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ORIGINAL ARTICLE

Pharmamedia and pectinase production by *Bacillus subtilis* Mz-12P

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Abstract

Bacterial isolates collected from the environment were screened for pectinolytic activity, and a strain with the highest activity was selected and identified as *Bacillus subtilis* Mz-12. The presence of pectin hydrolase, lyase, and esterase activities was confirmed. Pectinase was purified and characterized. Enzyme production was optimized with respect to temperature, pH, and growth medium. Enzyme stability and activity were characterized under different temperatures and pH values. The results showed that this strain was capable of producing high yields of pectinase in commercial medium (Pharmamedia) 24.6 U/mL compared to other media. The purified pectinase of 22.3 kDa produced was constitutive in nature. The isolated enzyme from this strain displayed a wide range of temperature and pH stability, with the optimal activity observed at pH 9.0 and 50°C. These results indicate that the *B. subtilis* Mz-12 strain is a favorable candidate for industrial enzyme production. The use of Pharmamedia is reported for first time for pectinase production.

KEYWORDS
Bacillus, characterization, optimization, pectinase, pharmamedia

1 | INTRODUCTION

Pectinase is widely present in nature and can be found in microorganisms such as bacteria, yeast, and fungi. These microorganisms produce pectinase as part of their natural metabolic processes. Pectinase enzymes derived from bacteria have gained significant attention due to their potential applications in various industries, particularly in food processing. Bacterial strains play a vital role in determining the yield and characteristics of pectinase enzymes. Factors influence the strain selection, including enzyme activity, substrate specificity, growth characteristics, and genetic stability. Pectic substances are polysaccharides derived from plants, consisting of a significant amount of galacturonic acid. Pectin and pectate are two types of pectic substances, with pectin being partially methyl esterified and pectate being free of methyl groups.[1] Pectinases, including pectinesterases (PE) and depolymerases, are enzymes responsible for degrading the α-1,4-glycosidic bonds between galacturonic acid subunits.[1,2] PE hydrolyzes methyl ester groups in pectin, while depolymerases break the main chain. Depolymerases can be categorized into polygalacturonases (PG) and lyases (PL). PG cleaves the glycosidic bonds through hydrolysis, while PL breaks the bonds via β-elimination at esterified D-galacturonic acid units.[3] Pectin lyases specif-

Abbreviations: DNSA, dinitrosalicylic acid; OM, optimized medium; M9, minimal 9 salt; YEP, yeast extract-pectin broth; LB, luri-britani; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; YEP, yeast extract-pectin broth.

ically target pectin, while pectate lyases are specific to pectate.[4-6] Pectinolytic enzymes, capable of breaking down pectins, have diverse industrial applications.[7-10] Acidic pectinases are used in various processes such as fruit juice and wine extraction, clarification, and liquefaction,[11] biomass liquefaction and saccharification, and protoplast isolation.[12] Conversely, alkaline pectinases find applications in degumming and retting of fiber crops, pretreatment of pectic wastewater from fruit juice industries, paper production, and oil extraction.[13] These enzymes also play a significant role in coffee and tea fermentation.[14]Typically, fungal sources provide acidic pectinases, while bacteria are the primary source of alkaline pectinases.[15,16] Both plant pathogenic and non-pathogenic bacteria and fungi contribute to the natural recycling of carbon compounds by producing pectolytic enzymes. These enzymes are involved in invading host tissues and decaying dead plant materials, supporting other microorganisms' recycling of carbon compounds.[17,18] Pectinases have vast potential for industrial applications, such as fruit juice clarification, winemaking, and bioconversion of plant cell walls into value-added products.[13] Additionally, pectinases can be used in the production of low-calorie fruit juices, functional foods, and the development of novel food-processing technologies.[16] They also play a role in the production of biofuels, biodegradable plastics, and the bioremediation of contaminated soil and water.[19] Alkaline pectinases derived from *Bacillus* spp. are particularly important.[20-23] Alkaline and thermostable pectinases are crucial for industrial applications.[24] The present investigation is based on pectinolytic activities of wild-type *Bacillus subtilis* strains isolated, identified, and the characterized enzyme was characterized with respect to temperature and pH. Production conditions were optimized, and the use of cottonseed derivative (Pharmamedia) was used for the first time for the production of pectinase.

2 | MATERIALS AND METHODS

2.1 | Screening for pectinase producers

Fifty bacterial strains randomly collected from soil and wastewater from a fruit juice industry area were tested for their ability to produce pectinase. This was accomplished by spotting the strains onto pectin-agar plates containing K_2HPO_4 (0.2% w/v), NaH_2PO_4 (0.1% w/v), $MgSO_4{\cdot}7H_2O$ (0.05% w/v), NH_4NO_3 (0.2% w/v), yeast extract (0.1% w/v), and pectin (1% w/v) at pH 7.2. Plates are incubated at 37°C for 24 h. Once the colonies had reached approximately 3 mm in size, the plates were flooded with either 1 M $CaCl_2$ or Lugol iodine. The appearance of halo zones around a colony indicated the presence of pectinase production. One strain was chosen for further investigation due to its ability to produce the largest zones of pectin degradation.

2.2 | DNA extraction PCR and ribotyping

Genomic DNA extraction was done using gene extraction kit (Biorad) from overnight incubated bacterial culture at 37°C in Luria–Bertani (LB) broth and with rotary shaking at 120 per minute. Amplification of 16S rDNA was performed successfully following the PCR method described by Alajlani[24] with forward primer 27f (5-AGAGTTTGATCCTGGCTCAG) and reverse primer 1522r (5-AAGGAGGTGATCCA(AG)CCGCA).

2.3 | Culture conditions

Selected strains were used for enzyme production assays in liquid medium. Liquid medium (YEP) containing (percent w/v): glucose, 1; K_2HPO_4, 0.2; NaH_2PO_4, 0.1; $MgSO_4{\cdot}7H_2O$, 0.05; NH_4NO_3, 0.2; yeast extract, 0.3; pectin, 1; pH 7.2 was inoculated with a suspension of 10^6cells/mL. Cultures were grown in 250 mL Erlenmeyers flasks with 50 mL of medium in a rotary shaker (150 rpm) at 37°C. After 24 h, the biomass was separated by centrifugation at 1000*g* for 20 min, and the supernatant was used to evaluate pectinase activity.

2.4 | Cup–plate assay of pectic enzymes

The "cup–plate" method for the assay of pectinases (hydrolase, lyase, and esterase) was peformed. General pectinase activity was assayed at a pH of 7, and the medium contained (percent, w/v): pectin, 1; Ionagar no. 2 (Oxoid), 1.5; 0.1 M potassium phosphate buffer, and pH 7. Pectinase activity was detected as breakdown of pectin indicated by a clear zone around the cup, which was recorded as millimeters of the cleared zone. Larger is the diameter the higher is the activity.

2.5 | Spectrophotometric assay

Pectinase activity was determined by measuring the amount of D-galacturonic acid released from pectin. The reaction mixture containing 0.5 mL of an appropriately diluted cell-free supernatant and 0.5 mL of 0.5% pectin (apple pectin in potassium phosphate buffer, pH 7.0) was incubated at 40°C for 1 h, and the final products were quantified using dinitrosalicylic acid (DNSA) reagent. One pectinase unit was defined as the amount of enzyme cat-

alyzing the formation of 1 μmol galacturonic acid per milliter per 1 minute under the assay conditions.

2.6 | Optimization of production conditions

The influence of growth conditions and medium on pectinases production was determined by growing the strain in aerobic conditions at 37°C for 24 h in five different media: yeast extract-pectin broth (YEP),[25] optimized medium,[26] Landy broth,[27] M9-minimal salt medium,[28] LB medium[29] and Pharmamedia (commercial medium).[30] M9 medium was also supplemented with several carbon sources: glucose, fructose, sucrose, lactose, maltose, mannitol, pectin, and glycerol, to test their influence on enzyme production. The YEP medium was used to grow the producer strains for 24 h, at different temperatures (30°C, 35°C, 40°C, 45°C, and 50°C), pH (5, 6, 7, 8, and 9), and aeration (150 rpm) versus static conditions in order to determine the best conditions for the production of the enzyme. The producer strain was incubated in various different conditions. The grown culture was centrifuged (10,000*g* for 10 min) to obtain culture-free supernatant that is tested to establish different pectinolytic activities.

2.7 | Pectinase purification and Physico-chemical characterization

An incremental saturation of ammonium sulfate (0%−60%, 60%−80%, and 80%−90%) was added to the crude enzyme. The precipitate collected subsequently further purified at 10 kDa cut-off dialysis membrane. Final, purification step is carried out in DEAE cellulose column. SDS page was performed to determine the enzyme size. Purification steps were carried out in cold environment and monitored for specific enzymatic activity.

Pectinase activity was determined in different pH (5–10) and temperature (30°C–70°C) ranges. The buffers used were sodium acetate (pH 5.0), potassium phosphate (pH 6.0–7.0), Tris-HCl (pH 8.0), and Gly-NaOH (pH 9.0–10.0). In addition, the influence of different temperatures (0°C–90°C) and pH values (5–11) on the stability of pectinase was determined. To check the temperature stability, the enzyme solutions were kept in the temperature range from 0°C to 90°C for 1 h. To check pH stability, the enzyme solutions were dispersed in 0.1 M buffer solutions with different pH values, and kept at 25°C for 24 h. An aliquot was assayed for residual activity. Pectinase activity at 20°C and pH 7.0 was assumed to be 100%.

3 | RESULTS

3.1 | 16S rDNA sequence analysis and phylogeny

Nearly complete 16S rDNA sequences (1495 nucleotides) were determined, and sequence homology was analyzed using BLAST-N (NCBI website). Strain Mz-12P showed more than 98% homology *B. subtilis* strain E9 (DQ474759), respectively. The sequence was submitted to GenBank and an accession number was obtained (OQ423207).

3.2 | Optimization of production conditions

Six different media: LB, yeast extract-pectin broth (YEP), optimized medium, Landy broth, M9 (minimal salt medium), and Pharmamedia (commercial medium), were tested for pectinase production. Maximum activity was observed in YEP and commercial medium (Figure 1). Strain Mz-12P showed maximum activity in M9 medium by using maltose/sucrose and pectin as a carbon source, while least enzyme production with lactose. Strain Mz-12P generally showed better activity in M9 medium (with all the nutrients tested) (Figure 2). The YEP medium was altered to various pH values ranging from 5.0 to 9.0 to investigate the effect on enzyme production. Illustrated in Figure 3, the optimal pH for the Mz-12P strain was determined to be 9.0, which resulted in maximum production. The impact of incubation temperature on enzyme production is depicted in Figure 3. As the temperature increased from 30°C to 50°C, enzyme production decreased, with the lowest production observed at 50°C. However, aeration did not significantly impact enzyme production, as determined by comparing shaking and stationary cultures.

3.3 | Puruification and Physico-chemical characterization

Ammonimum perciptation resulted in 1.8-fold purification (3.06 U/mg and a total protein content of 5.69 mg/mL), while DAED column resulted in 5.2-fold increase accounting for 16.36 U/mg. SDS-PAGE analysis confirmed the enzyme size to be 22.3 kDa. The effect of pH on pectinase activity was determined by using reaction mixtures at pH values ranging 5.0–10.0 (Figure 4). Pectinolytic activity was the highest at pH value 9.0. Optimal temperature for pectinase activity was determined by incubating the reaction mixtures at 30°C–70°C. Maximal activities were obtained

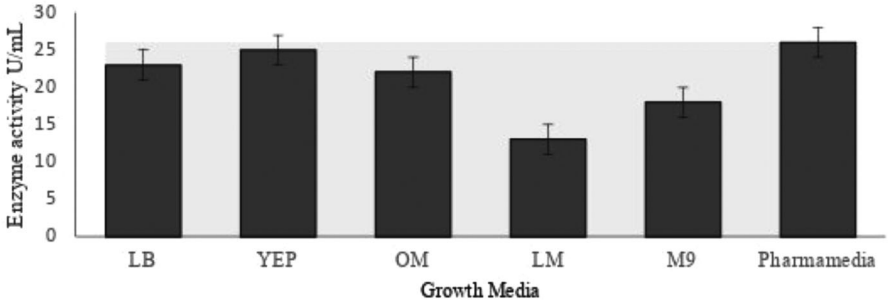

FIGURE 1 Pectinase production in different media (LB: Luria–Bertani; YEP: yeast extract pectin; OM: optimized medium; LM: Landy medium; M9: minimal 9 salt) and Pharmamedia. Bars represent standard deviations.

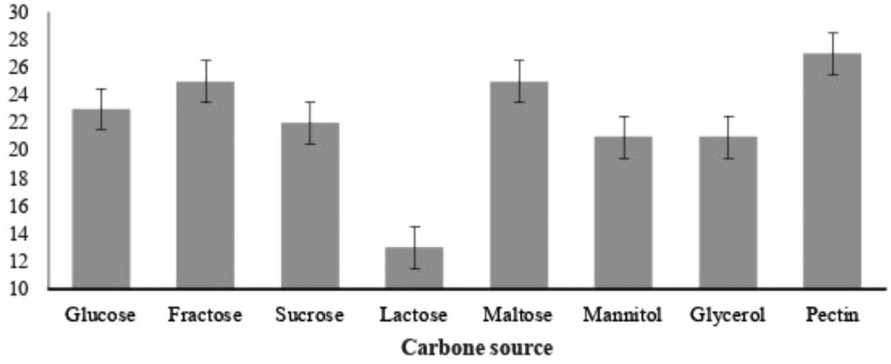

FIGURE 2 Effect of carbon source on pectinase production. Strains were grown in minimal medium (M9) supplemented with respective carbon source. Bars represent standard deviations in enzymatic activity in U/mL.

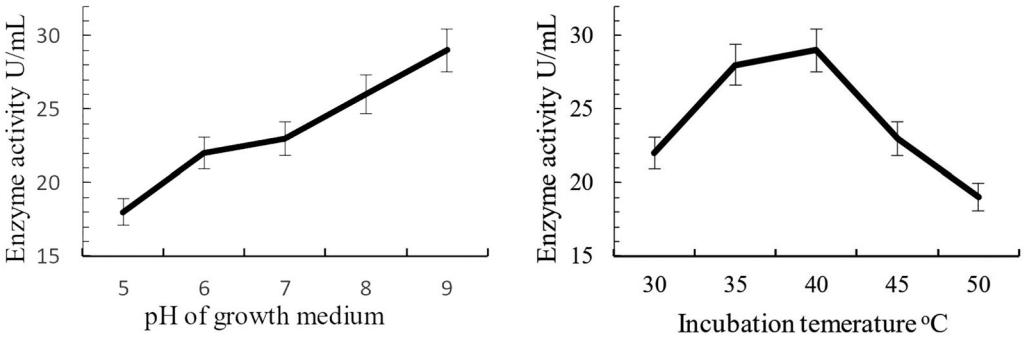

FIGURE 3 Pectinase production by *Bacillus subtilis* Mz-12P when grown in yeast extract pectin medium (YEP), adjusted to different pH and incubated at different temperatures. Bars represent standard deviations.

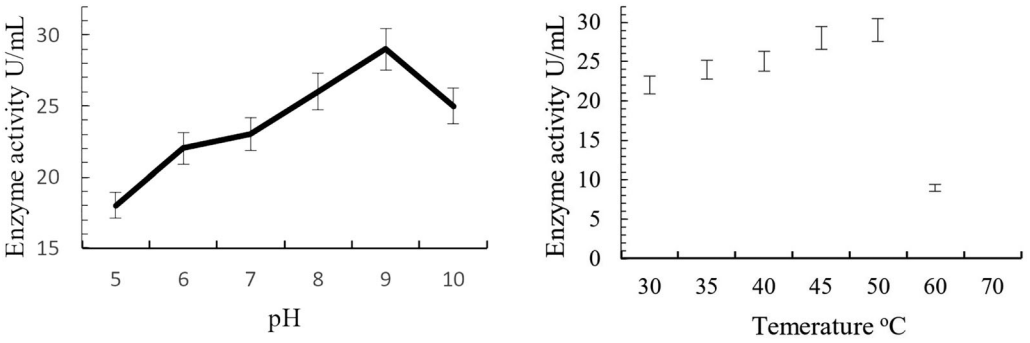

FIGURE 4 Effect of pH and temperature on pectinase activities from *Bacillus subtilis* Mz-12P. Bars represent standard deviations.

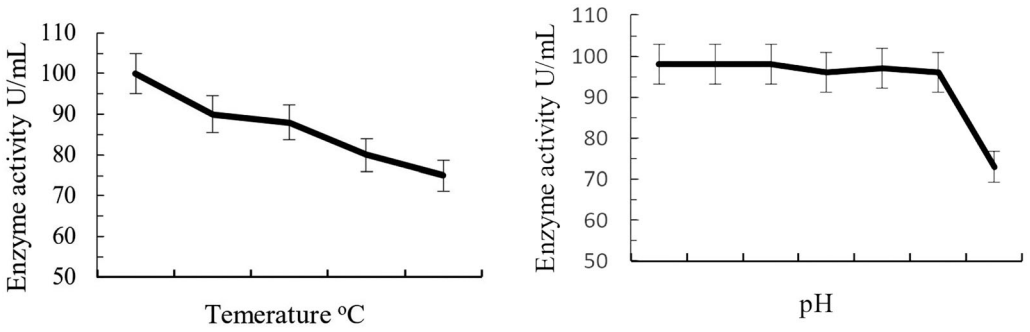

FIGURE 5 Stability of the pectinase from *Bacillus subtilis* Mz-12P against temperature (A), and pH (B). Bars represent standard deviations.

TABLE 1 Cup–plate assay of pectinolytic enzymes produced in yeast-extract pectin broth.[a]

	Activity (diameter in mm)			
Strain	**Pectinase**	**Pectin hydrolase**	**Pectin lyase**	**Pectinesterase**
Mz-12P	25.0	12.0	15.0	14.0

[a]General pectinase activity was assayed at pH 7.0; hydrolase activity was assayed at pH 5.2; lyase and esterase activities were assayed at pH 8.6.

at 50°C (Figure 4). To determine the effect of temperature on the stability of pectinase, enzyme solutions were incubated for 60 min at temperatures between 0°C and 90°C and the residual activity was assayed. Increasing temperature from 0°C to 90°C led to gradual decrease in activity. Enzymatic activity retained upto 82% of activity after 1 h at 90°C, respectively (Figure 5). Stability of the enzyme at pH values between 5.0 and 11.0 was assessed by incubating enzyme solutions for 24 h at 25°C in respective buffers. Both increasing and decreasing pH from neutral resulted in activity decline (Figure 5). Activity of strain Mz-12P exhibited largest spectrum of pH stability.

3.4 | Determination of specific enzymatic activities

Apart from general pectinase activity, specific activities (pectin hydrolase, lyase, and esterase) were determined by "cup–plate" assay (Table 1). All three types of pectinases were detected in these strains. This would provide an advantage over the production of single enzyme, and will provide a wider range of biotechnological applications.

3.5 | Pharmamedia as production medium

The medium provided an optimum pectinase activity with 26 U/mL. This is higher than all tested media (LB, YEP, OM, LM, and M9). We have noted even higher pectinase production of 32 U/mL upon supplementing Pharmamedia with 0.5% of fructose as a favorable source of carbon for *Bacillus* spp.

4 | DISCUSSION

Pectinase is an enzyme that breaks down pectin, a complex polysaccharide found in the cell walls of plants.[31] The role of pectinase in nature is well established: it has in the ripening of fruits, the softening of vegetables, and the decomposition of plant material.[32] Strain Mz-12P, isolated from wastewater from a fruit juice industry, could potentially be used in pectic waste water treatment and the fiber industry. Strains identified were studied for their potential to produce pectinases, and all three types of pectinase activity (hydrolase, lyase, and esterase) were observed. The strain was characterized and identified using 16S rDNA to be *B. subtilis* Mz-12P. The highest pectinase production was obtained in yeast extract-pectin broth medium, and pectin enhanced enzyme production in complex medium. Pectinase production was constitutive in the absence of pectin and optimal under mesophilic conditions with a slightly alkaline pH. It also exhibited high yield of pectinase when grown on Pharmamedia. The later medium is produced as a cottonseed derivative growth medium for microorganism and represent an excellent environmentally friendly medium. Pharmamedia has been described previously by Al-Ajlani et al.[30] as an optimum medium for the production of surfactin by *Bacillus* spp. The medium is prepared from cottonseed derivatives with favorable composition

that included 58% proteins, 24% carbohydrate, minerals, and vitamins. Interestingly, there is about 2.55% fiber leftover that might induce pectinases production and hence prove to be a superior medium for pectinase production. The environmental value of Pharmamedia is also of great value, as it is considered as ecofriendly medium and helps in cotton-oil production recycling.

Yeast extract-pectin broth was the best medium for pectinase production, as previously reported.[25] Pectinase production in the absence of pectin (in complex as well as minimal medium) proved its constitutive nature similar to what has been found in *Bacillus* GK-8.[32] Presence of pectin, however, enhanced the pectinase production in complex medium (YEP). Pectin as a sole source of carbon was optimal for enzyme production, explaining the advantage of cottonseed derivative medium. The stain produced the highest enzyme activity (30 U/mL) at a pH of 9.0, and has a wide temperature range for growth and enzyme production. This value is more than that reported for a highly alkaline pectinase producer *Bacillus pumilus* dcsr1 (25.33 U/mL).[33] Pectinases from the studied strains exhibited good stability at higher temperatures and extreme pH.[34] The pectinase from *B. subtilis* Mz-12P exhibited optimal activity at a maximum temperature of 50°C and pH of 9.0. Given its useful properties, this strain is a potential candidate for enzyme production at a commercial level.

DATA AVAILABILITY STATEMENT

The data for this article are available in GenBank and can be accessed with [OQ423207] from March 1, 2023. Data as well as material are also available from authors upon request.

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