VNU Journal of Science: Earth and Environmental Sciences
Journal homepage: https://js.vnu.edu.vn/EES

Original Article

Isolation of Cellulase Enzyme from Water Used in White Pepper Processing

Tran Ngoc Hung*

Thu Dau Mot University, 6 Tran Van On, Phu Hoa, Thu Dau Mot, Binh Duong, Vietnam

Received 27th August 2024
Revised 5th February 2025; Accepted 28th February 2025

Abstract: Cellulase, an enzyme that helps soften fruit peel, is produced by plants during the fruit ripening phase. Utilizing the immersing water from the white pepper processing to extract cellulase will help minimizing environmental waste and retrieving valuable compounds. Some factors affecting the cellulase yield from the immersing water of pepper were studied, including the degree of ripeness of peppercorn, immersing temperature and time. We then assess the precipitating yield of $(NH_4)_2SO_4$ and etanol, purify cellulase by gel Sephadex G-75 filtration, and determine the preliminary compound mass by electrophoresis method. In comparison with the unripe peppercorn, the ripe peppercorn releases more cellulase. The highest cellulase activity in the water of immersed pepper was recorded at 40 ºC for 48 hours, with an activity of 1.98 U/ g peppercorn. The yield of cellulase extraction was highest when precipitating with 70% ethanol, with a cellulase recovery of 48% and protein recovery of 37%. The results of gel filtration and electrophoresis showed that the cellulase mass was less than 75 kB.

Keywords: Electrophoresis, gel-Sephadex G-75 filtration, the water of immersed pepper, isolating cellulase.

1. Introduction

Cellulase is widely used in various application within the food industry, including beverage production, brewing, wine making, and fermented drinks. Commonly, microfungi have been the primary source of cellulase for industrial processing [1, 2]. However, cellulase isolated from plants could possess advantages when applied to this material, especially in the processing of agricultural products with the steps of peeling, such as coffee or white pepper.

Isolating and applying plant enzymes were carried out for many years. It were typically

* Corresponding author.
E-mail address: hungtngoc@tdmu.edu.vn

https://doi.org/10.25073/2588-1094/vnuees.5229

applying amylase of malt, isolating bromelin from stem and pineapple fruit, isolating papain from the latex of papaya fruit, isolating ficin from the latex of fig fruit, ect. However, the source of cellulase produced in the peel of various kinds of ripened fruit has received little attention of researchers.

The peel of pepper fruit possesses cellulose and pectin as its main components. During the natural ripening process, the fruit produces cellulase, which helps to soften its peel [3, 4]. Isolating cellulase from the peel of pepper fruit could help effectively reuse the source of agricultural by-product and reduce the hazards of environmental pollution from the white pepper processing factories. In addition, the cellulase preparation could be added to the peeling step for shortening the time of the white pepper process or other food processing. In this study, some factors affect cellulase yield from the pepper peel (green pepper and ripe pepper, immersing temperature and time) were assessed. Then, cellulase was partially purified by Sephadex G-25 column and estimated the molecular mass by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2. Methods

2.1. Materials

The mature pepper fruits (*Piper nigrum*, Indian genus) was collected at Dak Nong province, from 02/2022 to 04/2022. The fruit was carefully washed with tap water before separating the green pepper fruits and the red or yellow pepper (ripe pepper) fruits. Materials were used for tests within 24 hours.

2.2. Methods

2.2.1. Extraction of Cellulase from the Pepper Fruit

Pepper fruits immersed into water with the ratio of 1:1 (w/w) at 40 ºC. After 24 hours of immersion, the pepper fruit was manually rubbed to separate the peel from the core of pepper. The peel and sediments were removed by centrifuging at 5000 rpm for 10 min. After separating the core of pepper, extract was centrifuged at 5000 rpm for 10 min in centrifuge (Hettich EBA 20, Germany) to get clear extract. The extract was stored at 5–8 ºC until use.

2.2.2. Cellulase Activity Assay by DNS Method

Cellulase activity was measured by DNS (3,5-dinitrosalicylic acid) method through the amount of reducing sugars liberated during hydrolysis [5]. 0.3% solution of Carboxy Methyl Cellulose (CMC) was prepared in 1 N citrate buffer (pH 5.0) and was considered as substrate. 100 µL of crude enzymes and 1 mL of citrate buffer were added into the mixture of 1 mL of CMC solution. The mixture was incubated at 45 °C for 30 min. 3 mL of DNS solution was added to the solution to stop the reaction [5]. The treated samples were boiled for 3 min, cooled in water for color stabilization followed by addition of 5 mL H_2O. The optical density was measured at 540 nm (Bioway, Biochrom, England). The control tubes was added 3 mL of DNS solution followed by addition of the enzyme solution. One unit of cellulase activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 µmol of glucose within 1 hour of reaction [6].

2.2.3. Protein Determination by the Lowry Assay

Concentration of the protein was determined by Lowry assay using Folin reagent [7]. 0.4 mL of the sample solution was transferred to 15 mL test tubes. Then, 2 mL of C solution (49 mL of A solution (2% of Na_2CO_3 in NaOH N/10 solution): 1 mL of B solution (0.5% of $CuSO_4$ in 1% trisodium citrate)) was added to each sample tube. After 20 min of incubation at room temperatures in dark, 0.2 mL of Folin reagent was added to each tube and mixed immediately using vortex. The absorbance of solutions was measured at 750 nm. Albumine was used as standard. The stock solution of 10 mg/mL was prepared using distilled water. The stock solution was then diluted with distilled water to obtain 50, 100, 150, 200, and 250 µg/mL concentrations.

2.2.4. Statistical Analysis

All experiments were repeated three times, and data are presented as the mean ± standard deviation (SD). Analysis of variance was performed using Statgraphic Centurion 15.

2.3. Procedures

2.3.1. Assessing Cellulase Activity in Green Pepper Fruit and Ripe Pepper Fruit

Pepper fruits (unripe (green) or ripe fruits) were immersed into water with the ratio of 1:1 (w/w) at 40 ^{o}C. After 24 hours of immersion, the pepper fruit was manually rubbed to separate the peel from the core of pepper. The peels and sediments were removed by centrifuging at 5000 rpm for 10 min. The solution was used to determine the cellulase activity, the total protein content and the specific cellulasse activity. The kind of pepper fruit that has a higher cellulase activity was used for the next experiments.

2.3.2. Effect of Immersing Temperature on the Yield of Cellulase

The ripe pepper fruits were immersed into water at the different temperatures: 30 ^{o}C, 35 ^{o}C, 40 ^{o}C, 45 ^{o}C, 50 ^{o}C and 55 ^{o}C for 24 hours. The cellulase activity of extracts was determined by DNS assay. The immersing temperature showing the highest cellulase activity was used for the next experiment.

2.3.3. Effect of Immersing Time on the Yield of Cellulase

The ripe pepper fruits were immersed into water at the selected temperatures for the difference times: 12, 24, 36, 48 and 60 hours. The cellulase activity of extracts was determined by DNS assay.

2.3.4. Effect of Precipitating Agents on the Collecting Yield of Cellulase

To check the effect of precipitating agents on cellulase extraction, ammonium sulfate and ethanol were used.

Ammonium sulfate precipitation was performed by the method described by Englard and Seifter [8]. The ammonium sulfate was added in small quantities to the crude extract which was maintained at 5 ^{o}C until the different levels of saturation (70%, 80% and 90%) was reached. For ethanol, the absolute ethanol (99.9%) was added to the crude extract to get the final ethanol concentrations of 60%, 70% and 80%.

To maintain the temperature, ice buckets were used. The samples were incubated for 30 min to allow precipitation then centrifuged for 10 min at 5,000 rpm and 5 ^{o}C. Discarding the supernatant and re-dissolving the pellets in 0.01 M phosphate buffer (pH 7.0). This sample was used to measure the total protein content and cellulase activity.

2.3.5. Isolation of Cellulase by Sephadex G-25

The crude extract of cellulase from the pepper peel was obtained and separated through Sephadex G-75. Approximately 40 g of Sephadex G-75 gel was completely saturated with 30% ethanol, after which it was transferred carefully to the column (16 mm × 60 cm). Subsequently, the column was eluted with 0.01 M phosphate buffer (pH 7.0) for at least 3 bed-volumes. Then, 1 mL sample solution containing 5 mg/mL of protein was loaded onto the column. The column was then eluted with 0.01 M phosphate buffer at a flow rate of 0.5 mL/min, and the eluates were collected using an automatic fraction collector (2.0 mL/tube). The protein content of tubes was estimated by measuring the OD at 280 nm [9]. Based on the OD, the peaks was determined and pooled together into fractions. The total content of protein was determined by Lowry assay. The cellulase activity and specific activity of fractions were determined by DNS assay and the Agar well diffusion assay. The specific activity is the cellulase activity per mg protein.

2.3.6. Agar Well Diffusion Assay of CMCase Activity

Agar well diffusion assay was done according to the procedures outlined by Purkayastha and Dahiya [10]. The plates were poured the medium containing 0.3% CMC and 1.5% agar in sterile condition. Wells measuring 8 mm in diameter were then aseptically punched

into the agar with a gel puncher (6 equidistant wells per agar plate). Subsequently, 100 µL of the different fractions were pipetted into the wells. The fractions containing cellulase from the pepper peel were left to diffuse in the agar at room temperature for 2 hours. All the agar plates were then incubated (upright position) at 37 °C for 24 hours. Post-incubation, the halo zone diameters to the nearest millimeter (mm) were measured.

2.3.7. Estimation of Cellulase Molecular Mass by SDS-PAGE

One dimensional SDS-PAGE (1D SDS-PAGE) was performed following the method of Laemmli [11]. The polyacrylamide gel contained 0.1% (w/v) SDS using a Tris–glycine buffer, pH 8.8. Protein bands were observed by staining with Coomassie brilliant blue R-250.

3. Results

3.1. Assessing Cellulase Activity in Green Pepper Fruit and Ripe Pepper Fruit

The green pepper fruit and ripe pepper fruit were separately immersed into water at 40 °C for 24 hours. The immersing solution after removing the cores and the peels was used to determine the cellulase activity, the total protein content and the specific cellulase activity.

Table 1. Cellulase activity and protein content of the immersing solution of pepper fruit

Types of pepper fruit	Cellulase activity (U/g pepper fruit)	Protein content (mg/g pepper fruit)	Specific cellulase activity (UI/mg)
Green pepper	0.029 ± 0.02	5.2 ± 0.1	0.005
Ripe pepper	0.791 ± 0.10	12.1 ± 0.2	0.065

The mature pepper fruit, after harvesting, will produce enzymes to activate the ripenning process. A period of time during which enzymes were produced affected by many factors, such as the ripenning level of fruit. Commonly, these enzymes were only produced during a particular period of time [3]. Determining the cellulase activity at different states of ripeness plays an important role in receiving source of plant cellulase. The results showed the content of protein and cellulase activity isolated from the ripening pepper fruit was significantly higher than from the green pepper fruit. This difference may be due to the fact that cellulase was kept at a low level in the green pepper fruit. The amount of cellulase could significantly increase when the pepper fruit transfers to the ripening phase. Thus, the ripened pepper fruit was used to survey some factors that affect isolating cellulase from the peel.

3.2. Effect of Immersing Temperature on the Yield of Cellulase

Temperature could affect the cellulase yield of fruit and the solubility of compounds. The experiment was designed to assess the effect of the different temperatures (30-55 °C) on the cellulase yield.

The immersing temperature affects significantly the protein extraction yield. The protein content increased gradually corresponding to the immersing temperature, from 35 °C to 50 °C. This also led to an increase in cellulase activity. However, in another aspect, the increase in temperature probably inactivates the enzyme production in fruit and affects the structural stability of the enzyme. At the immersing temperature from 40 °C to 50 °C, the cellulase activity isolated from the pepper peel varied insignificantly, from 0.67 to 0.74 U/g pepper. Thus, the suitable temperature to isolate cellulase from the pepper peel was recorded at 40 °C with a specific activity of 0.054 U/mg. At this temperature, the cellulase activity increased quickly, but the miscellaneous protein was not much. The hardness of fruit peel was predominantly due to cellulose and pectin. A study by Mohammed et al., about factors that affect the ripenning process of Date fruit also

showed the fruit peel handled at 45-50 ºC was softer than handled at 55 ºC [12]. This indicated that cellulase was probably generated vigorously between 40 ºC and 50 ºC.

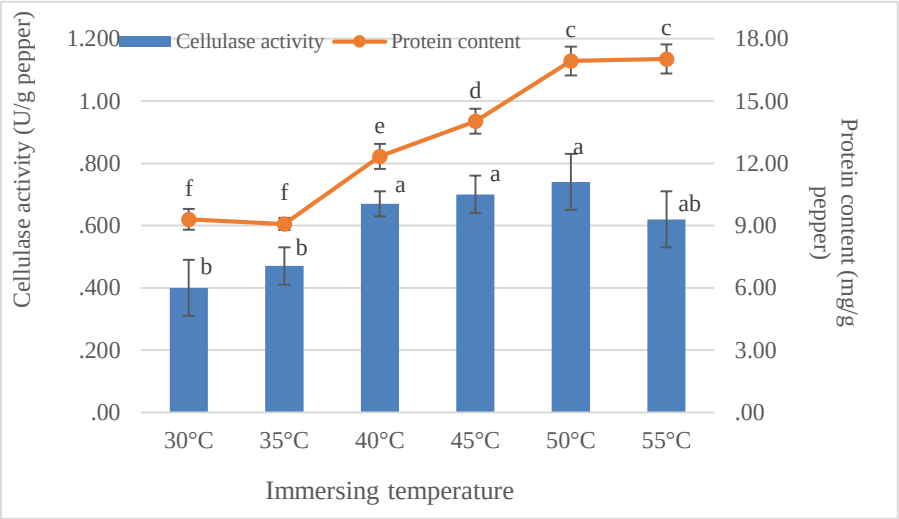

Figure 1. Effect of immersing temperature on the cellulase yield and the protein content. The means with different letters are significally different ($p<0.05$).

3.3. Effect of Immersing Time on the Yield of Cellulase

Although the immersing time helps to increase the rate of solubility, it also affects the structural stability of enzymes. The experiment was carried out at 40 ºC for the different immersing times (12-60 hours).

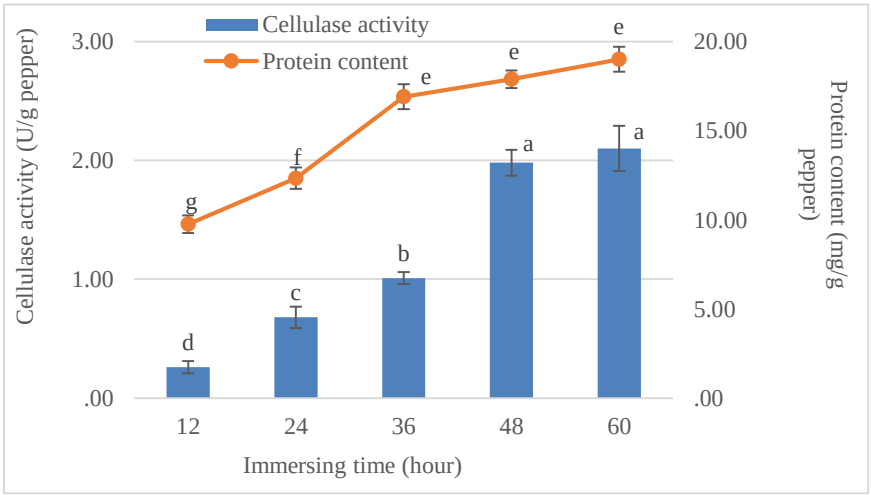

Figure 2. Effect of immersing time on the cellulase yield and the protein content. The means with different letters are significally different ($p<0.05$).

The protein content in the extract increased obviously when extending the processing time from 12 to 36 hours. After 36 hours, the protein content was not significantly different from the

36-hour time point. However, the cellulase activity increased vigorously over a period of 36 to 48 hours, reaching a specific activity of 0.111 U/mg at 48-hour (Figure 2). This result also showed the cellulase in the ripened pepper peel was produced and solubled abundantly in the extract after 48-hour of immersion at 40 °C. The strong hydrolysis of cellulase could soften the peel of pepper fruit, and making peeling easier. In line with current trend, the processing of white pepper using environmentally friendly method is becoming essential. Determining the optimal time and temperature for the immersion step can facilitate the easy separation of the core from the peel and reduce the overall processing time for white pepper. Addititionally, the immersion water used for the pepper can be reused to extract valuable compounds, thereby minimizing environmental hazards.

3.4. Effect of Precipitating Agents to the Collecting Yield of Cellulase

Cellulase extracted from the ripened pepper was precipitated with ethanol and ammonium sulfate at different concentrations. The yield of cellulase was showed in the Figure 3.

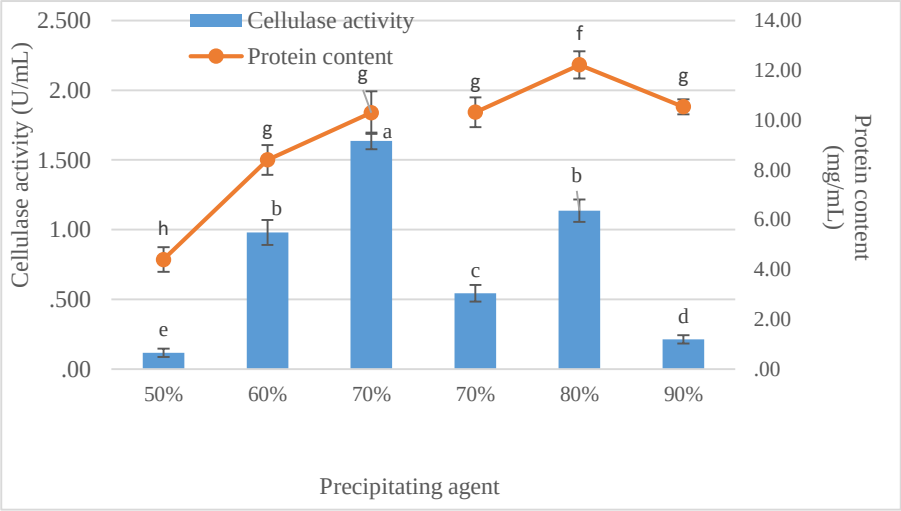

Figure 3. The precipitating ability of cellulase by ethanol and ammonium sulfate. The means with different letters are significally different (p<0.05).

The different agents significantly affected the isolating ability of cellulase in the pepper immersing solution. For $(NH_4)_2SO_4$, the 80%-saturation solution demonstrated higher cellulase and protein yield compared to the other treatments. However, the purifying yield of cellulase was better when using 70% ethanol, with a specific activity of 0.159 U/mg. While some earlier studies often used the 80%-saturation $(NH_4)_2SO_4$ solution to precipitate cellulase from microfungi [1] or from bacteria [6, 13]. The differences could be due to the structure of pepper cellulase.

3.5. Purification of Cellulase by Gel Filtration Chromatography

The crude cellulase received from precipitation with 70% ethanol was loaded onto a Sephadex G-25 column. The 20 mM phosphate pH 7.0 solution was used to eluate the protein and contained in 60 tubes (2 mL/tube). The OD 280 nm results from the tubes indicated the presence of six peaks (Figure 4). Tubes corresponding to each peak were combined into a single fraction: fraction 1 (tubes 1-4), fraction 2 (tubes 5-9), fraction 3 (tubes 10-20), fraction 4 (tubes 21-25), fraction 5 (tubes 26-30), and

fraction 6 (tubes 31-45). Most of the protein concentrated in fractions 2, 3, and 4 occupied 80.5% of the eluated protein content (Figure 5). However, the results of the agar well diffusion assay indicated that fractions 5 and 6 contained a high content of protein with halo diameter of 6 mm and 7 mm (fig. 5B) and cellulase activity of 0.030 and 0.035 U/mL (Table 2), respectively.

Fractions 3, 5, and 6 were continuously evaluated for the degree of purification by SDS-PAGE. After staining with Coomassie brilliant blue R-250, fraction 3 showed a protein band with a molecular mass of 75 kDa; both fraction 5 and fraction 6 had a band with a molecular mass less than 75 kDa. This result suggests that fraction 5 and 6 may contain the same protein. To achieve a higher level of purify, the ion exchange chromatography technique could be employed prior to SDS-PAGE. However, strategies to enhance cellulase activity should be considered, as the hydrolytic ability of enzymes often decreases significantly after each step of purification.

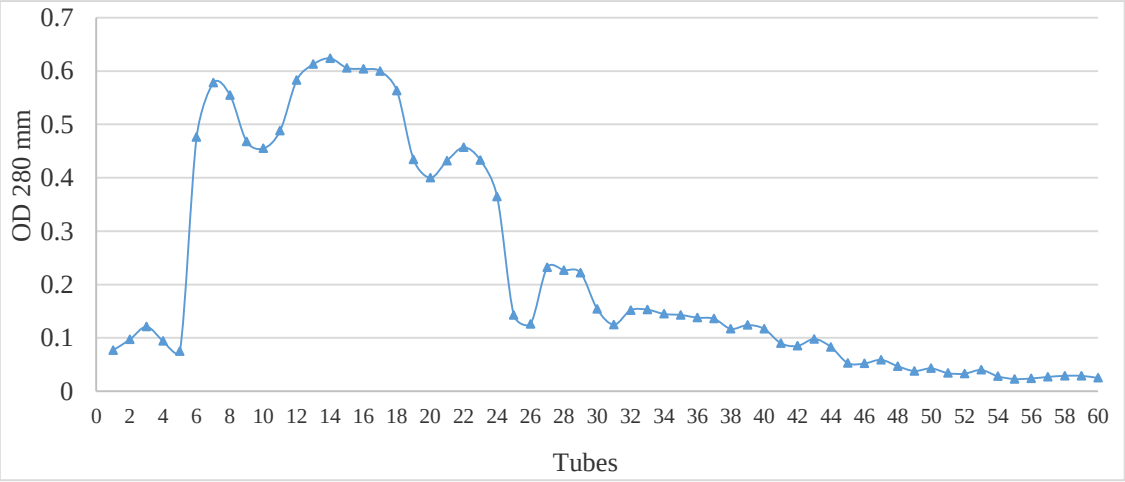

Figure 4. OD 280 nm value of eluted tubes from the Sephadex G-75 column.

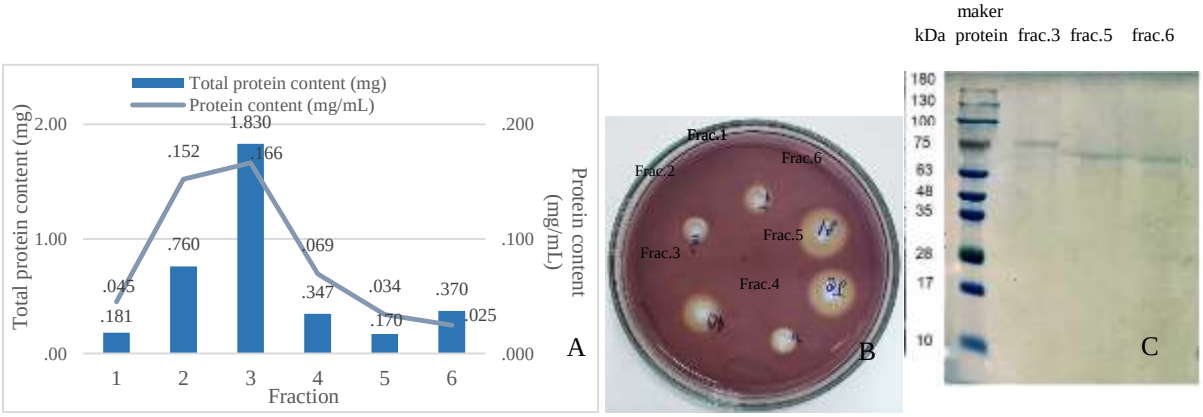

Figure 5. A) Protein content of fractions; B) CMCase zone of diameter of fraction; C) Molecular weight determinations by SDS-PAGE method. The migration positions of protein were indicated as 75 kDa (frac. 3) and <75 kDa (frac. 5 and 6).

Table 2. Cellulase activity of fractions from the Sephadex G-75 column

Fraction	Cellulase activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)
3	-	0.167	-
5	0.030	0.035	0.857
6	0.035	0.024	1.458

"-": not detect at the minimum rate of dilution.

4. Conclusion

Extracting cellulase from the peel of ripe pepper is better than green pepper. The highest cellulase activity was recorded at 40 ^{o}C for 48 hours of immersing ripe pepper, with an activity of 1.98 U/ g pepper fruit. Using 70% ethanol, the precipitating yield more than ammonium sulfate, with an 48% of cellulase activity of 48% and protein content of 37%. By Sephadex G-75 filtration and SDS-PAGE electrophoresis, the molecular mass of cellulase was estimated less than 75 kB.

References

[1] S. Raveendran, B. Parameswaran, S. B. Ummalyma, A. Abraham, A. K. Mathew, A. Madhavan, S. Rebello, A. Pandey, Applications of Microbial Enzymes in Food Industry, Food Technol Biotechnol, PMID: 29795993; PMCID: PMC5956270, Vol. 56, No. 1, 2018, pp. 16-30, https://doi.org/10.17113/ftb.56.01.18.5491.

[2] C. S. Karigar, S. S. Rao, Role of Microbial Enzymes in the Bioremediation of Pollutants: a Review, Enzyme Res, Article ID: 8051872011, https://doi.org/10.4061/2011/805187.

[3] S. Bhaskarjyoti, D. Kaushik, S. B. Sarat, Physiology of Fruit Development, Int. J. Curr. Microbiol. App. Sci., Vol. 9, No. 06, 2020, pp. 504-521, https://doi.org/10.20546/ijcmas.2020.906.066.

[4] H. Chen, Chemical Composition and Structure of Natural Lignocellulose, In: Chen, H.Z., Ed., Biotechnology of Lignocellulose: Theory and Practice, Springer, Netherlands, Dordrecht, 2014, pp. 25-71, https://doi.org/10.1007/978-94-007-6898-7_2.

[5] G. L. Miller, Use of Dinitrosalisylic Acid Reagent for Determination of Reducing Sugars, Anal. Chem., Vol. 31, No. 3, 1959, pp. 426–428, https://doi.org/10.1021/ac60147a030.

[6] K. Shanmugapriya, P. S. Saravana, M. M. Krishnapriya, A. Mythili, S. Joseph, Isolation, Screening and Partial Purifcation of Cellulose from Cellulose Producing Bacteria, Int. J. Adv. Biotechnol. Res., Vol. 3, No. 1, 2012, pp. 509-514.

[7] O. H. Lowry, N. J. Rosbrough, A. L. Farr, R. J. Randall, Lowry Protein Assay, J. Biol. Chem., Vol. 193, 1951, pp. 265.

[8] S. Englard, S. Seifter, Precipitation Techniques, in: M. P. Deutscher (Ed.), Guide to Protein Purification, Academic Press, San Diego, USA, 1990.

[9] Y. Shangguan, J. Ni, L. Jiang, Y. Hu, C. He, Y. Ma, G. Wu, H. Xiong, Response Surface Methodology-Optimized Extraction of Favonoids from Pomelo Peels and Isolation of Naringin with Antioxidant Activities by Sephadex LH20 Gel Chromatography, Curr. Res. Food Sci., Vol. 7, 2023, pp. 100610, https://doi.org/10.1016/j.crfs.2023.100610.

[10] S. Purkayastha, P. Dahiya, Phytochemical Screening and Antimicrobial Activity of Some Medicinal Plants Against Multi-drug Resistant Bacteria from Clinical Isolates, Indian J. Pharm. Sci., Vol. 74, No. 5, 2012, pp. 443-450, https://doi.org/10.4103/0250-474x.108420.

[11] U. K. Laemmli. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4, Nature, Vol. 227, 1970, pp. 680-685.

[12] M. Mohammed, A. Sallam, N. Alqahtani, M. Munir, The combined Effects of Precision Controlled Temperature and Relative Humidity on Artificial Ripening and Quality of Date Fruit, Foods, Vol. 10, No. 11, 2021, pp. 2636, https://doi.org/10.3390/foods10112636.

[13] B. Wu, S. Zheng, M. M. Pedroso, L. W. Guddat, S. Chang, B. He, G. Schenk, Processivity and Enzymatic Mechanism of a Multifunctional Family 5 Endoglucanase from *Bacillus subtilis* BS-5 with Potential Applications in the Saccharifcation of Cellulosic Substrates, Biotechnol. Biofuels., Vol. 11, No. 1, 2018, pp. 20, http://doi.org/ 10.1186/s13068-018-1022-2.