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Purification of Pectin Lyase Enzyme from *Bacillus pumilus* Bacteria by Three-Phase Partitioning Method (TPP), Nanoflower Preparation and Investigation of Fruit Juice Clarification

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Abstract: In this study, firstly, *Bacillus pumilus* bacteria were isolated from tomato vegetables and identified. Then, the new pectin lyase enzyme was purified from *B. pumilus*, characterized, and hybrid nanoflower pectin lyase (hNF-PL) was synthesized the first time in this study. For this purpose, PL enzyme was produced in a solid culture medium using *B. pumilus* bacterium, and PL was purified in 191.8 folds with a yield of 78.2% using the three-phase partitioning (TPP) technique. Using SDS-PAGE, PL enzyme was determined to have a single subunit, and molecular weight was defined as 32.88 kDa with gel chromatography technique. This is the very first study to easily immobilize purified PL enzyme onto nanoflower chitosan/calcium pyrophosphate hybrid NPs. The synthesized nanoflower hNF-PL structure was characterized by SEM, FT-IR, XRD, and TEM chromatographic methods. In the final phase of the study, the effects of the pure PL and hNF-PL enzymes on the clarification and cleavage rate of fruit juices obtained from black grape, pomegranate, peach, red apple, and plum were investigated. Under the light shed by this study determined that the hNF-PL enzyme clarified the fruit juices more effectively than the pure PL enzyme.

Keywords: pectin lyase (PL); *Bacillus pumilus*; three-phase partitioning (TPP); hybrid nanoflower pectin lyase (hNF-PL); fruit juice clarification.

1. Introduction

Structural polysaccharides that are present in plant walls and middle lamella are defined as pectic substances. These substances have a molecular weight of 30 -300 kDa. Most of the pectin is composed of D-galacturonic acid units [1]. Within the structure of pectin, galacturonic acid units are linked by α-(1,4) glycosidic bonds [2–5]. Pectinolytic enzymes break down pectins, a natural polymer of galacturonic acid [5]. Generally, these enzymes are produced in solid culture media. *A. niger*, *A. flavus*, *A. wentii*, *A. oryzae*, *Rhizopus liquefaciens*, *Geobacillus stearothermophilus* (Ah22), *Brevibacillus borstelensis* (P35), and *Penicillum* microorganisms are used in the production of PL enzymes. The biosynthesis of pectolytic enzymes is controlled by a catabolic or induction repression mechanism [5-7].

An important member of the pectinase enzyme complex is pectin lyase. Pectin lyase (PL, EC 4.2.2.10) is a microorganism that is the main source of PL enzymes, which are industrial enzymes that possess hydrolytic properties. 50% of them are obtained from fungi and yeast, 35% from bacteria, and the remaining 15% from plant or animal sources [7]. Pectin lyase enzymes known today are endogenous [8-10], and methylated galacturonic acid residues occur when they hydrolyze the glycosidic bond [11]. They prefer esterified pectin polymers and contain Ca^{2+} ions as cofactors [9]. While it is known that PL enzyme can be obtained from bacterial species belonging to the genera of *Aspergillus, Rhizopus*, *Saccharomyces*, *Bacillus, Kluyveromyces*, *Erwinia*, *Penicillium*, it is commercially obtained mostly from microorganisms species of the genera *Aspergillus, Penicillium, Erwinia*, *Aspergillus parasiticus* [10-15].

Pectinase is used in areas including but not limited to the food industry, processing of plant fibers, textile, paper industry [12–15], vegetable oil extraction [12–15], and the wine industry [16]. Pectinase enzymes are also used for fruit juice extraction and clarification and the prevention of gelation during shelf life in thick fruit juices [5,6,17,18].

To ensure continuous use or re-use of the enzymes by preserving the pure enzyme activity obtained by different methods, they are immobilized on organic or inorganic carriers by using chemical or physical methods [19-21].

When the enzyme is immobilized on the stationary material using the covalent immobilization technique, it may lose its enzyme activity due to its structurally affected active site. As of the 1990s, immobilized enzymes have been shown to protect their activities as much as do pure PL enzymes when immobilized onto nanoscale materials. In 2012, when the first organic-inorganic hybrid nanoflowers (NF) were found to retain the enzyme activity, a great interest has been drawn to the subject [21]. Nano-hybrid structures from nanomaterials are often called nano-flowers because of their flower-like appearance. Although these synthesized flower-shaped hybrid structures are on a micrometer scale *per se*, they are called nanoflowers just because the leaves that form them are nanoscale [21-23].

In this study, after isolation and molecular identification of *B. pumilus* bacteria from a tomato plant, PL enzyme was obtained by developing in a solid culture medium. Then, the extracellularly produced PL enzyme was purified by triple-phase separation and characterized. The pure PL enzyme was immobilized on an hNF-PL structure, and its usability in fruit juice clarification was investigated.

2. Materials and Methods

2.1. Bacteria isolation.

In this study, tomatoes from Erzurum local markets were used for microorganism isolation. A 10 g/mL of sampling was made from the work specimen under aseptic conditions, and it was homogenized with a 90 mL maximum recovery diluent (Merck 1.12535) solution by utilizing Stomacher homogenizer. Serial dilutions from 10^{-1} to 10^{-8} were prepared using the prepared maximum recovery diluent. Nutrient agar (Merck 1.05450) was spread from each dilution and then incubated at 34ºC for 48 hours. Nutrient agar medium was drawn from the typical colonies developed after incubation, and plotting was done by drawing 3 phases. This was continued until pure colonies were obtained. The purified colony was then transferred into the nutrient broth (Merck 1.05443) containing a 20% glycerol solution. It was then stored at -80 °C as stock culture.

2.1.1. Identification tests.

In this study, gram staining and catalase tests were performed on isolated microorganisms among general identification tests. According to the results of microbiological analysis, it was found to be gram-positive and catalase-positive.

2.1.2. Identification of microorganisms.

After isolation of microorganisms from tomatoes has been carried out, obtained pure cultures were identified at the species level. For this purpose, DNA analysis was performed before pure culture, and then 16S rRNA and 16S-23S rRNA gene (ISR) were identified by sequencing.

2.1.3. Genotype characterization of bacteria.

For the sequence analysis of the 16 S rRNA genes, universal primers LPW57 (5 W-AGTTTGATCCTGGCTCAG-3 ′) and LPW205 (5′-CTTGTTACGACTTCACCC-3 T) [22] were used. In the amplification of the ISR region, 16-1A (GTCGGAATCGCTAGTAATCG) and 23-1B (GGGTTCCCCCATTCGGA) [23] universal primers were used. Sequence analysis was made by Medsantek (Turkey) company. The results of the 16S rRNA and 16S-23S rRNA full sequence analysis of the study with other bacterial sequences in GenBank (http://blast.ncbi.nlm.nih.gov/blast.cgi) were compared, and the similarity ratio was determined [24]. It was determined that the isolate obtained was 99% similar to *Bacillus pumilus* bacteria in Genbank.

2.2. Pectic enzyme production with solid culture fermentation.

For enzyme production in microorganisms, the solid culture fermentation method was used [25]. Separate media were prepared by adding different amounts of pectin to 250 mL flasks (pectin ED 85% -93%) and 5 grams of bran and 10 ml broth solution ((0.14% $(NH_4)_2SO_4$, 0.2% KH_2PO_4 and 0.02% $MgSO_4$). Subsequently, these prepared media were sterilized in an autoclave at 121 °C for 20 minutes. Bacteria were planted in the medium after sterilization and incubated for 5 days at 34 °C in the oven. After incubation, 60 ml deionized water with 0.9% NaCl was added to the medium and mixed with the magnetic stirrer for 1 hour to allow the PL enzyme to pass to the solution phase. The supernatant (growth medium) and the precipitate (bran + microorganism) were separated by centrifugation at 10,000 x *g* (20 min at 4 ° C). PL activity and protein determination were performed in (pure extract) supernatants [26].

2.3. Purification of pectin lyase enzyme.

From *Bacillus pumilus* bacteria produced in solid culture medium, PL enzyme was purified and characterized by the three-phase partitioning (TPP) technique [27]. For this purpose, first n-butanol optimization and then ammonium sulfate optimization was carried out. Briefly; n-butanol and 8 g of ammonium sulfate were added to the reaction medium at a ratio of 1: 0.5, 1.0: 1.0, 1.0: 1.5, 1.0: 2.0. The mixture was stirred for 30 min in a magnetic stirrer. The resulting solutions were then centrifuged at 6000 x g for 10 min. Three-phase formation in the reaction media was observed, such as supernatant alcohol as the upper phase, precipitate middle phase, and water lower phase (Figure 1). The precipitate middle phase was dissolved in 0.05 M phosphate buffer (pH 8.0) with 1 mL and dialyzed against the same buffer for 3 hours.

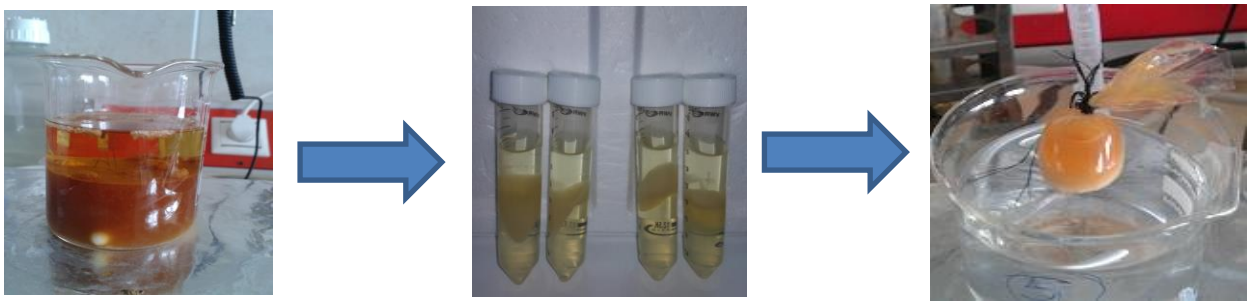

Figure 1. Purification steps of pectin lyase enzyme by the three-phase partitioning (TPP) method.

Enzyme activity was determined using pectin as a substrate at 550 nm. A blank sample was prepared by using phosphate buffer instead of the enzymes.

For ammonium sulphate optimization, pure extract and the optimum ratio of n-butanol (1.0:0.5%) were determined by adding 20%, 40%, 60% and 80% ammonium sulfate. The highest activity of ammonium sulfate was determined. As per this ratio, the optimum value was determined by a second ammonium sulfate precipitation. Purification of the PL enzyme was carried out according to the obtained parameters.

PL enzyme activity was determined by the method of colored derivation [28]. For pectin lyase activity measurement, the following procedure was followed. A 0.25 mL of the enzyme solution was added to 0.25 mL of substrate solution (1% pectin solution) and left at 50 °C for 10 minutes. Then 50 µL of 1 N NaOH was added to the 0.5 mL reaction mixture and incubated for 5 minutes in a water bath at 80 °C and then cooled to room temperature. A 0.6 mL of 1 N HCl and 0.5 mL of 0.04 M thiobarbituric acid solution was added to the cooled reaction mixture, incubated for a second time at 80 °C for 5 minutes, and cooled. The optical density of the obtained colored derivative was measured at 550 nm by using a UV-VIS spectrophotometer.

2.3.1. Protein determination.

Protein content was determined using the Bradford method. For this purpose, 100 µL of the enzyme was added to 5 mL of Coomassie brilliant blue solution. After vortexing, it was kept at room temperature for 10 minutes, and the absorbance value at 595 nm on the spectrophotometer was measured against the blank sample prepared using pure water instead of the enzyme [29]. Bovine Serum Albumin was used to determine the amount of protein in the enzyme samples.

2.3.2. SDS-PAGE electrophoresis.

To determine the subunit number of purified pectin lyase enzyme, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5% aggregate gel and 10% separation gel) was performed.

2.3.3. Determination of optimum pH and stable pH values.

The optimum pH values of pure and hybrid PL enzymes were determined by activity measurements with appropriate buffer systems at the pH range of 3-11. For this purpose; sodium acetate (pH: 3.0-5.0), phosphate (pH 6.0-8.0), Tris-HCl buffer (pH: 9.0-10.0) and sodium carbonate buffer (pH 11.0) were used. Activity measurements were made against a blank sample prepared with pure water instead of the enzyme [26].

Activity measurements were made between pH 3.0-11.0 to determine the pH stability of pure PL and hybrid PL enzymes. In different buffer systems (3-11), the reaction media prepared with pectin substrate were sampled for 1 week, and the stability of enzyme samples was determined [26].

2.3.4. Determination of optimum and stable temperature values.

In order to determine the optimum temperature value of the purified PL enzyme, activity measurements were made in the range of 0-90 ° C. Blank sample measurements were also determined using pure water instead of the enzyme at each temperature value. For determining the stable temperature of the enzyme; Activity measurements were made every 15 minutes for 2 hours in the range of 10-90 ºC. All measurements were performed against the blind sample [30].

2.3.5. Determination of V_{max} and K_M values.

Maximum velocities of pure PL and hybrid PL enzymes (V_{max}) and rate constants (K_M) were determined by activity measurements against 5 different concentrations of three different substrates (pectin, locus bean gum, and chitin). Vmax and K_M values of Lineweaver and Burk graphs were calculated for this purpose [31].

2.3.6. Effect of metal ions on pectin lyase activity.

Two different concentrations of Ca^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Hg^{2+}, and Fe^{3+} ions were measured on the activity of pure PL and hybrid PL enzymes. % Activity values were calculated from the findings [32].

2.4. Synthesis of nanoflower pectin lyase Ca^{2+} hybrid structure.

Nanoflower chitosan structure was synthesized by the ionotropic gelation method. Briefly, chitosan (CS) was dissolved in 1% acetic acid solution (2 mg mL^{-1}).

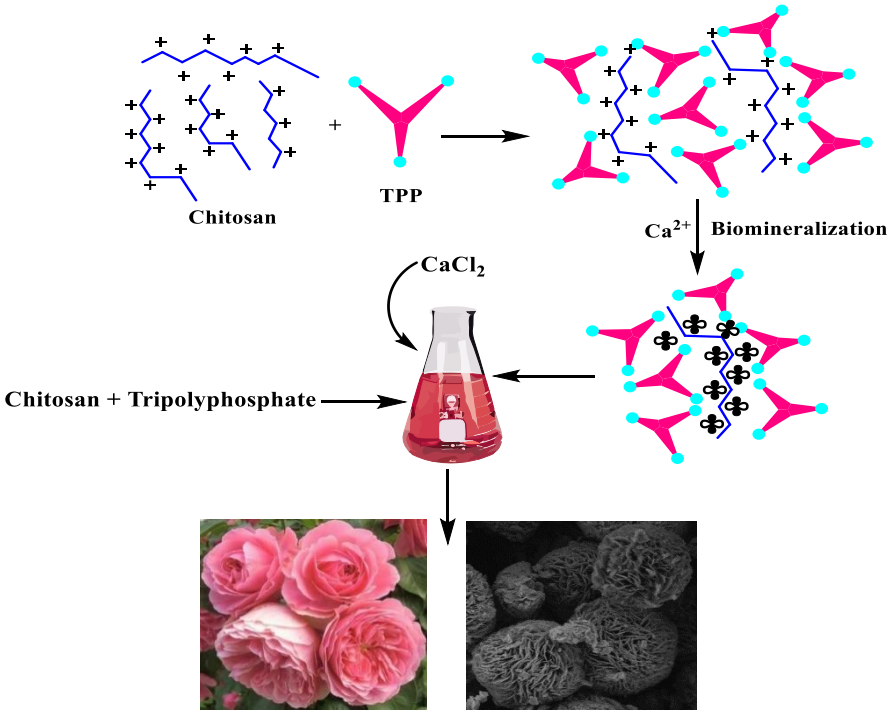

Figure 2. Synthesis steps of hNF-PL enzyme.

To the CS solution, 1.2 mL (125 mg mL^{-1}) of TPP solution was added and stirred for 10 minutes. Then, 4.2 mL of 100 mM $CaCl_2$ was added, and the mixture was stirred for a further 10 minutes at room temperature, and 2.5 ml of purified PL enzyme was added. One day after waiting at room temperature, the protein was determined in the supernatant. After centrifugation, the precipitate was washed with purified water to remove unbound proteins. The synthesized hybrid nano flower PL structure was dried at 37 °C and characterized (Figure 2) with pure PL enzyme [4].

2.5. SEM, EDX, XRD, FT-IR, and TEM characterization in hNF-PL enzyme.

SEM, EDX, FT-IR, and TEM chromatographic analyzes were used to characterize hNF-PL enzyme structures [33].

2.6. Applications of pure PL and hNF-PL enzymes in fruit juice clarification.

Fresh fruits (black grape, pomegranate, cornelian cherry, peach, red apple, and plum) bought from local markets were used in fruit juice clarification experiments. Pure PL and hybrid PL enzyme were added to each fruit juice medium and incubated for 5 hours at 50 °C. İn the fruit juices produced as a result of the clarification process, fragmentation rate and clarification rate was determined and recorded in percentages [26].

3. Results and Discussion

3.1. Characterization of LAB.

In this research, general identification techniques and molecular techniques were used for the identification of test isolates. As per general identification methods, the test isolate was rod-shaped, gram-positive, and catalase-positive. Obtained results were consistent with the results obtained from the literature [34,35]. In the molecular identification method, 16S-rRNA gene and 16S-23S rRNA intergenic spacer regions (ISR) were identified by means of sequence analysis. This sequence analysis results show that *B. pumilus* have a high percentage (99%) of resemblance to this isolate when compared to other bacterial series in GenBank. Parvathi *et al.* [36] also reported similar 16S rDNA gene sequence results as (99-100%).

Table 1. Purification and yield profile of PL enzyme purified from *B. pumilus* bacteria by TPP method

Steps	Volume (mL)	Activity (EU/mL)	TotalActivity (EU/mL)	%	SpecificActivity (EU/mL)	Protein Amount (µg protein/mL)	Purification Fold
Pure Extract	60	233.4±1.14	14007.3±3.24	100	0.17	1340.7±2.07	-----
n-Butanol (1:0.5)	20	210.2±2.12	4219± 1.59	90	1.09	192.4±1.09	6.41
1st Ammonium sulphate precipitation (60%)	20	187.5±0.44	3750± 1.41	80.3	4.57	41± 0.47	26.9
2nd Ammonium Sulphate precipitation (75%)	20	182.6±1.11	3652±1.17	**78.2**	32.6	5.6±0.16	**191.8**

3.2. Purification of the pectin lyase from B. pumilus.

PL enzyme was purified by using TPP method in three steps from *B. pumilus* the first

time. In the first step, the maximum PL enzyme activity value was determined in a 1:0.5 ratio (extract: n-butanol). In the second step, at the optimum n-butanol concentration, the 1st ammonium sulfate saturation was made between 20% and 80%. The highest activity was obtained at 60%. In the third step, 2nd ammonium sulfate saturation was performed at the optimum 1:0.5 n-butanol ratio and at 65-75% ammonium sulfate concentration, and the highest activity was determined at the rate of 75% ammonium sulfate. Purifications were carried out under these conditions. Using TPP method, PL enzyme from *B. pumilus* bacteria was purified with a yield of 78.2% in 191.8 folds (Table 1).

In another study, alkaline PL obtained from *Aspergillus flavus* MTCC 7589 bacteria by using traditional chromatographic methods (Sephadex G-100 gel filtration chromatography) was reported to have been purified 58.01 folds [37]. Extracellular PL enzyme produced by *B. pumilus* (P9) bacteria in solid culture fermentation was reported to be purified 36.36 folds using DEAE-cellulose anion exchange column chromatography [5,26]. Demir *et al.* (2011) reported that extracellular PL produced by *Geobacillus stearothermophilus* Ah22 bacteria in solid culture fermentation medium was purified 40.8 folds using DEAE-cellulose anion exchange column chromatography. In an experiment by [27] Sharma and Gupta, tert-butanol was added to the medium where ammonium sulfate is present, and the three-phase partitioning (TPP) technique was used to purify the pectinase enzyme from *Aspergillus niger* and tomato. According to the research findings, 76% yield, 10-fold purification, and 183% yield, and 9-fold purification were achieved from *Aspergillus niger* fungi and tomato, respectively.

SDS-PAGE analysis was performed to determine the number of subunits of the PL enzyme purified from *B. pumilus*, and a single band at 32.88 kDa level was observed. The molecular weight of the pure PL enzyme was also controlled by gel filtration chromatography (Figure 3).

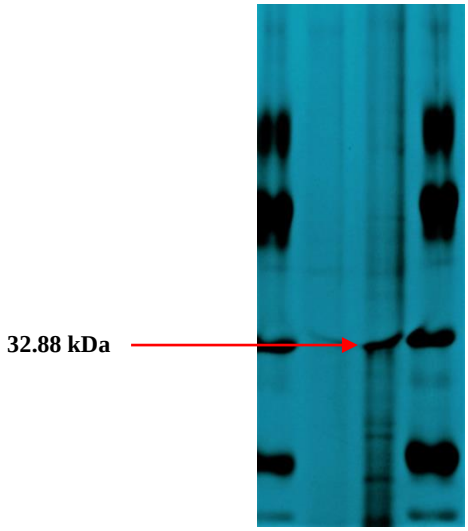

Figure 3. SDS-PAGE electrophoresis gel image of PL enzyme purified by TPP from *B. pumilus* bacteria.

PL enzyme from *B. pumilus* (P9), *Aspergillus flavus* MTCC 7589, and *Penicillum chrysogenum* bacteria was produced and purified using ion-exchange chromatography technique, and according to SDS-PAGE results; the molecular weight of the enzymes were determined as 25 kDa, 38 kDa, and 31 kDa, respectively [26,37,38].

3.3. Synthesis and characterization of the hNF-PL enzyme structure.

Hybrid structures with chitosan support material were generated to immobilize the PL enzyme that we obtained to provide a more stable structure. These hybrid enzymes were structurally characterized using SEM, TEM, XRD, and FT-IR chromatographic techniques.

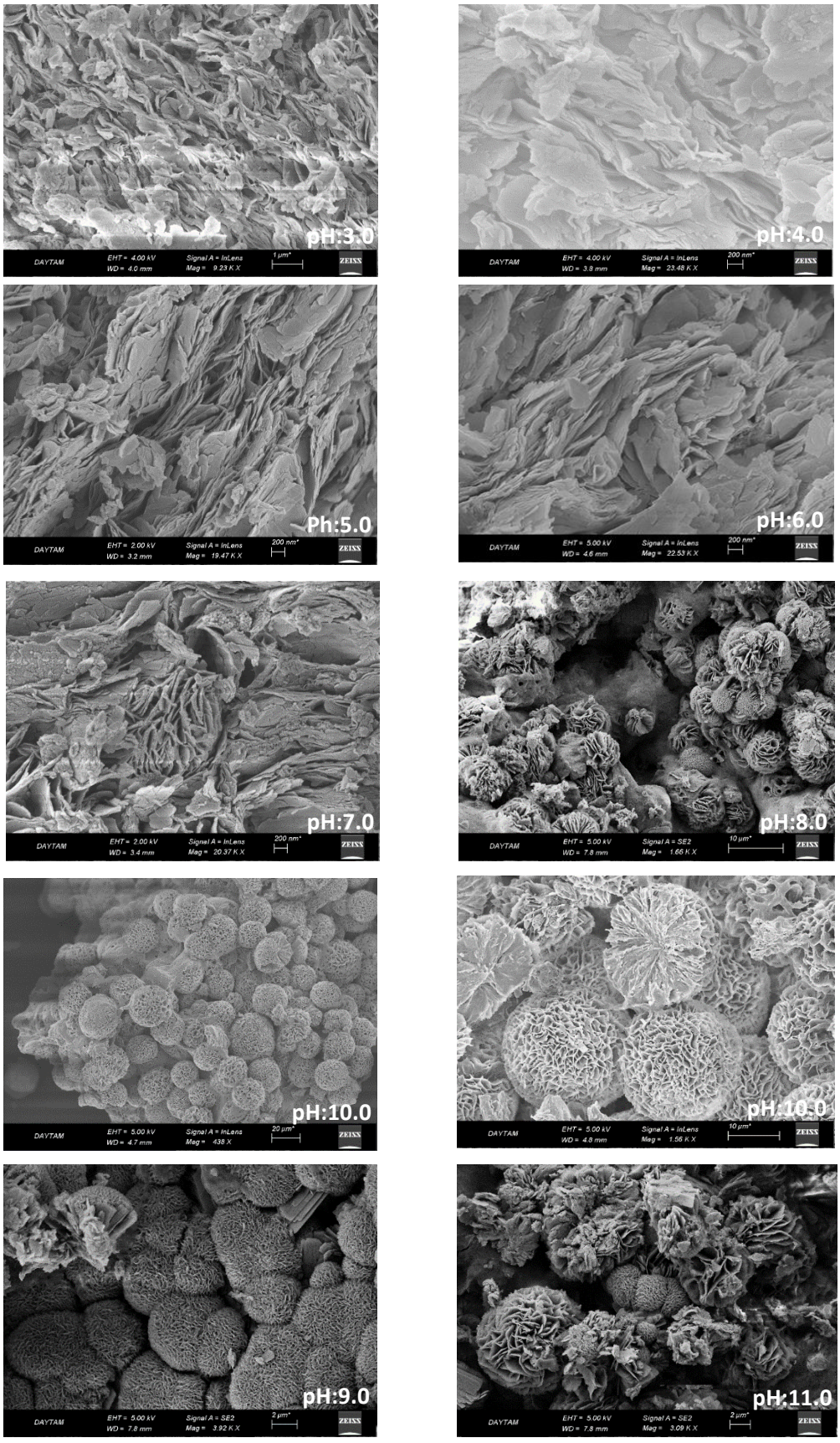

Figure 4. SEM images of hNF-PL structures at different pH (3-11).

3.3.1. Result of SEM analysis.

Hybrid nanoscale PL enzyme morphology was carried out using Zeiss brand Sigma 300 model scanning electron microscope (SEM). hNF-PL enzyme was synthesized at different pH values (Figure 4). As pH values approach pH 10, NF formation got better and reached the best condition at pH 10, beyond which the nanoflower structure was observed to have deformed. The results of EDX spectroscopy were given in Figure 5. It was determined that the nanoflower hybrid PL structure was composed of O (48.2%), P (18.6%), Ca (16.1%), and Na (6.1%). The atomic ratio of P to Ca was calculated to be 1.483.

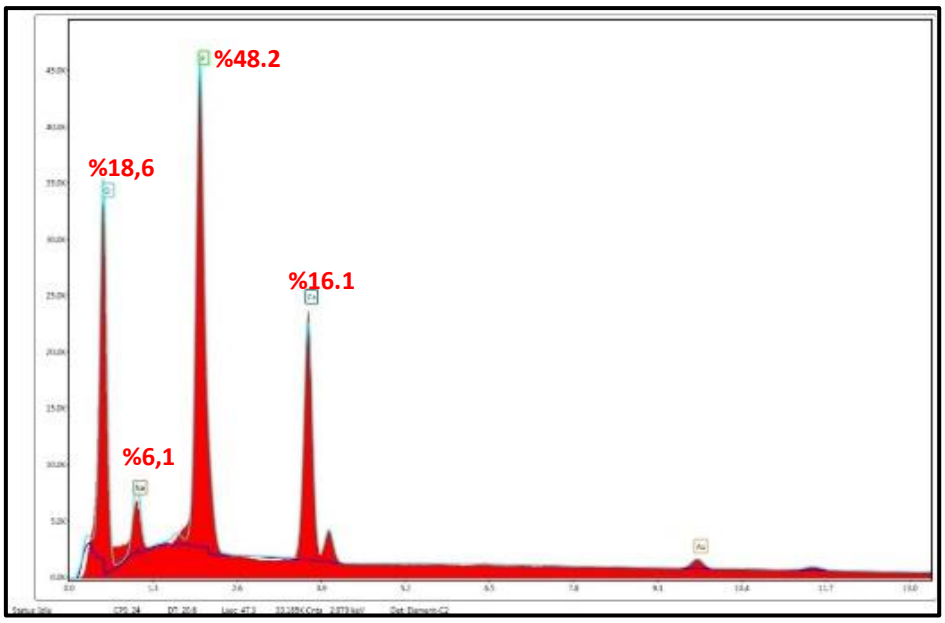

Figure 5. EDX image of hNF-PL structure.

In a study [4], by using catalase enzyme chitosan - tripolyphosphate (CS-TPP) nano-micro flowers (nano complexes) were synthesized by ionotropic gelation. In the SEM-EDX analysis, the presence of C, O, P, and Ca elements was determined. The atomic ratio of P to Ca was 1.448. The findings in this research support our results.

3.3.2. Result of XRD analysis.

The spectra of XRD analysis of the hNF-PL enzyme structure was shown in Figure 6. The peaks at 7.2° and 14.5° in the graph of the hNF-PL enzyme showed chitosan structure, and the peaks at 19.4°, 25.2°, 29.0°, 30.4°, 31.1°, 41.0°, 50.5°, 66.3°, and 71.3° showed tripolyphosphate, $Ca^{2+,}$ and carbohydrate units in the PL enzyme [39]. The obtained findings confirmed that the structure of the hNF-PL enzyme had formed successfully.

3.3.3. Result of FT-IR analysis.

The FT-IR spectrum of the hybrid NF PL enzyme structure was presented in Figure 7. The peak seen at 511 cm^{-1} in the FT-IR spectrum of hNF-PL enzyme belonged to the $P_3O_{10}^{5-}$ ion. $P_2O_7^{4-}$ ion gave the peak at 758 cm^{-1}. The peaks at the 918-758-692 cm^{-1} range belonged to POP, and 3483-1656 cm^{-1} range belonged to (O-H)(HOH). Our findings are consistent with the analysis of the chitosan calcium hybrid structure reported by Wang [39].

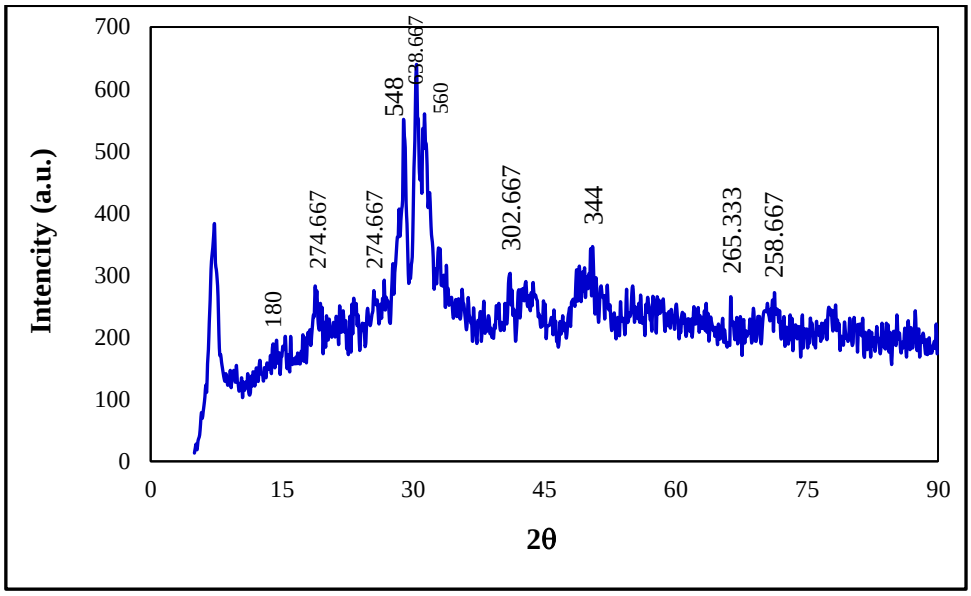

Figure 6. XRD analysis graph of hNF-PL enzyme structure.

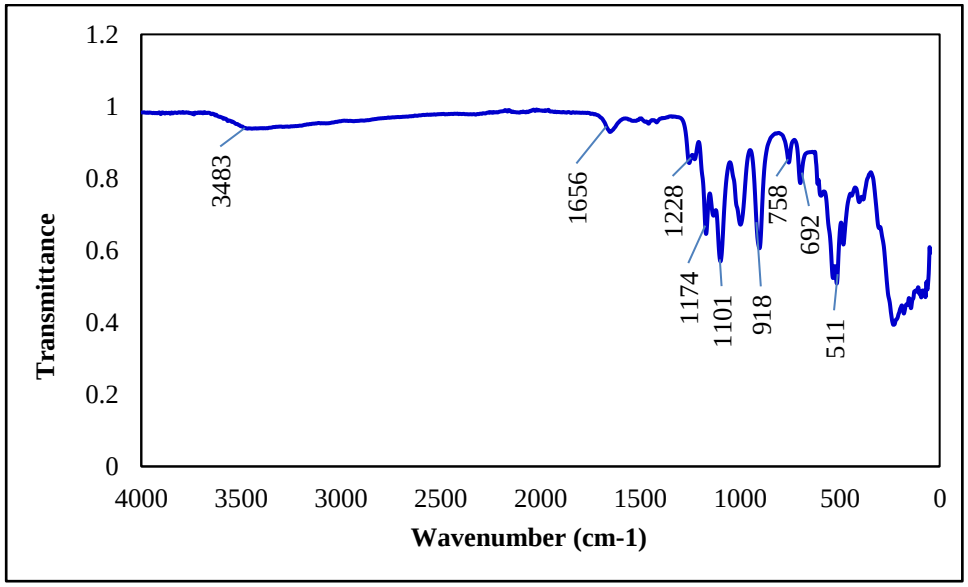

Figure 7. FT-IR analysis graph of hNF-PL enzyme structure

3.3.4. Result of TEM analysis.

TEM images of hNF- PL enzyme structures obtained using Transmission Electron Microscope (TEM) are given in Figure 8. According to the TEM images, it was determined that the synthesized nanoflower hybrid PL structures are composed of spherical-shaped nanoparticles and are highly porous and hierarchical structures.

Wang *et al.* [4], using catalase enzyme, have synthesized chitosan micro tripolyphosphate (CS-TPP) nanoflowers (nano complexes) by an ionotropic gelation method. They reported that the nanoflower structures of the TEM images they obtained consist of porous and hierarchical structures, and they support the findings we obtained.

3.4. Characterization of PL and hNF-PL enzymes.

3.4.1. Optimum pH and stable pH.

Enzyme activity measurements were performed between pH 3-11 in order to determine the optimum pH values and stable pH values of pure enzyme and hNF-PL enzymes. The results are given in Figure 9A. The optimum for both pure PL and hNF-PL enzymes was determined to be pH 8.0, whereas hNF-PL was more stable and active than the pure PL enzyme. It was determined that both pure PL and hNF-PL enzymes showed a sharp loss of activity at pH values below 7.0 and above pH 9.0.

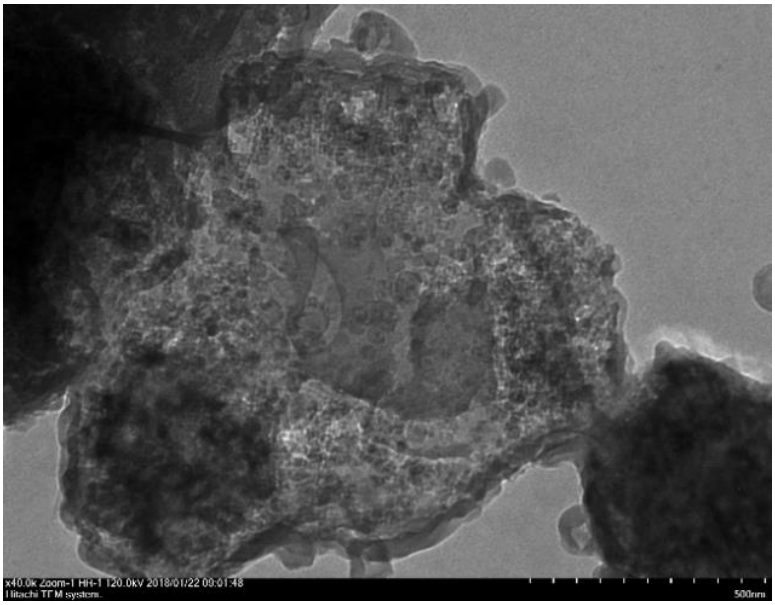

Figure 8. TEM analysis results of hNF-PL structure (pH: 10, room temperature).

To determine the pH stability of the pure PL and hNF-PL enzyme, daily activity measurements between pH 3.0-11.0 were carried out during 1 week (Figure 9B-C). Evaluating the findings, it was determined that pure PL enzyme and hNF-PL enzyme were more stable and had higher activity at pH 9.0 and pH 8.0, respectively.

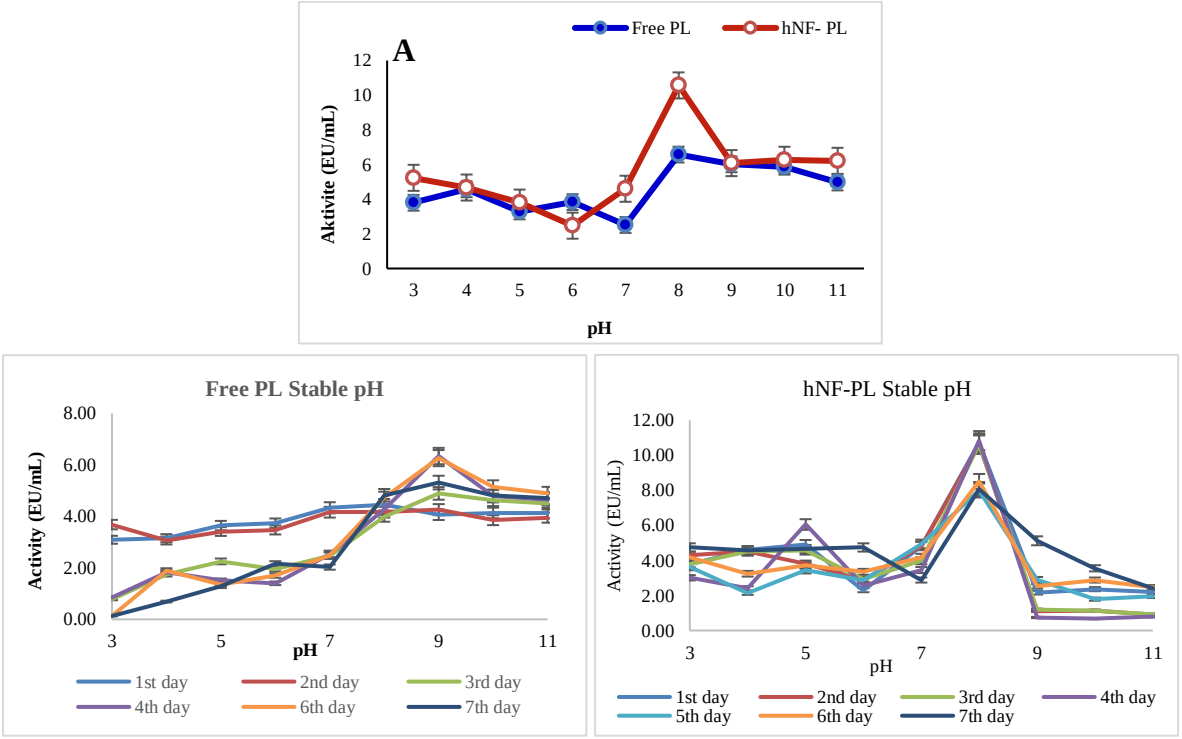

Figure 9. Effect of pH on activity (**A**) Optimum pH; (**B**) Pure PL enzyme stable pH; (**C**) hNF-PL enzyme stable pH.

3.4.2. Optimum temperature and stable temperature.

The effect of temperature on pure PL and hNF-PL enzymes between 10-90 °C was investigated. According to the results obtained, pure PL and hNF-PL enzymes showed maximum activity at 50 °C (Figure 10A).

When the effect of temperature on the stability of pure PL and hNF-PL enzymes (10 - 90 °C) was investigated for 2 hours, pure PL was determined to be most stable at 50 °C and hNF-PL at 60 °C. The figure for hNF-PL to be greater is attributed to its structural properties, which allows it to be more stable in higher temperatures (Figure 10 B-C).

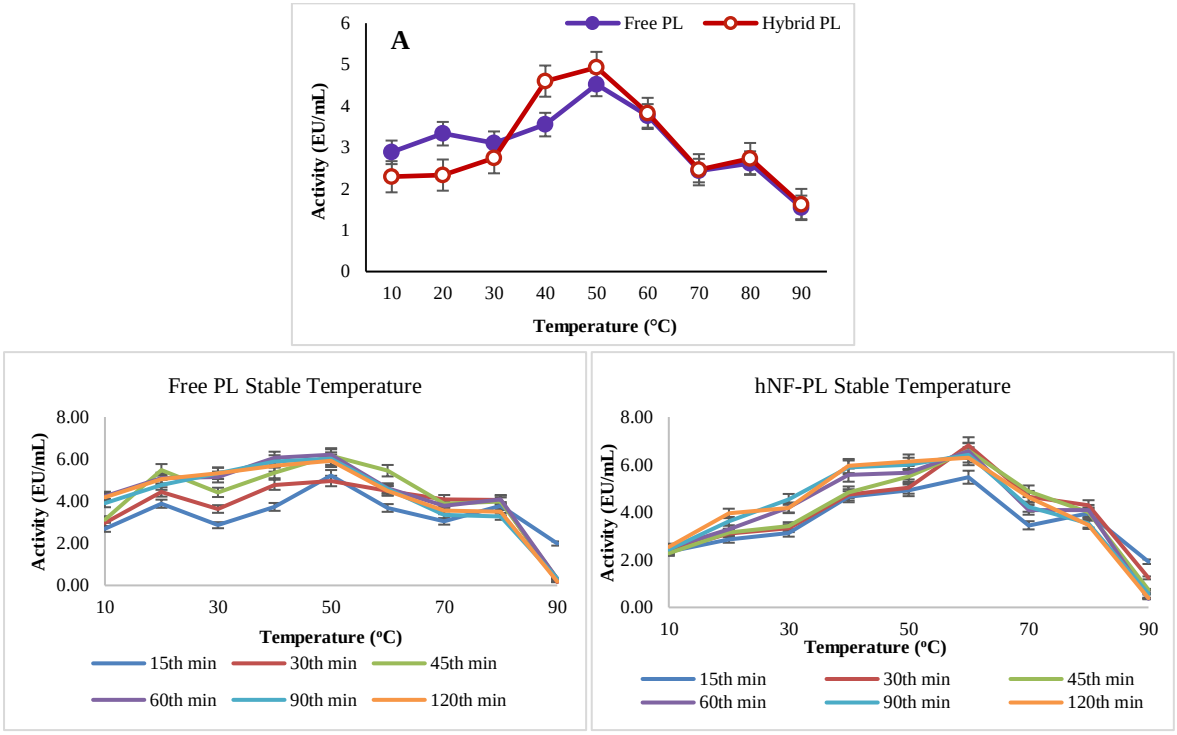

Figure 10. Effect of temperature on activity (**A**) Optimum temperature; (**B**) Pure PL enzyme stable temperature; **(C**) hNF-PL enzyme stable temperature.

The optimum pH values obtained from characterization studies of PL enzymes which were purified from *B. pumilus* (P9), *Aspergillus flavus* MTCC 7589, and *Aspergillus fumigatus* 2101 microorganisms, were determined to be pH 6.0, pH 8.0, and pH 8.0, respectively. Optimum temperatures were found to be 60 °C, 50 °C and 40 °C respectively [26,37,40].

3.4.3. The Effect of metal ıons on pure PL and hNF-PL enzymes.

The effects of Ca^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Hg^{2+}, and Fe^{3+} metal ions, in the concentration of 1 mM and 5 mM, on pure PL and hNF-PL enzyme activities were investigated and the results were given in Table 2 in percentage activity values. According to the findings, the presence of these metal ions activated both pure PL and hNF-PL enzymes.

Table 2. Effect of Ca^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Hg^{2+} Fe^{3+} metal ions on the activity of pure PL and hNF-PL enzyme

Chemicals	**% Pure PL Activity**		**% hNF-PL Activity**	
	1 mM	**5 mM**	**1 mM**	**5 mM**
Control	100	100	100	100
Ca^{2+}	160	186	173	198
Cu^{2+}	139	146	150	165
Fe^{3+}	161	184	144	167
Mg^{2}	133	146	148	157
Zn^{2+}	129	135	136	144
Hg^{2+}	0	0	0	0

Ca^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Hg^{2+} and Fe^{3+} metal ions activated pure PL enzyme by 60%, 39%, 33%, 29% and 61% respectively at the concentration of 1mM, and by 86%, 46%, 46%, 35% and 84%, respectively at 5 mM concentration. As for hNF-PL enzyme, 73%, 50%, 48%, 36% and 44% at a concentration of 1mM and 98%, 65%, 57%, 44% and 67% at 5 mM were achieved for the same. Hg^{2+} metal ion was found to completely inhibit both pure PL and hNF-PL enzymes. The highest activity in the hNF-PL enzyme was determined as 98% against Ca^{2+} metal ion.

In a previous study, the effect of various metal ions at a concentration of 10 mM on the PL enzyme purified from *Bacillus pumilus* (P9) was investigated. While Hg^{2+}, Mn^{2+}, EDTA, and SDS were found to inhibit PL enzyme completely, Cu^{2+}, Fe^{3+}, and Zn^{2+} inhibited metal ions by 89%, 32.7%, 12.1%, respectively. Ca^{2+} and Mg^{2+} metal ions were determined to activate by 32.1%, 19.2%, respectively [26].

In a study by Banu *et al.* [38], the effect of various metal ions at a concentration of 5 mM on the pectinase enzyme purified from the *Penicillum chrysogenum* bacteria was investigated. It was determined that Hg^{2+}, Cu^{2+}, and Co^{2+} metal ions inhibited the enzyme by 58.77%, 59.5%, and 58.5%, respectively, while Ca^{2+} metal ion activated the same by 3.56%. Also, Zn^{2+} and Mg^{2+} metal ions have been reported to inhibit enzyme activity by 16.21% and 26.56%, respectively.

In the study conducted by Cheng *et al.* [41] on the endo-polygalacturonase enzyme activity purified from the *Penicillium oxalicum* CZ1028 bacteria, the effects of various metal ions at 1 mM and 2 mM concentration on the enzyme activity were investigated. K^+, Mg^{2+}, and Ba^{2+} were slightly inhibited, whereas Cu^{2+} and Mn^{2+} very strongly inhibited the enzyme activity.

3.4.4. The effect of Vmax and KM on pure PL and hNF-PL enzymes.

Activity measurements were made at 5 different substrate concentrations for pectin, locust bean gum, and chitin substrates, and Lineweaver-Burk graphs were drawn by plotting the obtained 1/S values versus 1/V values. K_M and V_{max} values were calculated through these graphs (Table 3).

Table 3. V_{max} and K_M values of pure PL and hNF-PL enzymes for the substrates of pectin, locust bean gum, and chitin.

	Pectin		**Locust bean gum**		**Chitin**	
	V_{max}	**K_M**	**V_{max}**	**K_M**	**V_{max}**	**K_M**
	(µmol/Lmin)	**(mg/mL)**	**(µmol/Lmin)**	**(mg/mL)**	**(µmol/Lmin)**	**(mg/mL)**
Pure PL	17.98	0.94	1.3	0.88	1.42	0.64
hNF-PL	20.12	0.86	2.97	0.8	3.25	0.6

In a study conducted by Lu *et al.* [42], the exo-polygalacturonase enzyme from *Zygoascus hellenicus* V25 was purified, and the K_M and V_{max} values of the polygalacturonase were determined to be 5.44 mg/mL and 61.73 µmol/(min·mg), respectively. Nadaroğlu *et al.* [26] reported the K_M and V_{max} values as 0.298 mg/mL and 132.6 pmol/Lmin, respectively, for pectin lyase enzyme purified from *Bacillus pumilus* (P9). K_M and V_{max} values for PL enzyme purified from *Geobacillus stearothermophilus* Ah22 were determined to be 0.47 mg/mL and 355.3 µmol/Ldak, respectively, by Demir *et al.* in 2011 [5].

The hNF-PL enzyme was found to show more activity against pectin, locust bean gum, and chitin substrate than did pure PL enzyme. By using, The V_{max} and K_M values of hNF-PL enzyme were; 20.12 µmol/Lmin and 0.86 mg/mL, respectively for pectin substrate; 2.97

μmol/Lmin and 0.8 mg/mL, respectively for locust bean gum substrate; and 3.25 μmol/Lmin and 0.6 mg/mL, respectively for chitin substrate. As seen in Table 3, V_{max} and K_M values of hNF-PL differ from those of pure PL enzyme. For a pectin lyase enzyme in nanoflower structure, the V_{max} value is was observed to have increased while K_M value decreased, which is evidence for the increased affinity of the enzyme for pectin substrates. It is possible to interpret that the increase in V_{max} value was due to the fact that the hNF-PL enzyme was more stable than the other structure [50].

3.5. The results of pure PL and hNF-PL enzymes in the fruit juice production process.

The efficiency of pure PL and hNF-PL enzymes in fruit juice production and their usability in clarification processes were investigated. For this purpose, black grape, pomegranate, cornelian cherry, peach, apple, and plum obtained from the local markets of Erzurum-Turkey were pureed and treated with pure PL and hNF-PL enzymes. It was compared with the blank reaction medium prepared with pure water instead of an enzyme. When the amount of fruit fiber obtained after separation of the filtrate in the sample was compared, the highest rate of degradation of the fibers and cells in the samples was occurred in the apple sample by 38.3% for pure PL and 42.6% for hNF-PL (Table 4).

Table 4. Dry matter (D.M.) fragmentation rate of fruit purees added with pure PL and hNF-PL enzyme samples purified from *B. pumilus*, with reference to the control sample

Fruit amount (100 g)		Control	Pure PL	hNF-PL
Black Grape	**D.M. (g)**	3.8	3.5	3.4
	Decrease %	-	7.9	10.5
Pomegranate	**D.M. (g)**	4.2	3.2	3.1
	Decrease %	-	23.8	26.2
Cornelian cherry	**D.M. (g)**	5.2	4.8	4.2
	Decrease %	-	7.7	19.2
Peach	**D.M. (g)**	3.3	3	2.3
	Decrease %	-	9.1	30.3
Apple	**D.M. (g)**	4.7	2.9	2.7
	Decrease %	-	38.3	42.6
Plum	**D.M. (g)**	3.2	3	2.7
	Decrease %	-	6.25	15.6

According to the results of spectrophotometric measurements of the reaction media at 660 nm, the application in which the highest clarity rates were observed was the production of peach juice by 76.6% and 78.7% for pure PL and hNF-PL, respectively (Table 5).

Table 5. Reduction in absorbance at 660 nm in fruit juices obtained from fruit purees added with pure PL and hNF-PL obtained from *B. pumilus*

Fruit	% Reduction in absorbance at 660 nm		
	Kontrol	**Pure PL**	**Hybrid PL**
Black Grape	100	42	49
Pomegranate	100	32.8	42.5
Cornelian cherry	100	55.9	70.6
Peach	100	76.6	78.7
Apple	100	73.3	76.1
Plum	100	43	48.5

In a study conducted by Demir *et al.* [5], in the clarification experiments of fruit juices (apple, orange, peach, and banana) carried out by adding PL enzyme obtained from

Geobacillus stearothermophilus Ah22 bacteria, fragmentation rate in dry matter, in percentage to the control sample, in apple, orange, peach and banana juices were determined to be 30.8%, 53.3%, 34.6%, and 71.8% respectively [43]. The highest fragmentation rate was observed in banana juice, with a yield of 71.8%.

Sojitra *et al.* immobilized 3 enzymes, α-amylase, pectinase, and cellulose, on the amino-functional magnetic nanoparticle via the glutaraldehyde intermediate arm and used it in fruit juice clarification [44-46]. With the use of this magnetic immobilization system comprised of three enzymes, apple, grape, and pineapple fruit juice production with 150 min treatment yielded a reduction of turbidity by 41%, 46%, and 53%, respectively. Although three separate enzymes were employed in the combination, their results were lower than those achieved in this study [47-49].

4. Conclusions

Microbial enzymes are widely used in different industries because of their high yields, high stability, and economic advantages. Pectin lyase enzyme, which is in the commercial hydrolase enzyme group, and has a 70% industrial market share, is widely used in the food industry. In this study, 16S-RRNA gene and 16S-23S rRNA intergenic spacer regions (ISR) were identified by means of sequence analysis and isolated from tomato vegetables, and it was determined that the bacteria belonging to *B. pumilus* species was found to have a similarity of 99%. The extracellular production of the PL enzyme was achieved in a solid culture medium with *B. pumilus* bacteria, and the PL enzyme was first time purified by using the TPP technique with a high yield. In the immobilization of purified PL isoenzyme, hNF-PL was synthesized and characterized for the first time with the ionotropic gelation method. In the final stage of the study, using both free and hNF-PL enzymes, some fruit juices were clarified, and hybrid PL enzyme was found to be effective, hence increasing the yield more than free PL enzyme. The higher stability and effectiveness of hNF-PL are attributed to the greater surface area of its structure. In this respect, the hNF-PL structure is considered suitable to be used in different enzyme systems, in various industrial fields, especially in the food industry, without any inhibition.

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this study.

References

1. Mukhiddinov, Z.K.; Khalikov, D.K.; Abdusamiev, F.T.; A. C.C. Isolation and structural characterization of a pectin homo and ramnogalacturonan. *Talanta* **2000,** *53*, 171–176. https://doi.org/10.1016/S0039-9140(00)00456-2.
2. Levigne, S.; Ralet, M.-C.; Thibault, J.-F. Characterisation of pectins extracted from fresh sugar beet under different conditions using an experimental design. *Carbohydrate Polymer* **2002**, *49*, 145–153.
3. Mohare, Y.S.; Pratapwar, A.S.; Sakarkar, D.M.; Sheikh, A. Natural polymers used in sustained drug delivery systems. *American Journal of Pharmeceutical Research* **2013**, *3*, 4238–4261.
4. Wang, X.; Shi, J.; Li, Z.; Zhang, S.; Wu, H.; Jiang, Z.; Yang, C.; Tian, C. Facile one-pot preparation of chitosan/calcium pyrophosphate hybrid microflowers. *ACS Applied Materials & Interfaces* **2014**, *6*, 14522–14532. https://doi.org/10.1021/am503787h.
5. Cao, L.; Lu, W.; Mata, A.; Nishinari, K.; Fang, Y. Egg-box model-based gelation of alginate and pectin: A review. *Carbohydrate Polymer* **2020**, *242*, 116389. https://doi.org/10.1016/j.carbpol.2020.116389.
6. Demir, N.; Nadaroglu, H.; Tasgin, E.; Adiguzel, A. Gulluce, M.;Purification and characterization of a pectin lyase produced by *Geobacillus stearothermophilus* Ah22 and its application in fruit juice production. *Annals Microbiology* **2011**, *61*, 939-946. https://doi.org/10.1007/s13213-011-0217-6.
7. Demir, N.; Nadaroglu, H.; Demir, Y.; Isık, C.; Taskin, E.; Adiguzel, A.; Gulluce, M. Purification and characterisation of an alkaline pectin lyase produced by a newly isolated *Brevibacillus borstelensis* (P35), and its applications in fruit juice and oil extraction. *European Food Research and Technology* **2014**, *239*, 127–135. https://doi.org/10.1007/s00217-014-2198-8.
8. Auclair, J.; Gagné, F. The influence of polystyrene nanoparticles on enzyme clusters of fumarate, malate dehydrogenase and citrate synthase: a fractal analysis study. *Letters in Applied NanoBioScience* **2020**, *9*, 981–987. https://doi.org/10.33263/LIANBS92.981987.
9. Anisa, S.K.; Girish, K. Pectinolytic Activity of Rhizopus sp. and *Trichoderma viride*. *International Journal of Research in Pure and Applied Microbiology* **2014**, *4*, 28–31.
10. Sinitsyna, O.A.; Fedorova, E.A.; Semenova, M. V.; Gusakov, A. V.; Sokolova, L.M.; Bubnova, T.M.; Okunev, O.N.; Chulkin, A.M.; Vavilova, E.A.; Vinetsky, Y.P.; Sinitsyn, A.P. Isolation and Characterization of Extracellular Pectin Lyase from *Penicillium canescens*. *Biochemistry*, **2007**, *75*, 699–706.
11. Abd El-Rahim, W.M.; Moawad, H.; Hashem, M.M.; Gebreil, G.M.M.; Zakaria, M. Highly efficient fungal pectinase and laccase producers among isolates from flax retting liquor. *Biocatalysis and Agricultural Biotechnology* **2020**, *25*, 101570. https://doi.org/10.1016/j.bcab.2020.101570.
12. Yener, F. Pektinaz enziminin farklı iki destek üzerine immobilizasyonu ve karakterizasyonu, Yüksek Lisans Tezi. Çukurova Ü **2007**.
13. Favela-Torres, E.; Volke-Sepúlveda, T.; Viniegra-González, G. Production of hydrolytic depolymerising pectinases. *Food Technology and Biotechnology* **2006**, *44*, 221–227.
14. Ribeiro, D.S.; Henrique, S.M.B.; Oliveira, L.S.; Macedo, G.A.; Fleuri, L.F. Enzymes in juice processing: A review. *International Journal of Food Science and Technology* **2010**, *45*, 635–641. https://doi.org/10.1111/j.1365-2621.2010.02177.x.
15. Yang, G.; Chen, W.; Tan, H.; Li, K.; Li, J.; Yin, H. Biochemical characterization and evolutionary analysis of a novel pectate lyase from *Aspergillus parasiticus*. *International Journal of Biological Macromolecules* **2020**, *152*, 180–188. https://doi.org/10.1016/j.ijbiomac.2020.02.279.
16. Kashyap, D.R.; Vohra, P.K.; Chopra, S.; Tewari, R. Applications of pectinases in the commercial sector: A review. *Bioresource Technology* **2001**, *77*, 215–227. https://doi.org/10.1016/S0960-8524(00)00118-8.
17. Najafian, L.; Ghodsvali, A.; Khodaparast, M.H.H.; Diosady, L.L. Aqueous extraction of virgin olive oil using industrial enzymes. *Food Research International* **2009**, *42*,171–175. https://doi.org/10.1016/j.foodres.2008.10.002.
18. Chen, Q.; Jin, Y.; Zhang, G.; Fang, Y.; Xiao, Y.; Zhao, H. Improving production of bioethanol from duckweed (*Landoltia punctata*) by pectinase pretreatment. *Energies* **2012**, *5*, 3019–3032. https://doi.org/10.3390/en5083019.
19. Praveen, K.G.; Suneetha, V. A cocktail enzyme – Pectinase from fruit industrial dump sites: a review.

Biological Chemical Science **2014**, *5*, 1252–1258.
20. Garg, G.; Singh, A.; Kaur, A.; Singh, R.; Kaur, J.; Mahajan, R. Microbial pectinases: an ecofriendly tool of nature for industries. *3 Biotech* **2016**, *6*, 1–13. https://doi.org/10.1007/s13205-016-0371-4.
21. da Câmara Rocha, J.; da Silva Araújo, J.; de Paiva, W.K.V.; Ribeiro, E.S.S.; de Araújo Padilha, C.E.; de Assis, C.F.; dos Santos, E.S.; de Macêdo, G.R.; de Sousa Junior, F.C. Yellow mombin pulp residue valorization for pectinases production by Aspergillus niger IOC 4003 and its application in juice clarification. *Biocatalysis and Agricultural Biotechnology* **2020**, *30*, 101876. https://doi.org/10.1016/j.bcab.2020.101876.
22. Carr, J.G. ea, coffee and cocoa, In: B.J.B. Wood (Editor), Microbiology of fermented foods, London Elsevier Sci. Ltd. **1985**; 2, 133–154.
23. Uzuner, S.; Çekmecelioğlu, D. Gıda Atıklarının Pektinaz Enzimi Üretiminde Kullanımı. *Gıda / Journal Food* **2016**, *41*, 259–266. doi: 10.15237/gida.GD15076
24. Onem, H.; Nadaroglu, H. Preparation and Properties of Purified Phytase from Oakbug Milkcap (*Lactarius Quietus*) Immobilised on Coated Chitosan with Iron Nano Particles and Investigation of Its Usability in Food Industry. *Journal of Food Nutrition Research* **2014**, *2*, 938–945. https://doi.org/10.12691/jfnr-2-12-13.
25. Nadaroglu, H., Sonmez, Z. Purification of an endo-beta 1,4-Mannanase from Clitocybe geotropa and immobilization on chitosan-coated magnetite nanoparticles: Application for fruit juices. *Digest Journal of Nanomaterial and Biostructures* **2016**, *11*, 685–697.
26. Dal Magro, L.; Kornecki, J.F.; Klein, M.P.; Rodrigues, R.C.; Fernandez-Lafuente, R. Pectin lyase immobilization using the glutaraldehyde chemistry increases the enzyme operation range. *Enzyme Microbial and Technology* **2020**, *132*, 109397. https://doi.org/10.1016/j.enzmictec.2019.109397.
27. Lee, S.W.; Cheon, S.A.; Il Kim, M.; Park, T.J. Organic-inorganic hybrid nanoflowers: Types, characteristics, and future prospects. *Journal of Nanobiotechnology* **2015**, *13*. https://doi.org/10.1186/s12951-015-0118-0.
28. Woo, P.C.Y.; Fung, A.M.Y.; Lau, S.K.P.; Yuen, K.Y. Identification by 16S rRNA gene sequencing of Lactobacillus salivarius bacteremic cholecystitis. *Journal of Clinical Microbiology* **2002,** *40*, 265–267. https://doi.org/10.1128/JCM.40.1.265-267.2002.
29. Forsman, P.; Tilsala-Timisjärvi, A.; Alatossava, T. Identification of Staphylococcal and Streptococcal causes of bovine mastitis using 16S-23S rRNA spacer regions. *Microbiology* **1997,** *143*, 3491–3500. https://doi.org/10.1099/00221287-143-11-3491.
30. Garbers, I.M.; Britz, T.J.; Witthuhn, R.C. PCR-based denaturing gradient gel electrophoretictypification and identification of the microbial consortium present in kefir grains. *World Journal of Microbiology and Biotechnology* **2004,** *20*, 687–693. https://doi.org/10.1007/s11274-004-2624-3.
31. Taragano, V.M.; Pilosof, A.M.R. Application of Doehlert designs for water activity, pH, and fermentation time optimization for Aspergillus niger pectinolytic activities production in solid-state and submerged fermentation. *Enzyme and Microbial Technology* **1999**, *25*, 411–419. https://doi.org/10.1016/S0141-0229(99)00065-4.
32. Nadaroğlu, H.; Taşkin, E.; Adigüzel, A.; Güllüce, M.; Demir, N. Production of a novel pectin lyase from Bacillus pumilus (P9), Purification and characterisation and fruit juice application. *Romanian Biotechnological Letters* **2010,** *15*, 5167-76.
33. Sharma, A.; Gupta, M.N. Purification of pectinases by three-phase partitioning. *Biotechnological Letter* **2001**, *23*, 1625–1627. https://doi.org/10.1023/A:1011984517432.
34. Nedjma, M.; Hoffmann, N.; Belarbi, A. Selective and sensitive detection of pectin lyase activity using a colorimetric test: Application to the screening of microorganisms possessing pectin lyase activity. *Analitical Biochemistry* **2001**, *291*, 290–296. https://doi.org/10.1006/abio.2001.5032.
35. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.
36. Nadaroglu, H.; Adiguzel, A.; Adiguzel, G. Purification and Characterization Of B-Mannanase from *Lactobacillus plantarum* (M24) and Its Applications In Some Fruit Juices. *International Journal of Food Science and Technology* **2015**, *50*, 1158–1165. https://doi.org/10.1111/ijfs.12739.
37. Lineweaver, H.; Burk, D. The Determination of Enzyme Dissociation Constants. *Journal of American Chemical Society* **1934**, *56*, 658–666. https://doi.org/10.1021/ja01318a036.
38. Kolcuoğlu, Y.; Colak, A.; Faiz, O.; Belduz, A.O. Cloning, expression and characterization of highly thermo- and pH-stable maltogenic amylase from a thermophilic bacterium *Geobacillus caldoxylosilyticus* TK4. *Process Biochemistry* **2010**, *45*, 821–828. https://doi.org/10.1016/j.procbio.2010.02.001.
39. Ge, J.; Lei, J.; Zare, R.N. Protein-inorganic hybrid nanoflowers. *Nat. Nanotechnol.* **2012**, *7*, 428–432.

https://doi.org/10.1038/nnano.2012.80.
40. Buchanan, R.E.; Gibbons, N.E. Bergey‘s Manual of Determinative Bacteriology, Eight edition, U.S.A., **1974**.
41. Priest, F.G. Systematics and ecology of Bacillus. In *Bacillus subtilis* and other Gram-positive bacteria: Biochemistry Physiology and Molecular Genetics., Eds. A.L., Washington DC. American Society for Microbiology, **1993**.
42. Parvathi, A.; Krishna, K.; Jose, J.; Joseph, N.; Nair, S. Biochemical and molecular characterization of *Bacillus pumilus* isolated from coastal environment in Cochin, India. *Brazilian Journal of Microbiology* **2009**, *40*, 269–275.
43. Yadav, S.; Yadav, P.K.; Yadav, D.; Yadav, K.D.S. Purification and characterization of an alkaline pectin lyase from *Aspergillus flavus*. *Process Biochemistry* **2008**, *43*, 547–552. https://doi.org/10.1016/j.procbio.2008.01.015.
44. Rasheedha Banu A.; Kalpana Devi, M.; Gnanaprabhal, G. R. Production and characterization of pectinase enzyme from *Penicillium chrysogenum*, *Indian Journal of Science and Technology* **2010**, *3*, 377–381. https://doi.org/10.17485/ijst/2010/v3i4/29721.
45. Sojitra, U. V.; Nadar, S.S.; Rathod, V.K. A magnetic tri-enzyme nanobiocatalyst for fruit juice clarification. *Food Chemistry* **2016**, *213*, 296–305. https://doi.org/10.1016/j.foodchem.2016.06.074.
46. Rios-Corripio, G.; Welti-Chanes, J.; Rodríguez-Martínez, V.; Guerrero-Beltrán, J.Á. Influence of high hydrostatic pressure processing on physicochemical characteristics of a fermented pomegranate (*Punica granatum* L.) beverage. *Innov. Food Sci. Emerg. Technol.* **2020**, *59*, 102249. https://doi.org/10.1016/j.ifset.2019.102249.
47. Wang, J.; Wang, J.; Vanga, S.K.; Raghavan, V. High-intensity ultrasound processing of kiwifruit juice: Effects on the microstructure, pectin, carbohydrates and rheological properties. *Food Chemistry* **2020**, *313*, 126121. https://doi.org/10.1016/j.foodchem.2019.126121.
48. Liu, J.; Bi, J.; McClements, D.J.; Liu, X.; Yi, J.; Lyu, J.; Zhou, M.; Verkerk, R.; Dekker, M.; Wu, X.; Liu, D. Impacts of thermal and non-thermal processing on structure and functionality of pectin in fruit- and vegetable-based products: A review. *Carbohydrate Polymer* **2020**, *250*, 116890. https://doi.org/10.1016/j.carbpol.2020.116890.
49. Mansel, B.W.; Ryan, T.M.; Chen, H.-L.; Lundin, L.; Williams, M.A.K. Polysaccharide conformations measured by solution state X-ray scattering. *Chemical Physics Letters* **2020,** *739*, 136951. https://doi.org/10.1016/j.cplett.2019.136951.