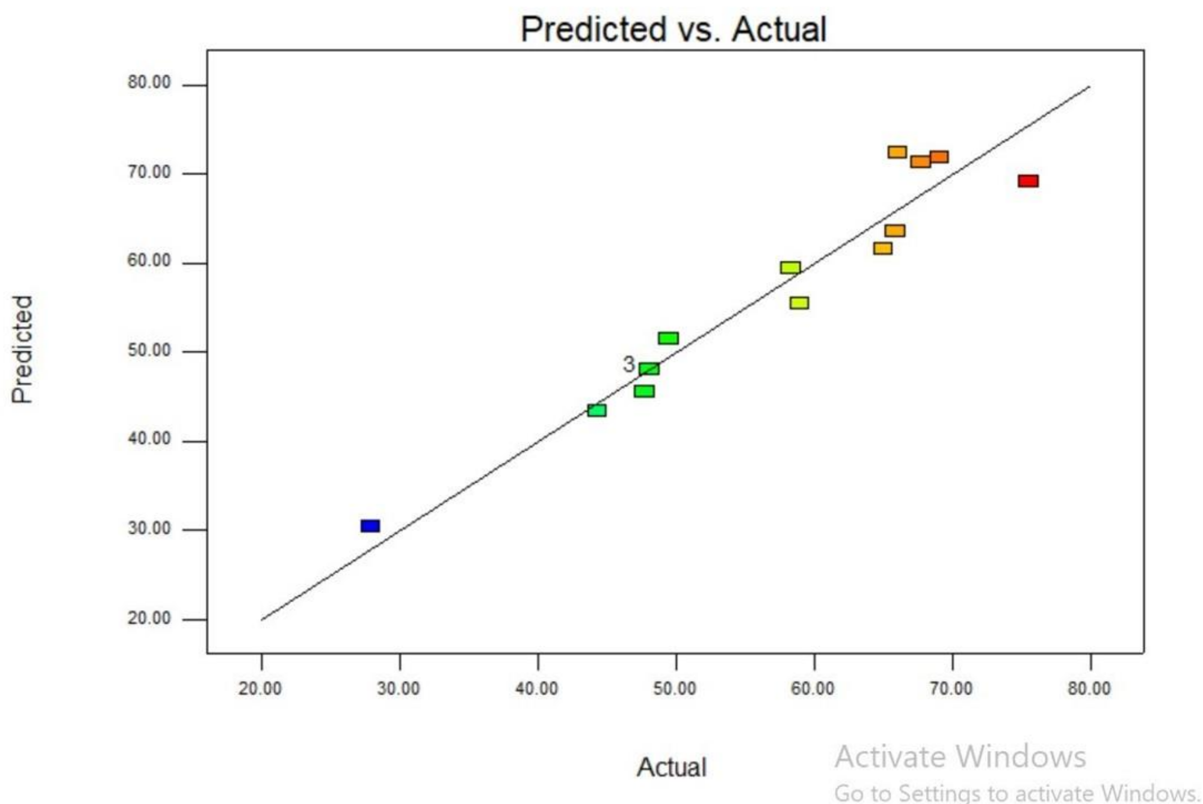


**Figure 8. Prediction profile of EndoPGase activity response of three factors (pH, substrate concentration and substrates).**

## Conclusions

In conclusion Bacillus subtilis was recognized as a promising candidate for Endopolygalacturonase production under submerged fermentation system. The enzyme activity was maximal at 50°C fermentation temperature and pH 5 in the presence of citrus peel as a substrate. The multiple factor at a time approach indicated both citrus peel and wheat bran as an ideal substrate 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 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, offers potential future use as promising cell factory in food, beverages, paper, and textile industries.

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