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Isolation and Characterization of Exopectinase from *Bacillus licheniformis* FMB9 Isolated from Agricultural Soil

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Abstract
Objectives: This study aimed to isolate and identify potential pectinase producing bacterium as well as optimization of its various parameters for maximum enzyme production.
Methods: A total of forty-three bacterial isolates were obtained from agriculture soil in Jeddah city using standard plate count method. Primary screening was done by hydrolysis of pectin on agar plate and measuring the clear zone after adding iodine-potassium iodide solution. Pectinase activity was determined by measuring the increase in reducing sugar formed by the enzymatic hydrolysis of pectin.
Results: Among the bacterial isolates, the isolate FMB9 exhibited higher pectinase activity in broth medium and was selected for further studies. The selected bacterial isolate FMB9 was identified as *Bacillus licheniformis* FMB9 with similarity level 97% to *B. licheniformis* AS10. The isolate was found to produce maximum pectinase at 37°C with pH 7 upon incubation for 72 hours, while cultured in production medium containing citrus pectin and yeast extract as carbon and nitrogen sources, respectively. The enzyme was purified using column chromatography and was characterized. It showed maximum activity at 45°C. *Bacillus licheniformis* pictinase was affected by pH values and optimum activity was at pH 5. The molecular weight was also determined and compared with other pectinases.
Conclusion: Pectinase produced from bacteria can be purified and used in many technological applications in food and medicine.
Keywords: Pectin; pectinase; *Bacillus licheniformis;* enzyme activity; 16s rRNA

1. Introduction

Pectin is an important component of the middle lamella and primary cell wall of higher plants. Pectins are high molecular weight acidic heteropolysaccharide primarily made up of α (1–4) linked d-galacturonic acid residues.[1] Degradation of pectin is difficult, and pectinases enzyme can help in the degradation of pectin.[2] Pectinases consist of a unique group of enzymes that catalyze the degradation of pectic polymers, they belong to the polysaccharidase family, also known as pectolitic or pectic enzymes, which contribute to the breakdown of pectins from various plants. In the current biotechnological period, pectinase is one of the increasingly used enzymes.[3] Pectinases, used since 1930, make up a significant part of industrial enzymes. There are different sources of pectinolytic enzymes; including bacteria, fungi, plant,[4] and no evidence was found in animal.[5] Microbial enzymes are routinely used in many environmentally friendly and economic industrial sectors.

Pectinases are enzymes which are widely distributed in microbes that are present in pectin enriched sites. The agro-industrial residues can be utilized in the industrial scale for low-cost and efficient pectinase production in an eco-friendly approach.[6]

Most pectin-degrading microorganisms are associated with raw agricultural products and with soil. Up to 10% of the microorganisms in soil have been shown to be pectinolytic.[7,8] These include, but are not limited to, bacteria in the genera *Pantoea*, *Achromobacter*, *Aeromonas*, *Arthrobacter*, *Agrobacterium*, *Enterobacter*, *Bacillus*, *Clostridium*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Xanthomonas*.[9-11] And many yeasts, molds, protozoa and nematodes.[12] Also pectolytic activity was found in a strain of *Leuconostoc mesenteroides*.[13]

Bacillus is one of the large genera of bacterial strains. It is a rod shaped, endospore bearing bacteria and belong to the family Firmicutes. The genus *Bacillus* covered a great diversity of strains and some of them are strictly aerobic, while others are facultative anaerobic. The *Bacillus* especially *B. licheniform* is capable of growing on a large diversity of nutrient sources because of synthesizing and secreting different hydrolytic enzymes and this quality makes the *B. licheniform is* an industrially important microorganism.[14]

In the current study, different bacterial strains were isolated from agriculture soil and screened for pectinase production. Finally, maximum pectinase producing bacterial strain was identified using conventional and molecular techniques.

2. Materials and Methods

2.1. Isolation of Bacteria

Several bacterial strains were isolated from soil samples collected from agriculture soil in Jeddah city. Isolation of bacteria was done by serial dilution plate method and incubated at 37°C for 24 hrs. The isolated colonies were selected to obtain pure bacterial cultures.

2.2. Screening of Pectin Producing Bacteria

Screening was performed to detect the presence of bacteria that degrades pectin. Pectin agar medium was prepared with (g/L): $NaNO_3$ 1.0, KCl 1.0, K_2HPO_4 1.0, $MgSO_4$ 0.5, yeast extract 0.5, citrus pectin 10 and agar 20 with pH adjusted to 7.0 (Kumar et al., 2012).Pure culture was inoculated by puncture in the medium and incubated for 48 hrs at 37°C. After incubation, iodine-potassium iodide solution was added to detect the clearance zone.[15]

2.3. Quantitative Screening of Bacterial Strains for Pectinase Production

The bacterial strains showing clear zone of hydrolysis on pectin agar medium were screened for pectinase production using pectin broth medium. The pure cultures were inoculated and incubated at 37°C for 24 h. After incubation, biomass was separated by centrifugation at 10,000 rpm for 15 min. The supernatant was used to evaluate pectinase activity.[16]

2.4. Enzyme Assay

Pectinase activity was measured by the estimation of the amount of galacturonicacids through the DNS method (Miller, 1959)[17] using 1.0% citrus pectin as a substrate and mono-Dgalacturonic acid as a standard. One unit of pectinase was defined as the "amount of enzyme required to generate 1 mole of galacturonic acid under standard assay conditions".

The enzyme activity (U/mL) was calculated according to equation:

$$\text{Enzyme activity (U/mL)} = \frac{(\mu\text{g galacturonic acid released} \times V)}{V \times 194.1 \times t}$$

where v is the enzyme broth volume used in the assay, 194.1 is the molecular weight of galacturonic acid, and t is the reaction time in min. One unit (U) is equivalent to 1 µmol product released per min.

Relative activity of the enzyme was calculated as the percentage by using the following formula:[18]

$$\text{Relative activity} = \frac{\text{Activity of the sample} \times 100}{\text{Maximum activity of the sample}}$$

2.5. Identification of Bacterial Strain

Morphological characteristics, biochemical characterization of the selected bacterial strain were studies for identification.[19] Molecular characterization 16S rDNA sequence analysis was performed for molecular based identification of selected isolate. The genomic DNA was extracted using the method reported by Chen and Kuo (1993).[20]

2.6. Optimization of Cultural Conditions for Pectinase Production by *Bacillus licheniformis* FMB9

The bacterial strain that showing high pectinolytic activity was selected. The effects of temperature, pH and incubation period on production of pectinase were studied for maximum pectinase production.

The bacterial isolate was subjected to different temperatures (25, 30, 37, 40 and 45°C) for 72 h then the enzyme activity was measured. After selection the best optimum temperature of pectinase production, the effect of pH value on pectinase production of the selected bacterial isolate were studded. The medium was prepared with different pH values (pH 5.0, 6.0, 7.0, 8.0 and 9) and incubated 72 h with agitation at 120 rpm at 37°C. After growth period, pectinase activity was measured. After selection the best pH of the medium, the effect of different incubation period (24 h, 48 h, 72 h, 96, and 120 h) was determined. After incubation the enzyme assay was measured.

2.7. Purification and Enzymatic Optimization

Production of pectinase by *Bacillus licheniformis* was carried out for 72 hrs and the enzyme was precipitated by 80% Ammonium sulfate in the refrigerator at 4°C, the precipitate was collected and centrifuged at the same temperature and the obtained crude enzyme was dialyzed at 4°C for two days with citrate buffer of pH 5. The obtained protein was frozen at −200°C and lyophilized in a lyophilizer. The enzyme was purified using sephadex G-100 and DEAE-cellulose columns chromatography where the enzyme was directly applied to the agarose column (1 × 30 cm.) and elution was carried out by liner gradient of NaCl (0.1–0.6 M). Many fractions (60), each one bout 5 ml, were collected and enzyme activity and protein content (A_{280}) were determined. Total protein was determined by the method of Lowery et al. (1951)[21] by using serum albumin as a standard.

2.8. Characterization of the Purified Enzyme

The purified enzyme was characterized at different pH (5–9), temperature (20–60°C) and substrate and enzyme concentrations. The molecular weight of pectinase that was purified was determined using a vertical gel electrophoresis including 12% separating and 5% stacking gel and low molecular weight protein was used as standard (Laemmli, 1970).[22] Protein bands were examined after staining with Coomassie Brilliant Blue R-250.

2.9. Statistical Analysis

The data for all experiments have been calculated from three replications, with the values presented as the mean ± SE standard error).

3. Results and Discussion

Pectinolytic enzyme can be derived from different sources.[23] However, pectinase producing microorganisms have due advantage over other sources because they can be subjected to genetic and environmental manipulations to increase yield.[24] It has been reported that most *Bacillus* sp, enhances the production of pectinase.[25]

Forty-three bacterial strains were isolated from agriculture soil in Jeddah city on nutrient agar. The pectinolytic activity was detected by visualizing a clear zone around the colony using potassium–iodide flooding method.[17] Among these isolates only twenty-five isolates showed pectinolytic activity on pectin agar medium (Table 1). The isolate FMB9 (Figures 1, 2) which was Gram positive bacterium, isolated from soil on nutrient agar medium demonstrated the largest hydrolysis zone around its colony, meaning the highest pectinase activity, compared to the other strains.

To identify the selected isolate FMB9, both traditional microbiological methods and modern molecular technologies were considered. On the basis of observed morphological, cultural, and biochemical characteristics, the colony of selected bacterial isolate FMB9 was large and the margin was undulate and had white creamy color. It cells were Gram positive, motile and producing endospore. This isolate had shown positive test for catalase, oxidase, starch hydrolysis, protease and can able to ferment glucose and sucrose whereas, negative for urease, citrate utilization, gelatin liquefaction and DNAase. The results obtained were compared with identification flowchart of Bergey's Manual.[19] The characteristics showed by this organism were fairly similar to *Bacillus licheniformis*. The details of biochemical characteristics of pectinase producing strain are given in Table 2. The phylogenetic tree generated using 16S

Table 1. **Activity of the bacterial strains isolated from agriculture soil for peptidase activity on pectin medium (mm) and in liquid medium (U.ml). Values are mean ± SD of 3 replicates**

Bacteria isolates	Diameter of clear zone (mm)	Pectinase activity (U/ml)
FMB 1	19.0 ± 2.3	1.191
FMB 2	10.3 ± 2.3	1.0 ± 0.3
FMB 3	33.0 ± 2.3	0.5 ± 0.3
FMB 4	14.3 ± 2.3	0.611
FMB 5	28.6 ± 2.3	0.6 ± 0.3
FMB 6	27.9 ± 2.3	1.2 ± 0.4
FMB 7	13.3 ± 2.3	0.4 ± 0.0
FMB 8	13.3 ± 2.0	0.6 ± 0.3
FMB 9	33.6 ± 2.8	1.4 ± 0.5
FMB 10	30.3 ± 2.0	1.2 ± 0.4
FMB 11	30.9 ± 2.0	0.7 ± 0.3
FMB 12	28.3 ± 3.3	0.7 ± 0.1
FMB 13	30.0 ± 3.3	0.7 ± 0.3
FMB 14	17.8 ± 2.4	0.8 ± 0.2
FMB 15	32.1 ± 2.9	0.6 ± 0.1
FMB 16	17.1 ± 2.7	0.5 ± 0.1
FMB 17	24.6 ± 2.8	0.7 ± 0.3
FMB 18	30.1 ± 2.9	0.7 ± 0.1
FMB 19	28.3 ± 2.0	0.6 ± 0.1
FMB 20	15.3 ± 2.1	0.5 ± 0.2
FMB 21	24.3 ± 2.0	0.7 ± 0.3
FMB 22	29.3 ± 2.3	0.9 ± 0.3
FMB 23	23.0 ± 1.4	0.6 ± 0.2
FMB 24	24.6 ± 5.1	0.7 ± 0.0
FMB 25	26.3 ± 2.5	0.5 ± 0.1

Table 2. **Morphological, physiological and biochemical characteristics of maximum pectinase producing bacterial strain**

Characteristics	Results
Temperature range	20–45°C
Optimum temperature	37°C
pH range	5.5–10.0
Optimal pH	6.5–7.5
Catalase	+
Oxidase	+
Urease	–
Protease	+
DNAase	–
Hydrolysis of starch	+
Gelatine liquefaction	–
Citrate utilization	–

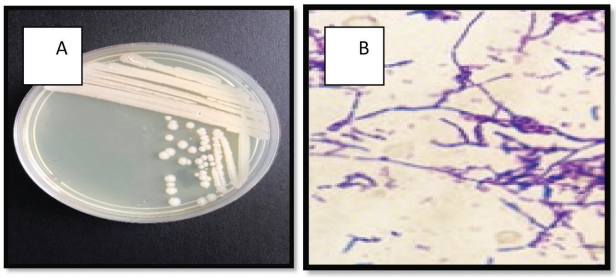

Fig. 1 **Isolate FMB9, A: On nutrient agar, B: Under light microscope after Gram staining.**

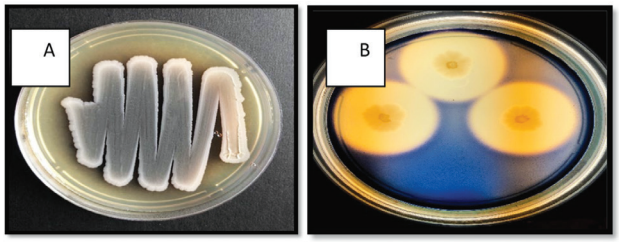

Fig. 2 **A: Isolate FMB9 growth on pectin agar medium, B: Qualitative screening of the isolate FMB9 for pectinase on pectin agar medium.**

rDNA gene sequences of the bacterial isolate showed that the bacterium has the highest homology (99%) with *Bacillus licheniformis* (GenBank Accession Number KJ729823.1) and designated as *Bacillus licheniformis* AS10. Figure 3 signifies the phylogenetic tree of the isolate FMB9 with the selected best homologous known bacterial strains.

For the production of pectinase in optimum cultural condition the bacteria strain was grown on pectin broth media. Factors such as temperature, pH and incubation periods were varied to analyze the optimum enzyme production by the bacteria strain.

Enzyme production went up with the increase of temperature up to 37°C and then declined (Figure 4). The maximum production which occurred at this temperature was 2.77 U/ml. This dramatically reduced to nearly 16.6% at 50°C. In the previous study of Aaisha and Barate (2016),[26] the highest pectinase production was observed from some *Bacillus* species at 37°C which is similar to our current study.

Enzyme activity also depends on the pH of the reaction mixture. Figure 4 depicts the effect of different pHs on the production of pectinase by *Bacillus licheniformis*. Maximum production (2.64 U/ml) was recorded at pH 7 (Figure 5). This finding is in accordance with other workers who reported that most of the *Bacillus* sp. that produce high amount of pectinase between pH 7 and 8 (Oumer and Abate, 2017).[27] At highly acidic and alkaline pH, enzyme production decreased by almost 61.37 % and 51.5%, respectively.

An attempt was made to determine the most favorable incubation period for enzyme production by the selected isolate and the highest enzyme production (2.852 U/ml) was recorded after 72 hours of incubation (Figure 6). The enzyme production gradually decreased to 0.361 U/ml at 120 hours of incubation which is almost 51% less than that of maximum. This might be due to the accumulation of waste products at prolonged incubation time with limited nutrient sources which consequently suppressed the growth of microorganism. According to Nawawi et al. (2017)[28] maximum pectinase

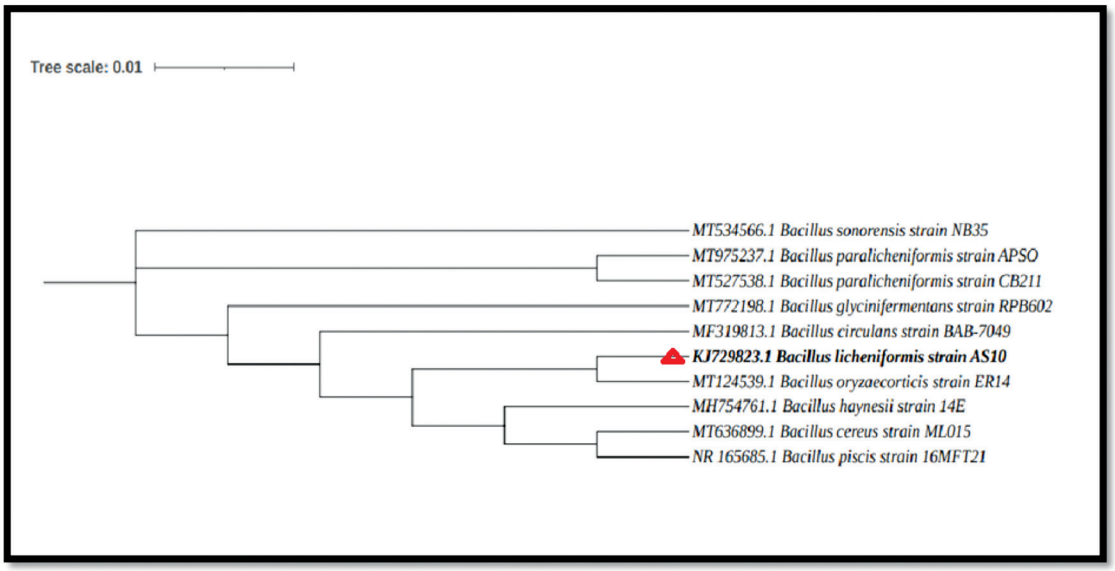

Fig. 3 **Phylogenetic tree of the identified bacterial isolate FMB9 based on the 16S rDNA sequences. The GenBank accession number is given in parentheses for each organism.**

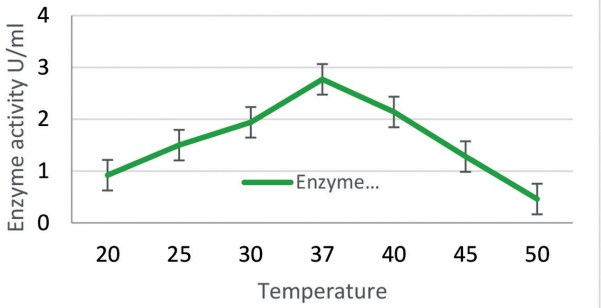

Fig. 4 **Effect of different temperature on pectinase production by *Bacillus licheniformis* FMB9.**

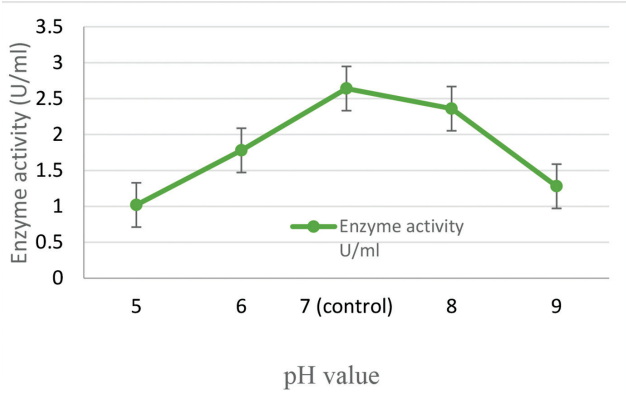

Fig. 5 **Effect of different pH on pectinase production by *Bacillus licheniformis* FMB9.**

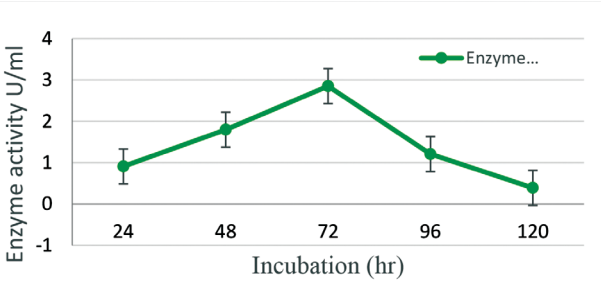

Fig. 6 **Effect of different incubation period on pectinase production by *Bacillus licheniformis* FMB9.**

production was determined from the *Bacillus subtilis* ADI1 after 72 hours of incubation which well agreed with our findings. On the other hand, this study is disagreeing with other study which reported that *Bacillus licheniformis* strain DY2 had maximum pectinase production after 44 hrs.[29]

The elution profiles of the crude enzyme of isolate FMB9 after using Sephadex G-100 and DEAE-cellulose columns chromatography are shown in Figures 7 and 8, respectively. For each column chromatography, the fractions with the highest pectinase activity have been collected, concentrated by lyophilization and the enzyme activity was determined. Molecular weight of the pure enzyme, detected using SDS-PAGE analysis, was ~43 kDa (Figure 9). According to the SDS-PAGE analysis, two extracellular pectinase of 60 and 64 kDa were obtained from *Bacillus subtilis* by Takcı and Turkmen (2016).[30] Molecular weights of the partial purified pectinase from various bacteria species were determined as follows: 37 kDa for *Paenibacillus xylanolyticus* and *Bacillus* sp. MFW7,[31,32] 89 kDa for *Bacillus cereus* NRC20,[33] 31 kDa for *Streptomyces* sp. GHBA10,[34] 106 kDa for *Bacillus* sp. DT7[35] and 66 kDa for *Bacillus* sp. MBRL576.[36] Higher molecular weight enzymes were isolated from *Kluyveromyces marxianus*[37] while an average molecular weight of 38–65 kDa, the enzymes (for both exo- and endopolygalacturonases) are separated from various microbial sources.[38] The purified enzyme showed maximum activity at 45°C and pH 5 (Tables 3, 4) in addition to 0.7 mM of the substrate (Figure 10). It was found that increase enzyme and substrate concentrations enhanced enzyme activity. In the presence of excess substrate, increasing enzyme concentrations increased the enzyme activity (Figure 11). Addition of Mg^{2+}, K^{+}, Zn^{2+} and Ca^{2+} ions significantly increased the enzyme activity while Na^{+}, Fe^{2+} and Cu^{2+} in addition to EDTA decreased the activity (Table 5).

Pectinases or pectinolytic enzymes that hydrolyze pectic substances are produced by living cells in the presence of

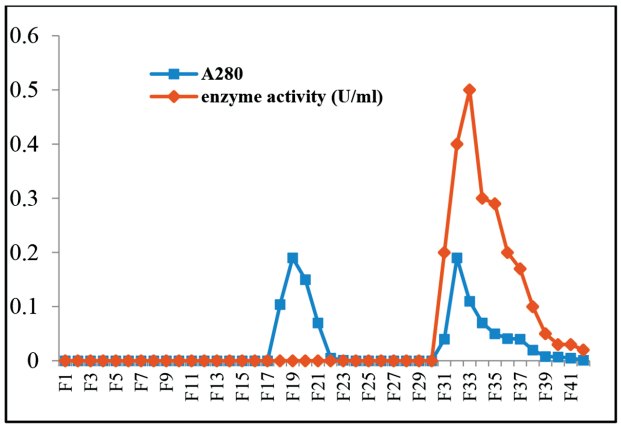

Fig. 7 **Elution profile of pectinase after sephadex G-100 chromatography.**

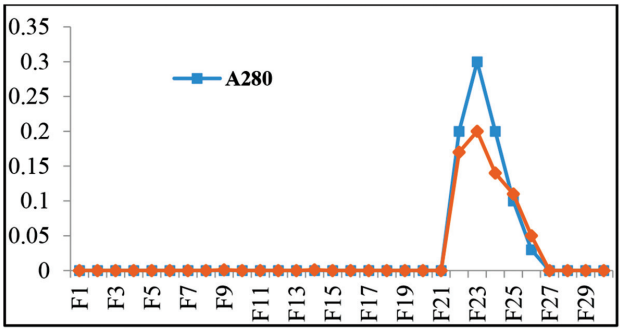

Fig. 8 **Elution profile of peptidase after DEAE-cellulose chromatography.**

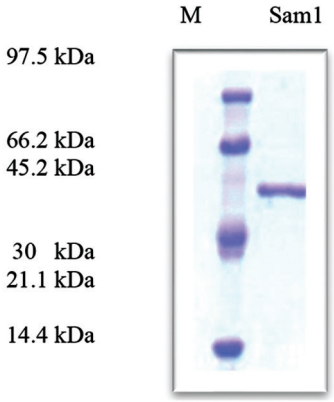

Fig. 9 **The molecular weight of purified pectinase isolated from isolate FMB9 by SDS-PAGE, M: protein standards employed were phosphorylase (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa), lane 2: Purified pectinase.**

pectin. They are classified into three groups, protopectinases, esterase, and depolymerases.[39] *Protopectinases* break down protopectin to soluble pectin, Esterase which removes methoxyl and acetyl esters from pectin forming polygalacturonic acid and *Depolymerases* which breakdown α-(1 → 4)-glycosidic bonds in units either by hydrolysis or by trans elimination.[38,40] Most of the polygalacturonase enzymes stimulate the rate of hydrolysis at an ideal pH ranging from 3.5 to 5.5 with a suitable temperature that ranges from 30 to 50°C. Several findings relating to various biochemical properties like molecular

Table 3. **Effect of different temperature on *Bacillus licheniformis* pectinase activity**

Incubation temperature (°C)	Enzyme activity U/ml	Relative activity (%)
20	0.99* ± 0.12	47.8
25	1.10 ± 0.11	53.1
30	1.80 ± 0.25	86.9
37 (control)	2.07 ± 0.12	100.0
40	2.14 ± -0.28	103.3
45	2.28 ± 0.16	110.1
50	2.00 ± 0.15	96.6
55	1.09 ± 0.22	52.6
60	0.40 ± 0.21	19.4

*Values are mean ± SD of 3 replicates.

Table 4. **Effect of different pH on pectinase activity from *Bacillus licheniformis***

pH value	Enzyme activity U/ml	Relative activity (%)
5	2.69 ± 0.09	119.0
6	2.50 ± 0.12	110.6
7(control)	2.26 ± 0.13	100.0
8	2.20 ± 0.11	97.3
8.5	2.11 ± 0.22	93.3
9	1.33 ± 0.05	58.8

Values are mean ± SD of 3 replicates.

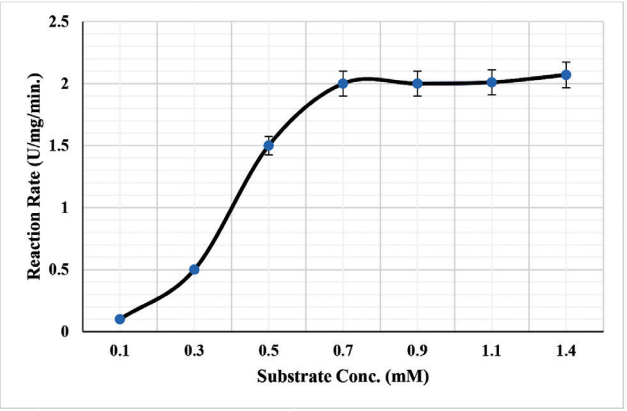

Fig. 10 **Effect of different substrate concentration on pectiinase activity.**

weight, pH, temperature, isoenzyme, isoelectric point, etc. are well-reported with respect to endopolygalacturonase in various bacterial and fungal species as compared to exopolygalacturonase and rhamnopolygalacturonase. As reported, almost all endopolygalacturonase as well as exopolygalacturonase enzymes are synthesized in acidic environmental conditions, whereas, some exopolygalacturonases are produced at high basic conditions (about pH 11.0) and by particular species including *Bacillus licheniformis, Bacillus* sp KSM-P410 and *Fusarium oxysporum*.[36] Regarding rhamnopolygalacturonases, it was stated that the enzymes are more stable and efficiently work at pH 4.0 and a temperature of 50°C.[41]

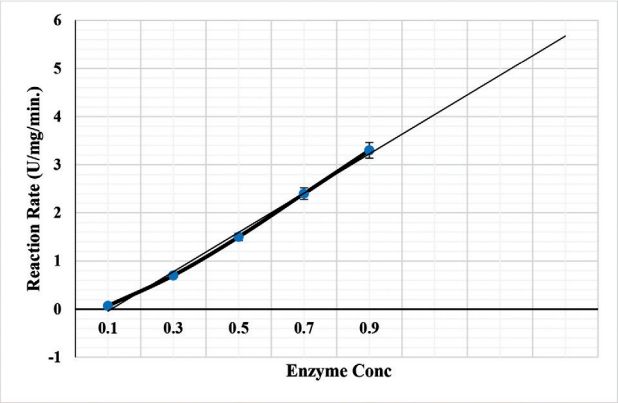

Fig. 11 **Effect of different enzyme concentrations on pectiinase activity.**

Table 5. **Effect of metal ions on pectiinase activity**

Metal ion (1 mM)	Relative activity (%)
Control	100
Na^{+}	90*
K^{+}	115
Mg^{2+}	110
Fe^{2+}	97
Cu^{2+}	90
Ca^{2+}	104*
Zn^{2+}	110*
EDTA	70

*Significant results using studied- *t*-test at $P < 0.05$ compared with control without addition.

Conclusion

In the present study, the extracellular pectinase was produced by using *Bacillus licheniformis* FMB9 isolated from agricultural soil in Jeddah city at Kingdom of Saudi Arabia. The strain showed maximum pectinase activity after 72 of growth at pH 7.0 and 37°C hrs. After purification, pectinase was characterized and molecular weight was determined.

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Conflict of Interest

None.

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