Journal of Bio-Molecule Research and Engineering
Vol. 1 | No. 1 | 22 – 30| 2022
https://www.e-journal.unair.ac.id/biome

Partial Purification of Thermophilic Pectinase from Local Isolate *Anoxybacillus flavithermus* TP-01

Kartika Dwi Asni Putri[1*], Galih Ayhusta Laras[1], Ismi Aulia Syafira[2], Afaf Baktir[2]
[1]Proteomic Laboratory, University-CoE-Research Center for Bio-Molecule Engineering, Universitas Airlangga, Surabaya 60115, Indonesia
[2]Department of Chemistry, Universitas Airlangga, Surabaya 60115, Indonesia
[*]Corresponding author: kartikaasni@gmail.com

ARTICLE INFO
Article history
Received 29th Dec 2021
Accepted 22nd May 2022
Keywords:
pectinase
enzyme purification
characterization
precipitation
dialysis

ABSTRACT
Pectinase is an enzyme that can hydrolyze substances of pectin. It has several applications in daily life, for example, in juice factories, wine factories, pulp and paper factories, coffee and tea factories that use fermentation. Pectinase consists of pectate lyase, pectin lyase, and polygalacturonase. The aim of this research is to partially purify pectinase protein by ammonium sulfate precipitation and dialysis. A preliminary test of pectinase activity used halo zone analysis. Partial purification was needed to optimize ammonium sulfate and get the optimal condition in 40–90% of the ammonium sulfate composition. Pectinase activity was measured using DNS method, and the concentration of protein was measured using Bradford method. The result of this partial purification of *Anoxybacillus flavithermus* TP-01 was 70%. Decreasing pectinase activity was resulted in this research due to the loss of cofactor. Based on the result, it can be concluded that the pectinase from *Anoxybacillus flavithermus* TP-01 was polygalacturonase.

1. Introduction

Naturally, microorganisms have a lot of potentials to produce many kinds of enzymes which have been exploited commercially over the years. Pectinase is one kind of enzyme that has great potential to be applied in industry. Pectinases are also enzymes whose discovery is the cause of the revolution in the economic and commercial sectors. It has a big role as an upcoming enzyme in several commercial industries, such as fruit juice, oil extraction, food industry, pulp and paper industry, fermentation of coffee and tea, etc [1]. Pectinases, also known as pectinolytic enzymes, are a diverse group of enzymes that hydrolyze or break down the pectin substance, which is found primarily in microorganisms and plants [2]. It has an important role in nature such as helping in the cell wall extension and softening of tissue during maturation and storage [3]. Pectinolytic enzymes also help in the decomposition and recycling process of plant waste material [4]. Generally, the food and fruit processing industries use a variety of enzymes to increase the quality of products and reduce production costs [5]. Enzyme is biomolecule that has function of a biocatalyst to accelerate various biological reactions [6]. So, it was applied in daily life and in many industries.

Pectin is an acidic heteropolysaccharide that is found inside the plant cell wall. It is a negative charge of high molecular weight of heteropolysaccharide that is composed of the main chain of rhamnose and side chains such as arabinose, xylose, and galactose [7]. Pectinase is used to break down the pectin

polymer into sugar monomers (e.g., galacturonic acid). The applications of pectinase have been used in several sectors of industry. Purified enzyme has several times the activity of crude enzyme, so purification and characterization of enzyme are the first and most important steps after optimizing enzyme production to reduce production costs in industries [8]. Furthermore, the kinetic enzyme assay could be used to learn about the naturally catalytic mechanism in metabolism, activity, and enzyme inhibition [9]. In this study, *Anoxybacillus flavithermus* TP-01 was isolated as a crude enzyme of pectinase from the hot spring Mount Pancar in Bogor, Indonesia. Partial purification means that the enzyme is partially purified. It is usually used for wild-type proteins that don't have tag purification. The wild types of pectinases P1 and P2 were partially purified by ammonium sulfate precipitation and dialysis. The purified product was checked for activity by DNS reaction.

2. Materials and methods

Luria Bertani (LB) agar medium was used for the isolation and maintenance of bacterial cultures. LB Medium was prepared by dissolving 1% NaCl, 1% tryptone, 0.5% yeast extract, and 2% bacto agar in 20 mL of distilled water. The pectin (MP Biomedicals) agar media was used for the primary and subsequent screening of pectinolytic bacteria isolates. The solution was sterilized at 121 °C for 15 minutes.

2.1. Screening and selection of potential isolates

Liquid samples were inoculated into LB medium, incubated at 60 °C and 150 rpm for 16–18 hours, and subcultured into LB solid medium. The isolate was inoculated into pectin agar medium using an inoculating needle. The isolate was grown in a flask, and then the solid medium was incubated at 60 °C. Colonies were characterized by measuring the size of the clear zones that were produced in the agar medium that contained starch and iodine crystals. The pectinase index value of every colony was indicated by the presence of halo on solid media which is determined by comparing the diameter of halo zone and diameter of the colony. The isolate with the maximum clear zone was further studied and selected as the potential single strain. The purpose of halo test is to determine whether the isolate has pectinolytic activity. From this test, the pectinolytic index was obtained with the following equation 1.

$$\text{Pectinolytic index} = \frac{\text{Diameter of halo zone (mm)} - \text{Diameter of bacterial colony (mm)}}{\text{Diameter of bacterial colony (mm)}} \quad (1)$$

2.2. Production of crude enzyme

Liquid samples were inoculated into LB medium and incubated at 60 °C and 150 rpm for 12 hours. Culture bacteria were prepared by adding inoculum medium as much as 1% of the total volume of medium production and incubated at optimum temperature, 150 rpm, 60 °C for 12 hours in the orbital shaker incubator (YINDER LM-510RD). The supernatant obtained was centrifuged for 15 minutes at 3500 rpm at a low temperature of 4 °C.

2.3. Determination of enzyme activity

The enzyme activity was determined using DNS method, which quantifies the amount of reducing sugar produced by the hydrolysis of a 1% (w/v) pectin substrate suspension in phosphate-citrate buffer (pH 6.0). According to this method, the reaction mixture (containing 100 µL of enzyme supernatant and 100 µL of substrate suspension) was incubated at optimum temperature (60 °C) for 30 minutes. After the incubation, 600 µL of DNS reagent was added. It was kept in a boiling water bath for 15 min. The reaction mixture was incubated in an ice bath for 20 minutes to stop the reaction, and left at room temperature for 30 minutes before measuring the absorbance at 550 nm with an UV-1800 UV-Vis

Spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per minute at 60 °C [10][13].

2.4. *Determination of protein concentration*

The protein concentrations of pectinase enzyme were determined using the Bradford method with standard solutions, Bovine Serum Albumin (BSA). Vortex 20 uL sample and 1 mL Bradford solution, and measuring the absorbance at 595 nm with an UV-1800 UV-Vis Spectrophotometer. The BSA standard curves were made from the absorbance data of the BSA standard solutions in various concentrations, ranging from 0.2–1 mg/mL at 0.1 intervals [11].

2.5. Precipitation with ammonium sulfate

Crude enzyme of pectinase was added by various concentration of ammonium sulfate (10–100%) to determine the optimum concentration. First, the ammonium sulfate was fractionated on a small scale into various concentrations and dissolved in 1 mL crude enzyme homogenously. The mixture was centrifugated at 6000 rpm at 4 °C for 20 minutes to collect the precipitate. The precipitate was dissolved with phosphate citrate buffer pH 6.0 and the enzyme activity was determined for each concentration. The concentration with the highest enzyme activity was the optimum concentration of ammonium sulfate to precipitate the crude enzyme.

Enzyme production on a larger scale was continued by incubating the crude enzyme in the ice bath, and ammonium sulfate was poured slowly and little by little while being stirred. The mixture solution was incubated overnight at -20 °C and then centrifuged at 6000 rpm at 4 °C for 20 minutes. The precipitate was dissolved in a pH 6.0 phosphate citrate buffer.

2.6. Dialysis

The cellophane tube was filled with the enzyme fraction as much as 1:3 of the total volume of the cellophane tube and immersed in a buffer solution of phosphate citrate pH 6.0 with the concentration of half of the buffer to dissolve the precipitate. The immersion process needed to be stirred continuously and gently. Buffers were changed every 3 hours, 6 hours, and 12 hours (overnight). The process was carried out until the ammonium sulfate in the enzyme solution was dissipated.

2.7. Determination of pectinases activity with specific substrate

The determination of the activities of these three enzymes was carried out using a thiobarbituric acid (TBA) test [12]. Enzyme activities were calculated from the increase in the absorbance of the supernatants at 550 nm (A550) (PEL and PL) or 515 nm (A515) (for PG). One unit of activity was defined as an increase of 1 A550 or 1 A515 unit in 1 hour at experimental conditions.

2.8. *Determination of PEL enzyme activity*

A total of 100 µL of enzymes extract was added to 100 µL of reagent containing 50 mM citrate phosphate buffer pH 5.0 and 6.0, 0.5 mM CaCl2, and 0.2% pectin (60% esterified) substrate. Then 3 mM EDTA was added to inhibit any contaminating PEL activity and incubated at 60 °C for 1 h. Added 2 volume (200 µL) of 0.5 N HCl and four volumes (400µL) of 0.01 M TBA, incubated at 100 °C for 1 h, and centrifuged. The absorbance was measured using UV-VIS spectrophotometer at 550 nm.

2.9. *Determination of PL enzyme activity*

A total of 100 µL of enzymes extract was added to 100 µL of reagent containing 50 mM citrate phosphate buffer pH 5.0 and 6.0, 0.5 mM CaCl2, and 0.2 % pectin (60% esterified) substrate. Then 3 mM EDTA was added to inhibit any contaminating PEL activity and incubated at 60 °C for 1 h. Added

2 volume (200 µL) of 0.5 N HCl and four volumes (400 µL) of 0.01 M TBA, incubated at 100 °C for 1 hour, and centrifuged. The absorbance was measured using UV-VIS spectrophotometer at 550 nm.

2.10. *Determination of PG enzyme activity.*

A total of 100 µL of enzymes extract was added to 100 µL of reagent containing 50 mM citrate phosphate buffer pH 5.0 and 6.0, 0.5 mM CaCl2 and 0.2 % PGA substrate. Then 3 mM EDTA was added to inhibit any contaminating PEL activity. The absorbance was measured using UV-VIS spectrophotometer at 515 nm.

3. Results and discussion

3.1. Isolation and screening of pectinase activity

The isolates used in this study were a collection from Proteomic Laboratory, Research Centre for Bio-Molecule Engineering, Universitas Airlangga. Both isolates were identified as spore-producing bacteria from the genus Anoxybacillus, namely *Anoxybacillus flavithermus* TP-01 (P1 and P2) [13]. *A. flavithermus* TP-01 is a local Indonesian isolate originating from Mount Pancar hot springs, Bogor, Jawa Barat, Indonesia.

A. flavithermus TP-01 was spotted on a medium plate containing pectin substrates with 1% concentrations. The isolates were then incubated at 60 °C for 16–18 hours. The presence of pectinase activity was indicated by a clear area around the isolate after being given crystal iod. The screening of pectinase was performed by selective medium using agar plate that contained pectin. The screening result was displayed by the hydrolysis of pectin substrate and the hydrolysis zone was observed using potassium iodide solution. The hydrolysis area was seen to be clear because of the production of pectinase by bacteria and made the pectin incapable of binding with iodine that could formed a complex. The largest hydrolysis area indicated the most pectinase activity [8].

The screening results showed that the P2 isolate had a positive pectinase activity with the largest halo index of 0.75 obtained at pectin 2% compared with P1 isolate with a 0.70 of halo index (**Figure 1**).

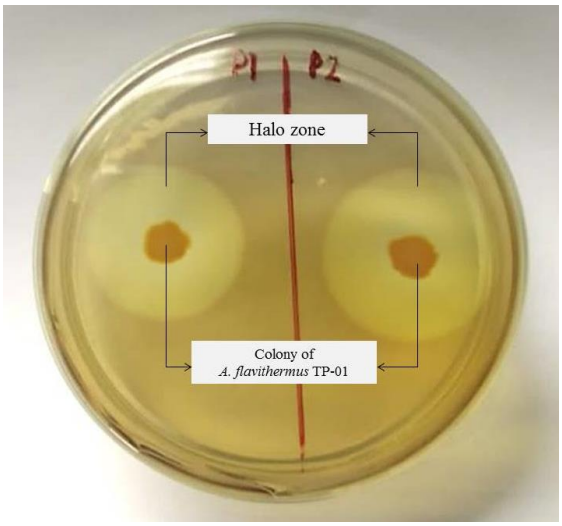

Figure 1. Pectinase production halo or clear zone was formed around the colony of *A. flavithermus* TP-01

3.2. Ammonium sulfate precipitation

Precipitation of the pectinase enzyme as crude extract with ammonium sulfate has the purpose of separating the pectinase enzyme from other proteins that cannot be separated by centrifugation. The crude extract still contained impurities such as non-target protein, so further purification was required. Ammonium sulfate precipitation is one of the partial purification methods of protein in solution form. The solution form of protein is bound to water molecules by hydrogen bond through their ionic or polar groups. When the high concentration of ammonium sulfate is added, its small ionic will compete with protein to bind the water molecules. This treatment has the purpose of removing the water and decreasing its solubility. The main factors affecting the protein precipitated are the number of polar groups, temperature, pH, MW of protein, and the position of polar groups.

Table 1. Value calculated according to protein purification, R.K. Scopes,1993 [14]

	Final percent saturation to be obtained																
	20	**25**	**30**	**35**	**40**	**45**	**50**	**55**	**60**	**65**	**70**	**75**	**80**	**85**	**90**	**95**	**100**
Starting percent saturation	**Amount of ammonium sulfate to add per (grams) liter of solution at 20 °C**																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	547	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	38	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

Optimization of ammonium sulfate deposition is needed to determine the optimum ammonium sulfate percentage which is used to precipitate pectinolytic protein at P2 isolate. Small-scale optimization of ammonium sulfate uses 1 mL of crude pectinase extract in a microtube which is then added with ammonium sulfate at a percentage of 40–90%. Then, each percentage that has been left idle for about 12 hours carries out an activity test to determine its optimum activity. Optimum results were obtained for ammonium sulfate deposition of 70%, as shown in **Figure 2**, so that it can be continued on a large scale, namely as much as 200 mL was deposited with ammonium sulfate with a percentage saturation of as much as 70%.

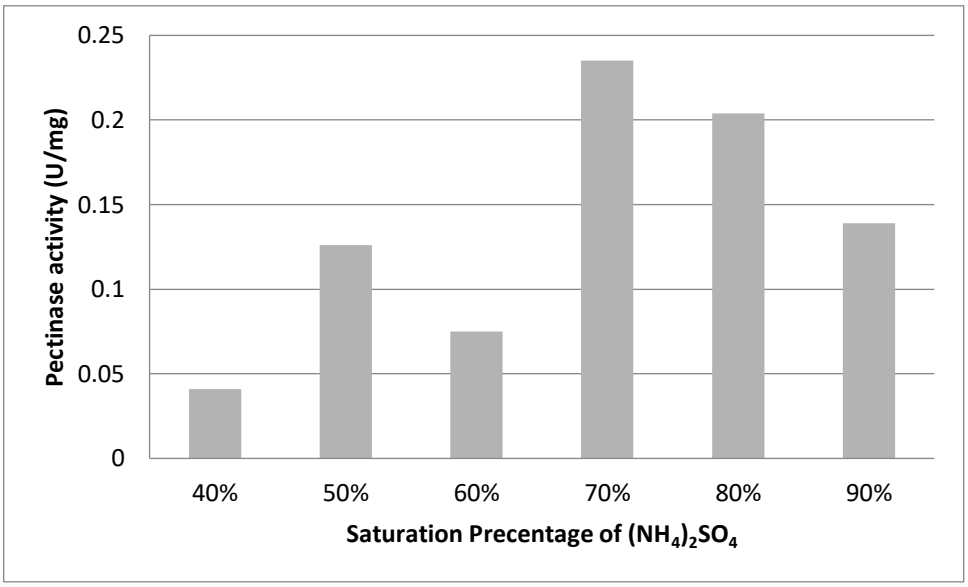

Figure 2. Optimization of ammonium sulfate percent saturation

Low activity is caused by the salting in, where the salt ion at a low salt concentration will protect the protein molecule so that the protein is still largely dissolved. While in high concentrations, salting out occurs, where salt ions increase the electric charge around the protein and decrease the solubility of the protein [15]. In precipitation at 70% saturation concentration, some of the enzymes were precipitated with ammonium sulfate salts. This was shown by the highest activity. The activity of the pectinase enzyme on the pectin substrate resulted from deposition with 70% ammonium sulfate salt, of which the specific activity is 0.0233 U/mg.

3.3. Dialysis

Dialysis aims to attract ammonium sulfate salt or other substances present in the enzyme solution and can interfere with the enzyme activity. The tube used for this process is a cellophane tube which has a semipermeable membrane so the ammonium sulfate and other small particles can penetrate the membrane, while the target pectinase protein, which is larger in size, remains in the tube. The enzyme solution that has been put in the cellophane tube is soaked and stirred with a magnetic stirrer in a phosphate citrate buffer pH 6.0. The dialysis buffer was changed every 3 hours, 6 hours, and 12 hours and tested to see whether there was still ammonium sulfate salt in it. The presence of ammonium sulfate salt in the buffer can be detected by taking a little of the buffer solution and adding several drops of Nessler buffer and heating it. If no brown color is formed, the dialysis process has been completed. The result of this dialysis process is an enzyme solution of 8 mL with an activity of 0.254 U, a protein content of 0.585 mg/mL, and a specific activity of 0.434 U/mg, shown in **Table 2**.

Table 2. Partial purification (dialysis) of pectinase enzyme that produced by *A. flavithermus* TP-01

Purification step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yields (%)	Purification (fold)
Crude enzyme	200	0.259	0.265	0.977	100	1.000
Dialysis	8	0.254	0.585	0.434	98	0.444

3.4. Determination of pectinase activity with specific substrate

The specific substrate activity test of the pectinase enzyme aims to determine the type of enzyme pectinases contained in bacteria *A. flavithermus* TP-01. Pectinase can be divided into four types: protopectinase, polygalacturonase, pectin lyase (PL), and pectinesterase (PE). It has protopectalase that has a role in promoting the dissolution of pectin. Based on the catalytic mechanism, it was divided into two types, they are type A pectinase and type B pectinase. Type A plays on the inner polygalacturonic acid region of protopectin. Besides, type B plays on the outer polysaccharide chains of the cell wall component and galacturonic acid chain. Pectin substrate is used to determine the activity of the PL enzyme, and polygalacturonic acid is used to determine the activity of the PEL and PG enzymes.

Pectate lyase (PEL) is an enzyme that has the function of cleaving the glycosidic linkages on polygalacturonic acid to produce an unsaturated product (D-4,5-D-Galacturonate) in the trans-elimination process. It acts on the substrate in a random manner, which is classified as exo-PEL and endo-PEL [16]. Pectate lyase needs a special cation, Ca^{2+}. So, it needs ethylene diamine tetraacetic acid (EDTA) as a chelating agent. Besides, Pectine Lyase (PL) is an enzyme that has the role of catalyzing the random cleavage of pectine. It hasn't required a special cation like Ca^{2+}, but it can be simulated by several cations [17]. Naturally, the lyases of fungi have optimum activity in acidic or neutral medium, whereas the lyases of bacteria are more active in alkaline medium. The last one, Polygalacturonase (PG), has a role in catalyzing the hydrolysis reaction of a-1,4-glycosidic linkages in polygalacturonic acid that produces D-galacturonate. It belongs to the glycosyl hydrolase family 28 [18]. PG has the same role as PMG as a hydrolase enzyme that acts in exo and endo mode. Endo-PG (EC 3.2.1.15) has the function to catalyze the random cleavage of substrate, and Exo-PG (EC 3.2.1.67) has the function to catalyze the hydrolytic cleavage at substrate's non-reducing end to produce mono-galacturonate or di-galacturonate [1].

The product as a result of the cleavage of PEL and PL enzyme was an oligomer of galacturonic acid which has a double bond and can be read on an aspectrophotometer at 550 nm. The product of the PG enzyme was an oligomer of galacturonic acid without double bonds, which was measured on a spectrophotometer at 515 nm. The results of pectinases enzymes with specific substrate were shown in **Table 3**, of 0.069; 0.088; and 1.740 U/ml for PL, PEL, and PG enzymes, respectively. These results indicated that the type of pectinase contained in *A. flavithermus* TP-01 bacteria was the PG enzyme.

Table 3. Pectinase enzymes specific substrate activity on *A. flavithermus* TP-01

Enzyme	Specific substrate	Enzyme activity (U/ml)
PL	Pectin	0.069
PEL	Polygalacturonic acid	0.088
PG	Polygalacturonic acid	1.740

3.5. Discussion

Recently, microorganisms have high potential in industrial applications as biotechnological sources like enzymes. Therefore, the number of extracellular enzymes explored in microorganisms is high [19]. The exploration of pectinase organisms is not only high in environmental sources but also in genetic manipulation or molecular engineering as another source to increase yield [8]. This research looked into the production of pectinase microorganisms from *Anoxybacillus flavithermus* TP-01 isolated from hot springs in Mount Pancar, Bogor, Indonesia. Several methods are used to isolate pectinase producing microorganisms, such as the streak plating technique, serial dilution, and pour plating. Pectinases have several main roles in industry, especially in food industries. This enzyme is helpful in the processes of wine fermentation, fruit juice extraction, jam and jelly making, and pickling. Furthermore, this enzyme

is also used in the pulp and paper industry, wastewater treatment, bioenergy, bio-scouring, oil extraction, protoplast fusion technology, etc.

The high number of pectinase applications in the industry emphasizes the importance of screening microorganisms for pectinase. They were selected based on their enzyme activity, novelty properties, characterization, and large scale of production [14]. This study screened the pectinase of microorganisms into plate agar that contained substrates of pectin and polygalacturonic acid. It has the function of identifying potential isolates that have the highest enzyme activity. This result could be observed by the diameter of the halo that formed. Pectinase can be isolated from several sources, such as bacteria, fungi, fruit, and plants. In our research, the source of this pectinase microorganism was hot spring water, while in other research screenings the bacterial strain was isolated from samples of vegetables and soil [16].

Pectinase is classified as a heterogeneous group of enzymes. They are divided into three groups: (1) pectin lyase; (2) polygalacturonase; and (3) pectin esterase. Polygalacturonases have been reported to be produced by many organisms, such as *Aspergillus niger*, *Neurospora crassa,* and *Baccilus* sp. [20]. In this research, the potential isolates for pectinase production were screened using a specific substrate for pectinase. The high potential of the pectinase enzyme was shown by the halo area that formed. It was observed by measuring the diameter of the halo. The diameter range of the halo indicated the level of enzyme activity. The pectinase activity was determined based on measuring the amount of reducing sugar by colorimetric methods. This method used the 3,5-dinitrosalicylic acid reagent method. Based on the assay of characteristics and procedure, both pectinases were identified as polygalacturonases.

The first partial purification method that is used to purify the wild-type enzyme from any debris to get a purified enzyme is ammonium sulfate precipitation. In the first purification method, the 70% ammonium sulfate precipitate showed high specific activity. Ammonium sulfate precipitation is a common and simple method to introduce as an initial step. Ammonium sulfate was chosen because it is cheap, soluble in water, and able to become much more hydrate by interacting with other water molecules than another ionic solvent. It happens because salt (ammonium sulfate) competes with the protein for binding with water molecules. It also increases the surface tension of water and makes the protein tighter. The protein surface area reduction caused a lower number of interactions between proteins and water, so it allows more hydrophobic interaction between protein molecules. It's caused aggregation and then precipitated. The saturated solution selectively precipitates the proteins based on the salting out mechanism of the crude extract [21]. The second step could be continued by dialysis with the purpose of increasing the purity factor and specific activity.

4. Conclusions

Pectinase from *Anoxybacillus flavithermus* TP-01 was successfully purified by 70% ammonium sulfate precipitation and dialysis and was classified as the polygalacturonase type.

Acknowledgements

The researchers thank and appreciate the efforts of Indonesian Ministry of Research, Technology, and Hinger Education for providing the research grant.

References

[1] D. Kashyap, P. Vohra, S. Chopra, and R. Tewari, "Applications of pectinases in the commercial sector: a review," *J Bioresource technology*, vol. 77, no. 3, pp. 215-227, 2001.
[2] W. JR., "Microbial pectinolytic enzymes," in *Microbial enzymes and biotechnology*, K. C. Fogarty WM, Ed. 2nd ed. London: Elsevier Science Ltd, 1990, pp. 133–76.
[3] O. P. Ward, M. Moo-Young, and K. Venkat, "Enzymatic degradation of cell wall and related plant polysaccharides," *Critical reviews in biotechnology*, vol. 8, no. 4, pp. 237-274, 1989.
[4] K. Kubra, S. Ali, M. Walait, and H. Sundus, "Potential applications of pectinases in food, agricultural and environmental sectors," *J Pharm Chem Biol Sci*, vol. 6, no. 2, pp. 23-34, 2018.
[5] M. Chandrasekaran, *Enzymes in Food and Beverage Processing*. CRC Press, 2015.
[6] P. K. Agarwal, "Enzymes: An integrated view of structure, dynamics and function," *J Microbial cell factories*, vol. 5, no. 1, pp. 1-12, 2006.
[7] B. M. Yapo, P. Lerouge, J.-F. Thibault, and M.-C. Ralet, "Pectins from citrus peel cell walls contain homogalacturonans homogenous with respect to molar mass, rhamnogalacturonan I and rhamnogalacturonan II," *J Carbohydrate Polymers*, vol. 69, no. 3, pp. 426-435, 2007.
[8] M. Siddiqui, V. Pande, and M. Arif, "Production, purification, and characterization of polygalacturonase from Rhizomucor pusillus isolated from decomposting orange peels," *J Enzyme research*, vol. 2012, 2012.
[9] K. K. Meena, M. K. Jaipal, and U. Singh, "Production kinetics and characterization of pectinase enzyme from Aspergillus niger," *J South Asian Journal of Food Technology Environment*, vol. 1, no. 2, pp. 131-135, 2015.
[10] G. L. Miller, "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Analytical chemistry*, vol. 31, no. 3, pp. 426-428, 1959.
[11] C. Kielkopf, W. Bauer, and I. Urbatsch, "Methods for measuring the concentrations of proteins. 2020; Cold Spring Harb," ed: Protoc, 2020.
[12] S.-P. Lei, H. Lin, L. Heffernan, and G. Wilcox, "Evidence that polygalacturonase is a virulence determinant in Erwinia carotovora," *Journal of Bacteriology*, vol. 164, no. 2, pp. 831-835, 1985.
[13] G. A. Laras, M. N. Handayani, L. N. Yamani, N. N. Purwani, and N. N. T. Puspaningsih, "Identification and characterization of thermophilic and pectinolytic bacteria isolated from gunung pancar hot spring bogor, Indonesia," *J Asian Journal of Microbiology, Biotechnology Environmental Sciences*, vol. 19, no. 3, pp. 496-508, 2017.
[14] F. M. Rombouts and W. Pilnik, "Pectic enzymes," in *Economic Microbiology, Vol. 5: Microbial Enzymes and Bioconversions*: Academic Press, 1980, pp. 227-282.
[15] K. C. Duong-Ly and S. B. Gabelli, "Salting out of proteins using ammonium sulfate precipitation," in *Methods in enzymology*, vol. 541: Elsevier, 2014, pp. 85-94.
[16] S. Mehta, R. Mitali, S. Nilofer, and P. Nimisha, "Optimization of physiological parameters for pectinase production from soil isolates and its applications in fruit juice clarification," *J Journal of Environmental Research Development*, vol. 7, no. 4A, pp. 1539-1546, 2013.
[17] R. S. Jayani, S. Saxena, and R. Gupta, "Microbial pectinolytic enzymes: a review," *J Process Biochemistry*, vol. 40, no. 9, pp. 2931-2944, 2005.
[18] P. Coutinho, "Carbohydrate-active enzymes: an integrated database approach," *J Recent advances in carbohydrate bioengineering*, 1999.
[19] R. Scopes, "Protein Purification New York," ed: Springer-Verlag, 1982.
[20] M. A. Bekri *et al.*, "Azospirillum irakense produces a novel type of pectate lyase," *J Journal of bacteriology*, vol. 181, no. 8, pp. 2440-2447, 1999.

[21] Q. Wang, Z.-X. Gao, N. Zhang, Y. Shi, X.-L. Xie, and Q.-X. Chen, "Purification and characterization of trypsin from the intestine of hybrid tilapia (Oreochromis niloticus× O. aureus)," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 1, pp. 655-659, 2010.