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KINETIC MODELING STUDIES OF ENZYMATIC PURIFICATION OF GLUCOMANNAN

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Abstract. [1] Purification of glucomannan by hydrolising starch – the main contaminant – was studied. Hydrolysis removed 88.7 % of starch. The highest glucomannan content was found to be 73.35 %. The sample showed the comparable infrared spectra to those of the commercial glucomannan. The kinetics of enzymatic hydrolysis was evaluated using the Michaelis-Menten model.

Keywords: α-amylase, *Amorphophallus oncophyllus*, glucomannan, hydrolysis, purification, starch.

1. Introduction

Amorphophallus oncophyllus is a type of tuber that grows wildly and recently being commercially cultivated in the forest edge of Indonesian. The tuber contains calcium oxalate, which is associated with itching and irritations in the mouth that makes the tuber underutilized for human consumption. Being a member of the philodendron (arum) family, the native *A. oncophyllus* flour (NAoF) was reported to contain glucomannan up to 60 % [1]. Unfortunately, most of the Indonesian farmers sell the tubers as dried chips or low-quality tuber flour [2]. Low glucomannan content reflects a low economic value of those products. The international standards have ratified that the top grade glucomannan flour should contain minimum 70 % of starch. Hence, it is important to explore glucomannan purification method in order to improve the economic value.

Glucomannan of *Amorphophallus* sp. is a linear heteropolysaccharide composed of β-1,4-linked D-mannose and D-glucose monomers with 1 to 1.6 of glucose/mannose ratio and certain short side branches at the C-3 position of the mannoses through β-1,6-glucosyl units. The acetyl groups along the backbone are located at the C-6 position on average every 9–19 sugar units. Glucomannan is widely utilized as food ingredients, including emulsifier and stabilizer as well as functional foods and drug excipient [1, 3, 4].

Purification methods of glucomannan from NAoF strongly affect the properties and structure of the purified glucomannan, which finally influence the scope of applications of glucomannan. Therefore, it is important to develop an efficient and convenient isolation method to produce high purity glucomannan. The isolation of glucomannan is commonly conducted using dry (mechanical) and wet methods. The mechanical purification methods include blowing and sieving, which result in flour with low glucomannan content [5]. Water, ethanol, and 2-propyl alcohol are common solvents used for impurities removal in wet methods [6]. However, the latter method demands a high volume of solvent. Due to the weakness of these previous methods, another alternative method was proposed.

Starch, cellulose, and nitrogen-containing materials are the main impurities, which encapsulate glucomannan granules [6]. High glucomannan content can be achieved by maximizing the removal of impurities. Starch is the highest impurity of NAoF, which seriously affects the purity and quality of glucomannan, such as reducing viscosity and increasing turbidity [7]. Hence removing of starch is expected to be the most efficient method to increase the quality of glucomannan. As far as literature survey being conducted, only α-amylase has been used to enzymatically modify starch and its derivatives by cleaving α-1,4 glycosidic [8]. Due to the specific properties of the enzyme, α-amylase is expected to only cleave α-1,4 glycosidic linkage of starch and leaves α-1,4 glycosidic of glucomannan uncleaved. Application of enzymatic method on glucomannan purification was still not fully studied. Hence, the objective of this work was to study the effect of enzymatic hydrolysis of starch on glucomannan purification from NAoF. In addition, the kinetics of enzymatic hydrolysis was evaluated using the Michaelis-Menten model.

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2. Experimental

2.1. Materials

The main material used in this study was NAoF obtained from the local farmer (Sidoharjo, East Java-Indonesia). The content of starch and glucomannan in the flour were 7.92 and 66.3 %, respectively. α-Amylase (EC 3.2.1.1) was isolated from *Bacillus subtilis* and working at pH 6.0–7.0 with 4,000 U/g activity (Suntaq International Limited). *B. subtilis* is one of the bacteria which has been used to produce enzymes for human and animal feed [9]. 2-Propyl alcohol (IPA) solution (70 %) was used for washing the suspension after the hydrolysis. Other chemicals used in this work were of analytical grade and used directly without pre-treatment.

2.2. Enzymatic Hydrolysis

The enzymatic hydrolysis was conducted based on the method of Wardhani [1]. A flour suspension (400 ml) was prepared by diluting a predetermined amount of NAoF in distilled water at 343 K to obtain a certain concentration of the flour (1.5, 2.0, 2.5, and 3.0 % w/v). Starch and water in the flour suspension were allowed to react with the assistance of *α*-amylase (0.03 g/g flour). The enzyme concentration referred to the best concentration of Wardhani [1]. The suspension was maintained at pH 6.8 during the hydrolysis. Each of the suspension was conducted for different period of hydrolysis (0, 25, 50, 75, 100 and 150 min) under continuous stirring at 350 rpm. Once the reaction completed, 2-propyl alcohol (200 ml, 70 %) was added to the suspension and stirring was continued at 350 rpm for 30 min. After filtration of the suspension, a creamy solid cake was obtained. The cake was then dried and powdered. The obtained powders were subjected for further analyses.

2.3. Analytical Methods

The powder samples obtained from enzymatic hydrolysis were subjected to starch determination using Fehling solution to obtain the glucose concentration. The conversion factor (0.9) was used to obtain starch concentration [10]. Reducing sugar was determined using Somogyi-Nelson method with D-glucose as a standard [11]. Glucomannan determination was conducted following the method of Chua based on 3,5-dinitro-salicylic acid colorimetric assay with D-glucose as a standard [4]. The sample was extracted using formiat–NaOH before hydrolysis using sulfuric acid. Both the extract solution and the hydrolysate solution were subjected to colorimetric reaction and the absorbances were read at 550 nm. Glucomannan concentration (*GM*) was calculated as:

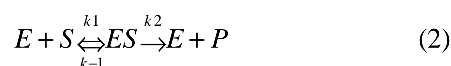
$$GM\,(\%) = 5000\varepsilon\cdot(5T - T_0)/m \qquad (1)$$

where ε is the correction factor (0.9); m is a sample weight, mg; T and T_0 are glucose in hydrosilate and in extract sample, respectively.

The analyses were done in triplicates. In addition, the powder sample with the highest glucomannan was subjected to Fourier Transform Infrared Spectrometry (FTIR) for functional groups characterization and Scanning Electron Microscope (SEM) for particles microstructure observation. FTIR spectrum of the samples was recorded under dry air condition at room temperature in the range of 4000-400 cm^{-1} by using IR Prestige Shimadzu. The peaks were assigned by comparison with the literature data. The powder granules morphology was observed using SEM apparatus (FEI Inspect S50) at a certain magnification. Prior to SEM analysis, a dry sample was placed on a stub and coated with gold.

2.4. The Kinetics Modeling

The Michaelis-Menten model was applied to study the kinetic model of enzymatic starch hydrolysis on NAoF using *a*-amylase at the first 25 min of the reaction. The Michaelis-Menten mechanism was developed based on the interaction between the enzyme (*E*) and the substrate (*S* – starch), leading to an intermediary enzyme-substrate complex (*ES*), from which the reaction product (*P* – reducing sugar) is generated (Eq. (2)).



$$E + S \underset{k-1}{\overset{k1}{\Leftrightarrow}} ES \overset{k2}{\rightarrow} E + P \qquad (2)$$

where k_1 is a reaction rate constant for enzyme-substrate linking; k_{-1} is a reaction rate constant for enzyme-substrate complex dissociation, and k_2 is a reaction rate constant for product generation.

Three stages of reaction can be identified in the Michaelis-Menten mechanism [12]:

i. The first stage is a rapid bi-molecular reversible one, leading to the formation of an enzyme-substrate complex.

ii. The second stage is the reverse of the first one, the intermediate complex, having a relatively short lifetime.

iii. The third stage leads to the final product by the irreversible conversion of enzyme-complex, while the free enzyme goes again to the first stage, linking another substrate molecule.

The kinetic constants, *i.e.* K_m and V_{max}, were determined from an initial rate of hydrolysis production at various starch concentrations by optimization of their

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values by fitting the model to the experimental data using solver facilities of Ms-Excel. The model was statistically validated through the R^2 value.

3. Results and Discussion

The effort to increase glucomannan content of NAoF through the removal of starch as the main impurities of the flour has been conducted by an enzymatic hydrolysis. The purified flour was determined for starch, reducing sugar, and glucomannan content.

3.1. Starch Content

The effect of time and flour concentration on starch contents of hydrolyzed products is depicted in Fig. 1a. The figure shows that the starch content decreased in line with time at all NAoF concentrations. At longer reaction time, the enzyme was able to create more active sites for the substrate to be cleaved resulted in lower starch concentrations in the flour. This result was in line with that reported by Simsek and El [13]. As expected, Fig. 1a also shows that hydrolysis of NAoF at higher flour concentrations results in lower starch content. This phenomenon reveals that the enzyme concentration (0.03 g/g NAoF) used in this research provides enough active sites to promote hydrolysis of the starch in the range of 1.5–3 % of NAoF. This result was supported by Wardhani who found that increasing the enzyme concentration up to 0.03 g/g NAoF showed a positive effect in reducing the starch [1]. However, reverse effect was observed when the enzyme concentration was over than that one. This could be due to increasing starch competitor compounds which were produced during the starch hydrolysis [1].

At all initial NAoF concentrations studied in this work, about a half of the starch content could be removed in the first 50 min, and then followed by a slower starch removal rate. During enzymatic hydrolysis, the initial period corresponds to the rapid hydrolysis of the most amorphous part of the starch granules, whereas in the latter stage, the more crystalline parts are slowly degraded [14]. In addition, the high concentration of reducing sugar as the hydrolysis product in the reaction medium during the hydrolysis of starch leads to a significant decrease of the hydrolysis rate due to its competition with the starch in occupying the same active sites of α-amylase [15]. As a result, the degradation rate decreased after 50 min. Moreover, it was observed that after 100 min of hydrolysis, the suspension system became more viscous. This increase in viscosity limited molecules movements resulted in lower opportunities of the starch granules to contact with an enzyme which subsequently reduces the starch degradation. A similar trend was reported by Hera [16], Zhang [17], and Zheng [18] who found there is no significant difference of starch hydrolysis rate after 90–100 min.

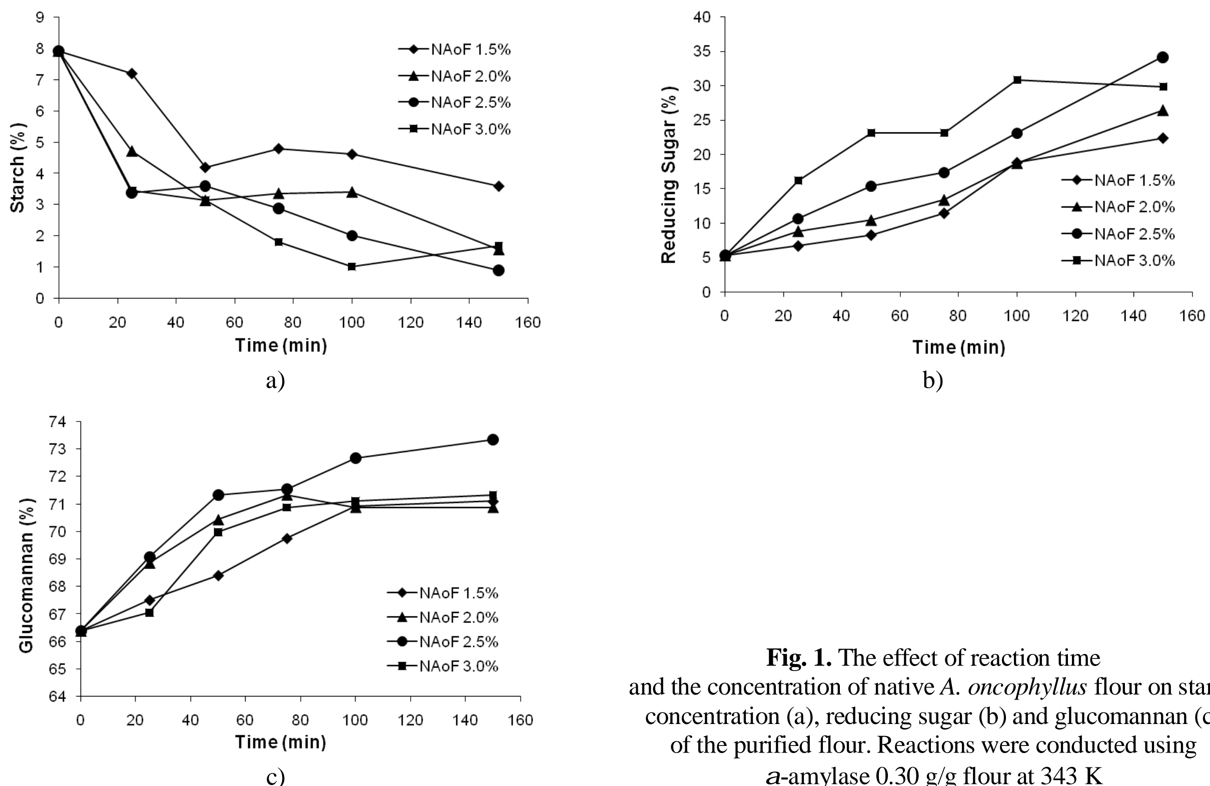

Fig. 1. The effect of reaction time and the concentration of native *A. oncophyllus* flour on starch concentration (a), reducing sugar (b) and glucomannan (c) of the purified flour. Reactions were conducted using α-amylase 0.30 g/g flour at 343 K

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In this study, the lowest starch concentration was observed at hydrolysis using 2.5 % w/w NAoF concentration for 150 min. In this condition, about 88 % of the initial starch was hydrolyzed. The removal of starch in this research was lower than the previous report Nurjanah [19] who managed to reach 96 % of starch conversion. The difference in results is possibly due to thermostable α-amylase which allows to be applied at a higher temperature (368 K) and converts more starch in a shorter time. Fig. 1a also shows some residual starch was still detected at 150 min which was difficult to be completely hydrolyzed. Lopez [20] reported that 90 % starch hydrolysis conversion was achieved within 2 h. However, the total hydrolysis is still not achieved even after 48 h of incubation time.

3.2. Reducing Sugar

Depending on the chain length, hydrolysis of starch results in reducing sugar and other derivative products. Fig. 1b shows that concentration of reducing sugars increased with the reaction time at all initial concentration of NAoF. A similar result was reported by Rodriguez and Bernik [21] who found that reducing sugar still significantly increased even after 240 min during hydrolysis of high amylose maize starch Hylon VII using α-amylase from human origin Type XI. Higher initial NAoF concentration leads to a higher production of reducing sugar at all reaction times. This result agreed well with Khawla [22] who hydrolyzed potato peel flour. However, the reducing sugar of 3 % NAoF decreased after 100 min which could be due to the possibility of reducing sugar degradation as a consequence of prolonged time [23] and high concentration of reducing sugar during the hydrolysis of starch led to a significant decrease of the hydrolysis rate [15]. In addition, increasing the viscosity of the system might also reduce the effectiveness of enzymatic hydrolysis. Wu and Zhong [24] reported that viscosity of glucomannan still maintains to increase even after 6000 s of stirring. The highest reducing sugar (20.34 %) was obtained from the reaction of 2.5 % w/w NAoF concentration for 150 min.

3.3. Glucomannan Content

Fig. 1c presents the effect of reaction time and initial concentration of NAoF on glucomannan content of hydrolyzed *A. oncophyllus* flour. In general, a longer reaction time resulted in higher glucomannan content. The rise in glucomannan purity was a result of α-amylase activity, which specifically attacked only the α-1,4-glycosidic linkage of the starch. Since glucomannan is a heteropolysaccharide molecule consisting of D-mannose and D-glucose linked by β-1,4 bond, it was not hydrolyzed. The enzyme helped in cleaving and removing starch coating the glucomannan granules and subsequently led to release the glucomannan granules. Fig. 1c shows the highest glucomannan purity (73.35 %) was attained at enzymatic hydrolysis of 2.5 % w/w flour for 150 min. The glucomannan purity increased about 10 % from NAoF. In this condition, the purified flour sample contained 0.9 % of starch and 20.34 % of reducing sugar. Nurjanah [19] reported higher increase of glucomannan content from 28.75 to 80.53 %. Meanwhile, Mulyono [25] obtained the glucomannan content increased from 31.99 to 93.75 %. Both authors used NAoF with lower initial glucomannan content than that used in this study. NAoF with lower glucomannan content may suggest that NAoF contains higher starch content. The higher starch content in the initial NAoF showed the higher potential of the impurities removal, which subsequently resulted in a higher increase in glucomannan content.

3.4. Enzymatic Hydrolysis Kinetic Model

Fig. 2 shows the correlation between the initial starch concentrations of NAoF and the initial rates of a product formation according to the Michaelis-Menten model. The constants of the model were determined by a nonlinear regression method. The optimized value of K_m and V_{max} were 32.84 and 0.905 g/l·min, respectively.

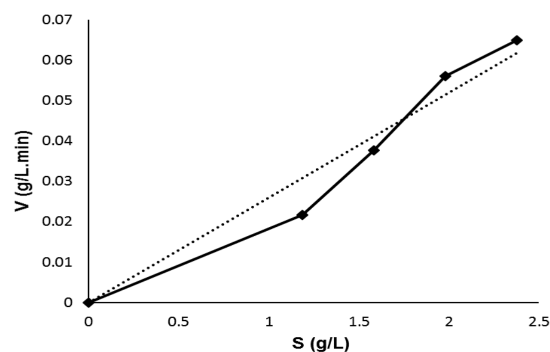

Fig. 2. Plotting between initial starch concentration in the suspension system and initial velocity of reducing of *A. oncophyllus* flour suspension data (solid-line) and model according to Michaelis-Menten equation (dot-line)

Michaelis-Menten constant (K_m) is the concentration of substrate at which the enzyme active sites are filled half-full. Thus, K_m measures a substrate concentration required for significant catalysis reaction to occur. High K_m value indicates a weak bond between the enzyme and the substrate in the formation of the complex compounds ES or low apparent affinity of the enzyme for the substrate. Hence, the enzyme requires a higher substrate concentration to reach 50% saturation. In this study, K_m value was higher than the concentrations of starch in the system observed (1.19–2.37 g/l). This

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implied that a constant rate of complex dissociation is faster than that of complex production (Eq. (2)). In this condition, the Michaelis-Menten equation for starch hydrolysis in the glucomannan enzymatic purification becomes:

$$V = \frac{V_{\max}[S]}{K_m} \quad (3)$$

Eq. (3) suggests that the rate of the reaction is proportional to the substrate concentration. Hence, the enzymatic starch hydrolysis in glucomannan purification followed the first-order kinetics.

Meanwhile, maximum velocity (V_{max}) is a theoretical maximum rate of the reaction, which is never achieved in reality. It shows the number of substrate molecules converted to the product by the enzyme per unit time when the enzyme active site fully filled with the substrate. V_{max} values are proportional to the rate constants of the product formation k_2 [12]. As expected, the V_{max} value obtained in this study was much higher than the rate constants of the product formation obtained from the experiments (0.031–0.061 g/l·min). The statistical parameter showed a good agreement ($R^2 = 0.956$) between the experimental data and modeling results using the Michaelis-Menten model.

3.5. FTIR Analysis

Comparison of IR spectra of NAoF, the purified sample (obtained from hydrolysis of 2.5 % substrate concentration for 150 min) and the commercial glucomannan (Patrick Holford, 98 % glucomannan content) in the wavelength range of 4000–400 cm^{-1} is presented in Fig. 3. The spectra were in good agreement with those reported by An [3] and Chua [4]. The IR spectra of all samples demonstrated similar peak ranges of wavelength but difference in the intensity of the absorbance. In general, the absorbance values of the purified flour sample were between NAoF and commercial glucomannan.

All samples showed a peak attributed to broad bands located at 3000–3700 cm^{-1}, which indicated the presence of O–H groups of glucomannan [17]. These hydroxyl groups were laid on characteristically by methyl groups located at ~2900 cm^{-1} which assigned to –CH stretch vibration and 1720 cm^{-1} which attributed to C=O stretching vibration. The existence of β-1,4 linked glucose and mannose of glucomannan was indicated by the carbonyl (C=O) stretch vibration located at ~1650 cm^{-1} [26]. While peaks at ~1150 and ~1050 cm^{-1} referred to C–O–C stretch vibrations from ether groups in the pyranose rings, which indicate the presence of β-1, 4 glucosidic and β-1,4 mannosidic linkages in glucomannan [27].

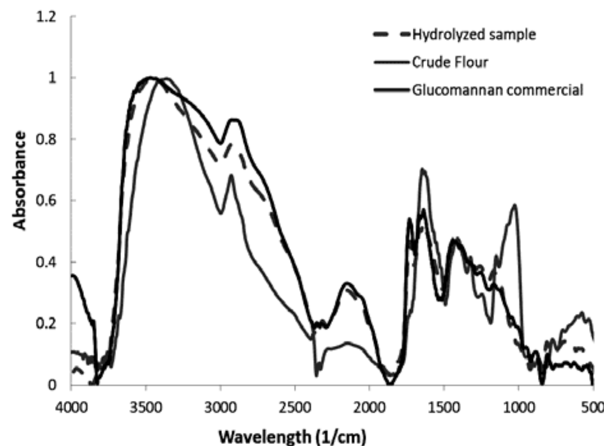

Fig. 3. Comparison of IR spectra of the native *A. oncophyllus* flour (grey solid-line), the purified flour (grey dash-line), and the commercial glucomannan (black solid-line)

3.6. Granules Morphology

The morphological observation was conducted to investigate the effect of hydrolysis on the microstructure of glucomannan granules. Fig. 4 (top) shows the granules morphology of NAoF observed at different magnifications. The NAoF granules displayed polygonal shapes with an average size of 600 µm. The figure indicates the presence of exposed biomaterials covering some parts of NAoF surface as a result of grinding and milling of *A. oncophyllus* dried chips into flour (Fig. 4, top left). These biomaterials were likely to be the impurities, which covered up the glucomannan granules. This fact is in accordance with the results [6] reported that glucomannan granules were encapsulated by impurities including starch, cellulose and nitrogen-containing material.

Meanwhile, Fig. 4 (bottom) shows the morphology of the hydrolyzed *A. oncophyllus* flour sample at 100 and 450 magnifications. After enzymatic hydrolysis, the hydrolyzed flour granules were irregular in shapes with an average size of 900 µm. The hydrolyzed flour granules degraded on their external part leading to the formation of a number of hollows. This phenomenon indicates that hydrolysis took place by exo-corrosion, and it was not uniform for all flour granules in which some regions were much more susceptible to enzymes attack than the others. The hydrolysis resulted in sharper edges and the cleaner surface of the flour granules than that of NAoF. Franco and Ciacco [27] also reported the enzymatic attack on the large granules of cassava and corn starches (> 16 µm) as indicated by remarkable corrosion of granule surface, primarily in the radial direction. The removal of impurities by enzymatic hydrolysis resulted in the significant increase of glucomannan content in the flour.

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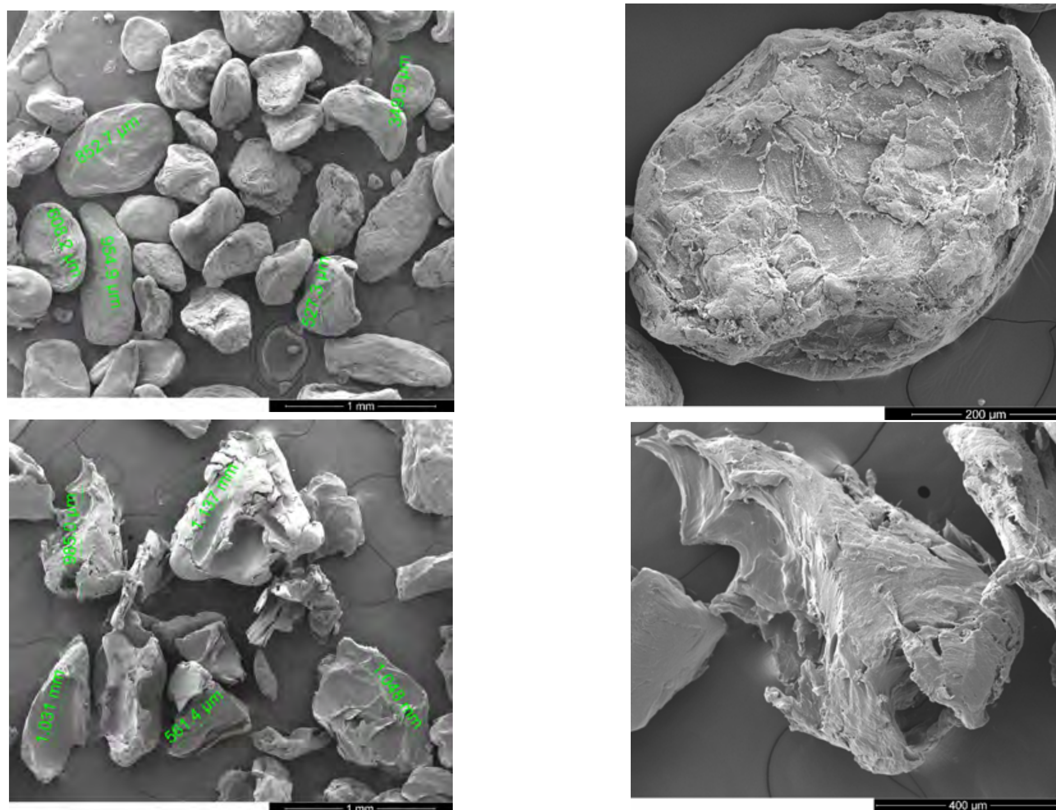

Fig. 4. Granule morphology of native *A. oncophyllus* flour (top) and the purified sample (bottom), at 100 (left) and 450 (right) magnifications

4. Conclusions

The overall results suggested that hydrolyzing 2.5 % w/w of NAoF for 150 min successfully removed 88.7 % of starch and increased 10% glucomannan concentration. The final product contained 73.35 % of glucomannan, 0.9 % of starch, and 20.34 % of reducing sugar. K_m and V_{max} values of Michaelis-Menten model were 32.84 g/l and 0.905 g/l·min, respectively with R^2 = 0.956. The enzymatic starch hydrolysis in glucomannan purification followed the first-order kinetics. The hydrolyzed flour sample with the highest glucomannan showed a comparable IR spectrum to that of commercial glucomannan. The morphology analysis confirmed the removal of NAoF impurities from the flour granules as well as the rupture of NAoF granule after hydrolysis.

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КІНЕТИЧНІ МОДЕЛЬНІ ДОСЛІДЖЕННЯ ЕНЗИМАТИЧНОГО ОЧИЩЕННЯ ГЛЮКОМАННАНА

Анотація. *Вивчено процес очищення глюкоманнана гідролізом крохмалю – основного забруднювача. Встановлено, що гідролізом усувається 88,7 % крохмалю. Одержано найвищий вміст глюкоманнану 73,35 %. Проведено порівняльні дослідження ІЧ-спектрів дослідженого і комерційного глюкоманнана. За допомогою моделі Міхаеліса-Ментена описано кінетику ензиматичного гідролізу.*

Ключові слова*: α-амілаза, Amorphophallus oncophyllus, глюкоманнан, гідроліз, очищення, крохмаль.*

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