Synthesis of microfibrillated cellulose from solid residue of seaweed processing industry and its applications in alginate-based hydrogels for papain enzyme carriers

Novitri Hastuti[1,2*], *Aisiyah* Tazkiatunnisa[3], *Safrina Dyah* Hardiningtyas[3], *Wahyu* Ramadhan[3], *Gustan* Pari[1], *Dian Anggraini* Indrawan[1] , *Erlina Nurul* Aini[1] , and *Heru Satrio* Wibisono[1]

[1] Research Center for Biomass and Bioproducts, National Research and Innovation Agency (BRIN), 16911, Bogor, Indonesia
[2] Research Collaboration Center for Nanocellulose, Universitas Andalas, 25163 Padang, Indonesia
[3] Faculty of Fisheries and Marine Science, IPB University, 16680 Bogor, Indonesia

Abstract. The seaweed processing industry generates cellulose-rich solid residues. Cellulose, a natural polymer, exhibits advantageous physical properties when employed as a scaffold or filler in specific matrices. Enzymes used in biotechnology encounter challenges related to their performance, influenced by both the surrounding environment and the release process. The objective of this study is to convert cellulose obtained from the solid residue of seaweed processing into microfibrillated cellulose (MFC) through hydrochloric acid hydrolysis. The MFC will then be incorporated into an alginate-based hydrogel matrix to serve as a carrier for the papain enzyme. The characterization results indicate that the presence of MFC derived from the solid residue of the seaweed industry at concentrations of 0.5% and 1% significantly affects the swelling behavior of the hydrogel compared to the hydrogel without MFC. The spectrophotometric analysis revealed that the incorporation of 0.5% MFC exhibited greater enzyme immobilization capabilities in comparison to other treatments. The study's findings support the utilization of cellulose derived from solid residues in the seaweed processing industry, highlighting its potential for diverse applications.

1 Introduction

Indonesia is rich in potential marine resources, including red seaweed as a source of gelatin and carrageenan which is an export commodity [1-5]. The Indonesian seaweed industry has rapidly expanded over the last twenty years and now contributes to the livelihoods of 267,000 coastal households [6]. The expanding agar market promotes the expansion of seaweed-based agar production [7]. Typically, agar production is accompanied by a substantial quantity of agar industrial waste residues, which are usually only thrown away as organic waste [1].

* Corresponding author: novi043@brin.go.id

These waste residues are predominantly made up of fiber cellulose, agar, and perlite filter aids [8]. Numerous research investigations have been conducted to examine the utilization of byproducts derived from the agar industry, which mostly relies on seaweed. These researches have explored several applications of these leftovers, such as their potential as biofuels, activated carbon, and as a valuable source of cellulose fiber for the production of polymeric film materials [9–12].

Among the numerous studies that have been conducted, interest has been drawn to the application of cellulose derived from seaweed-based industrial residues. Approximately 150 years ago, cellulose was first utilized as a basic chemical material [13]. Cellulose reactions and properties are governed by intermolecular interactions, cross-linking reactions, chain lengths, chain-length distribution, and the arrangement of functional groups along the polymer chains [13]. Cellulose is presently being converted into microfibrillated cellulose (MFC) or cellulose nanofibers in an effort to advance its use as a natural polymer in nanotechnology [14]. MFC is presently extensively employed in the reinforcement of polymeric composites [15, 16], microbeads for vitamin delivery [17], nanoemulgel for emulsifier applications [18], and also used as porous ultrafine fiber membrane for enzyme immobilization [19, 20]. Hence, enzymes are frequently restricted to single-use applications, and the use of MFC in enzymatic catalysis is particularly intriguing for the aforementioned purposes.

Papain is an enzyme that is extensively utilized in the manufacturing sector, including the pharmaceutical industry, meat tenderization, leather, paper and pulps, cosmetics, stabilizers (beer), sugar syrup liquid from starch production, coagulation in dairy products (cheese), peptide manufacturing, and detergent production [21]. However, the spatial configuration of papain undergoes alterations in response to harsh environmental conditions, resulting in its denaturation or inactivation [22], and the inability to recover free papain from the products results in reduced reusability [23].

Therefore, the objective of this research is to fabricate microbeads as papain enzyme immobilizers using MFC derived from the solid residual of the seaweed industry, which is produced via hydrochloric acid hydrolysis and integrated with an alginate matrix. The study aims to generate novel insights into the utilization of solid residue derived from the seaweed-processing industry, with the objective of transforming it into value-added goods rather than allowing it to remain as unproductive organic waste.

2 Methods

2.1 Experimental apparatus and materials

The experimental equipment used in this research included a beaker, petri dish, spatula, stirrer, oven (Memmert TC-96-AD-A, Germany), stirrer with a magnetic bar, water bath (Memmert WNB Series, Germany), micropipette, scales (Ohaus PX224), Scanning Electron Microscopy instrument (Zeiss Type EVO 50), and UV-Vis spectrophotometer (Rayleigh Vis-723G, Beijing, China). The primary material used in this research was agar solid waste obtained from CV. Agar Sari Jaya, Malang, East Java. Other materials used were bovine serum albumin substrate (Sigma, Missouri, USA), distilled water, NaOH (Merck, Germany), H2O2 (Solvay, Thailand), H2SO4 (Merck), CaCl2 (Merck), and Bradford solution (Merck).

2.2 Cellulose extraction from agar-solid residue

The agar-solid residue was subjected to a treatment involving the addition of a 10% NaOH solution. The ratio of NaOH solution to solid residue is 8:1 by weight/volume (w/v), followed

by a three-hour cooking period at a temperature of 100 °C. The residual agar waste pulp underwent a water-washing process till achieving a neutral pH. Furthermore, a pretreatment process was conducted on the pulp using a 0.1 M hydrochloric acid (HCl) solution for a duration of 30 minutes in order to remove any contaminants present. The bleaching procedure involved the utilization of a 3% hydrogen peroxide (H_2O_2) solution for a duration of 2 hours within a water bath maintained at a temperature of 70 °C. The solid substance that underwent bleaching was subjected to neutralization until it reached a state of pH neutrality. It was subsequently designated as agar waste cellulose.

2.3 Synthesis microfibrillated cellulose by HCl hydrolysis

A bleached fiber from the cellulose extraction step (see 2.2), as much as 10 g, was hydrolyzed using 400-500 mL of 3 M HCl at 80 °C for 2.5 hours. Hydrolysis was stopped by adding 500 mL of cold water. The hydrolysis results were then filtered and washed until the pH was neutral. The fibers obtained via HCl hydrolysis, which have been neutralized, undergo mechanical treatment during a 30-minute grinding process using a blender with water addition. The cellulose suspension was then centrifuged at 4000 g at 22 °C for 15 min. The supernatant obtained was taken as microfibrillated cellulose from agar solid residue.

2.4 Syntesis of alginate beads containing MFC and papain enzyme

MFC solutions with concentrations of 0.5% and 1% (b/v) were prepared in the initial step. The MFC solution was mixed with a papain enzyme solution (100 U/mL) and then mixed again with a 4% sodium alginate solution. Alginate beads were synthesized by dispensing them through a 100-µL micropipette into a 3% calcium chloride solution ($CaCl_2$). The alginate beads containing MFC and papain enzyme were filtered, dried, and stored at 4°C for subsequent analysis. Alginate beads analysis encompasses the observation of their morphology, swelling ratio, and enzyme release profile. Beads consisting solely of alginate were fabricated for the purpose of serving as a comparison in the experimental setup.

2.4.1 Swelling ratio

The hydrogels were immersed in a 10 mL aqueous solution for a duration of 28 days during which they experienced swelling. The water was removed through the process of filtration and tissue cleansing. The swelling ratio (%) was determined using the formula:

$$\textit{The swelling ratio } (\%) = \frac{(w-w_0)}{w_0} x100 \quad (1)$$

where: W = Mass of hydrogels after swelling, W0 = Mass of dried hydrogels.

2.4.2 Enzyme release measurement

Experiments were performed to assess the enzymatic release from alginate beads containing MFC and papain enzyme by quantifying the anticipated quantity of enzyme bound inside the beads that would be liberated. This quantification was achieved by employing the Bradford method and utilizing bovine serum albumin (BSA) as a reference standard for protein content characteristics. The protein content has been established as a determining factor for the proportion of enzyme release. The protein concentration of an immobilized enzyme is assessed using the Bradford technique. A protein standard solution with a concentration of 2 mg/mL was prepared by dissolving 0.01 g of Bovine Serum Albumin (BSA) in 5 mL of sterile distilled water. The initial stock solution underwent dilution to produce solutions with

concentrations ranging from 0.01-0.1 mg/mL. The Bradford method for quantifying soluble proteins was conducted by mixing 0.1 mL of immobilized enzyme solution with 5 mL of Bradford's solution in a test tube. The measurement of absorbance length was conducted using a spectrophotometer at a specific wavelength of 595 nm subsequent to the incubation of the solution in a homogenous vortex for a duration of 5 minutes. The determination of the concentration of immobilized enzymes can be performed using the following calculation method:

$$y = ax + b \tag{2}$$

where: y : Sample absorbance, a : Slope, b : Intercept, x : Concentration of immobilized enzymes (mg/mL).

Determination of release of the enzyme was carried out after one day. The enzyme was immobilized in the hydrogel matrix and incubated in a 10 mL sodium phosphate buffer solution in the refrigerator. Table 1 describes the composition of hydrogels.

Table 1. Hydrogel formulations as scaffolds for the immobilization of enzymes.

Sample	Alginate 4%	MFC content		Enzyme	$CaCl_2$
		0.5% MFC	1% MFC	100 U/mL	3%
N0	3 mL	-	-	3 mL	6 mL
N1	2 mL	2 Ml	-	2 mL	6 mL
N2	2 mL	-	2 mL	2 mL	6 mL

3 Results and discussion

3.1 Cellulose characterization

Based on the results of our analysis, it was observed that the agar solid residue exhibited a cellulose content of 60.77% and a lignin content of 14.72%. Following the alkali extraction process utilizing sodium hydroxide (NaOH) and subsequent bleaching, the resulting cellulose content was determined to be 69.09%, while the lignin concentration was found to be 6.11% [24]. Characterization of extracted cellulose from agar solid residue under *Scanning Electron Microscope* (SEM) observation (250x magnification) as shown in Figure 1.

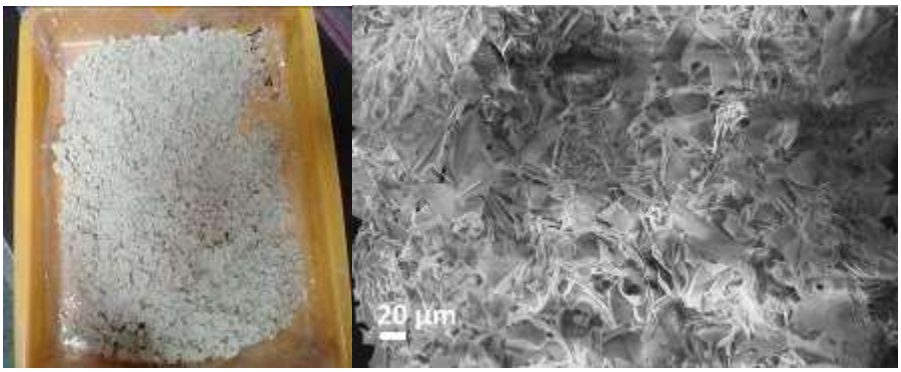

Fig. 1. Morphology of cellulose fibers from agar solid residue after alkali treatment and bleaching: (left) macro appearance and (right) under SEM observation 500 x magnification.

Image of cellulose of solid agar residue after being treated with 3M hydrochloric acid for 2.5 h at 80 °C under SEM observation (500x magnification) as in Figure 2. SEM observations have revealed a morphological transformation of cellulose fibers of agar solid residue, wherein the initial large sheets (Figure 1) underwent a change and became fine fibers (Figure 2). The obtained outcome aligns with the findings of a previous study that employed a 3M HCl solution to perform the hydrolysis of palm oil residue, specifically oil palm empty fruit bunches (OPEFB). Hydrochloric acid (HCl) effectively facilitated the degradation of cellulose, resulting in the production of cellulose nanocrystals characterized by aspect ratios within the range of 23-29 [25].

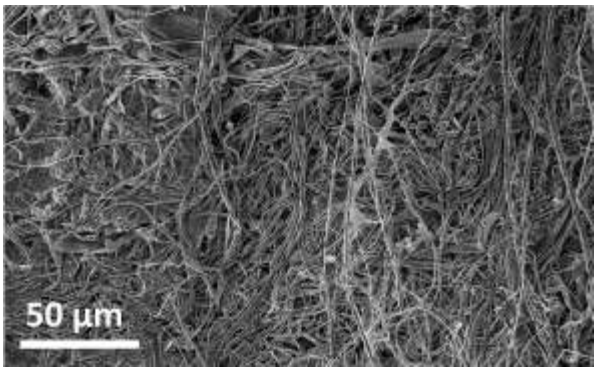

Fig. 2. SEM image of cellulose fiber of agar solid residue after HCl hydrolysis (MFC) 500x magnification.

3.2 Swelling ratio of alginate beads containing MFC and papain enzyme

Using a micropipette, a mixture of 4% (w/v) alginate, MFC, and papain enzyme was dropped into a 3% (w/v) $CaCl_2$ solution. Figure 3 depicts the appearance of the beads.

Fig. 3. Visual appearance of alginate beads containing MFC and papain ezyme.

In order to determine the resultant profile of the beads, observations were conducted using a 3D stereo microscope. The findings are depicted in Figure 4. Figure 4 illustrates that the beads consisting of alginate alone exhibit greater clarity compared to those containing MFC. The presence of delicate fibers was seen in beads that MFC.

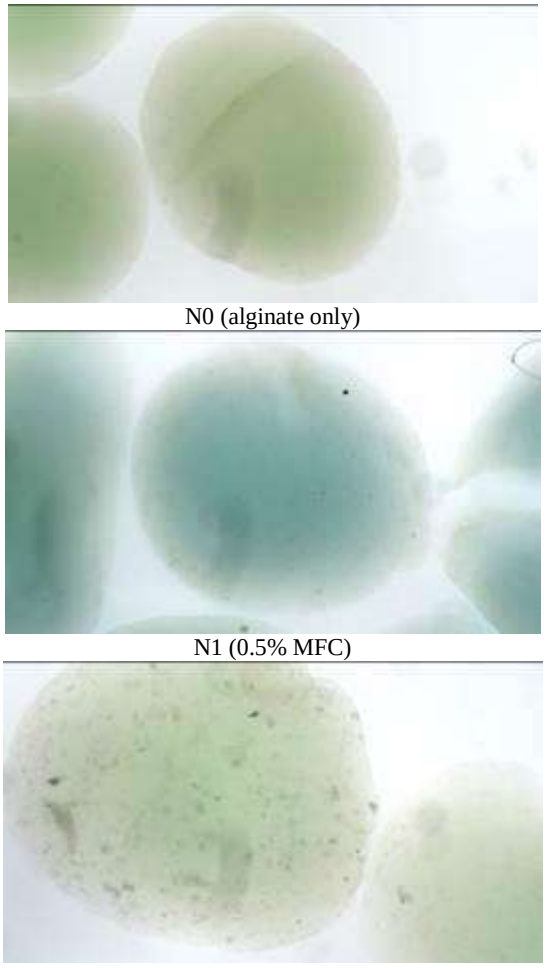

Fig. 4. Cross section of alginate beads and alginate beads containing MFC under stereo microscope (magnification 20x).

The process of cross-linking alginate and MFC using $CaCl_2$ as the crosslinker is illustrated in Figure 5. Hydrogen bonds between the hydroxyl groups of cellulose and the carboxyl groups of alginate are believed to influence the mechanical properties of hydrogels [26]. The formation of a three-dimensional network between nanocellulose and alginate via Ca^{2+} ions facilitated a more favorable microenvironment for the fermenter bacteria *Clostridium saccharoperbutylacetonicum*, as demonstrated in our prior research utilizing nanocellulose and alginate with $CaCl_2$ as a crosslinker [27].

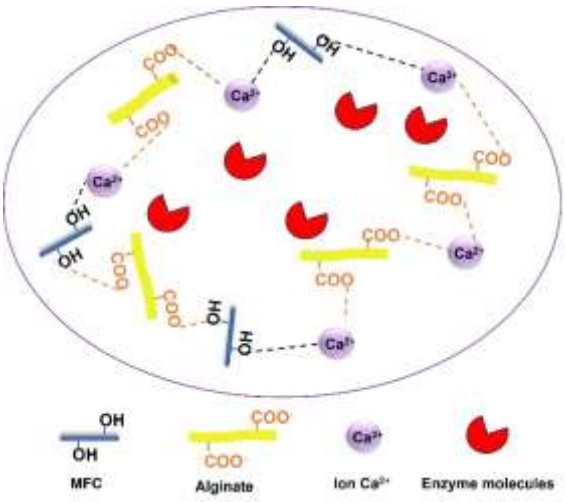

Fig. 5. Ilustration of crosslinking between alginate, ion Ca^{2+} and MFC.

The findings from the 30-days observation of alginate-MFC beads revealed evidence of swelling, the swelling ratio as depicted in Table 2. The findings from the hydrogel swelling observations indicate that the hydrogel composed only of alginate exhibited the highest swelling ratio following a 28-day observation period. According to previous research, it has been suggested that water tends to initially diffuse towards the amorphous regions and the external sites of the fibrils. These regions are more easily accessible to water molecules. On the other hand, the sites located at the inner surface and within the crystallites become involved only after the matrix has undergone significant swelling [28,29]. It can be inferred that beads formed only of alginate have a greater presence of amorphous regions in comparison to beads supplemented with MFC.

Table 2. Swelling ratio of alginate beads containing MFC made of solid residue of seaweed industry.

Day of observation Sample	Day 7 (%)	Day 14 (%)	Day 28 (%)
N0 (alginate only)	0	133	168
N1 (alginate containing MFC 0.5 %)	0	50	67
N2 (alginate containing MFC 1%)	33	33	133

3.3 Profile of enzyme release

The enzyme release profile of alginate and alginate-MFC hydrogels is determined by first establishing a standard curve, as depicted in the Figure 6. The equation derived from creating a standard curve was employed as a formula to ascertain the concentration of immobilized enzyme over a 24-hour period of observation.

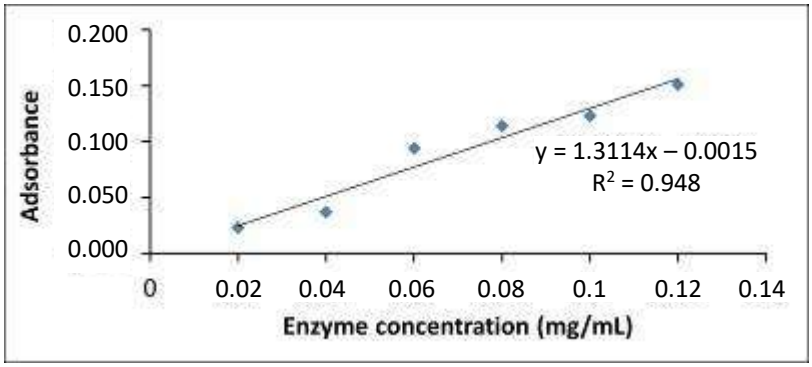

Fig. 6. Standard curve for determination of enzyme release from hydrogels.

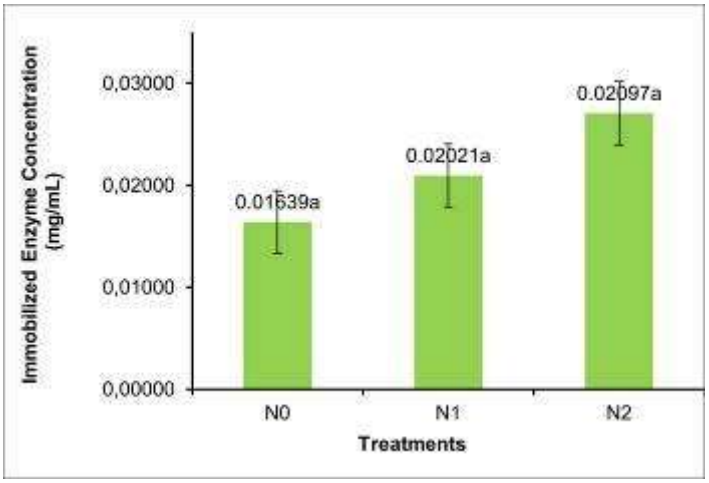

Fig. 7. Immobilized enzyme concentrations from each treatmentN0 (without MFC); N1 (MFC 0.5%) and N2 (MFC 1%).

Figure 7 demonstrates that including MFC into the alginate-based hydrogel matrix improved the immobilization of the papain enzyme. However, further statistical analysis revealed no significant difference. The incorporation of MFC into the alginate beads matrix results in chemical interactions that enhance the mechanical characteristics of the microbeads [17]. Due to the presence of robust polar groups in both MFC and alginate molecules, they are capable of interacting with one other through the establishment of hydrogen bonds [30]. When using a cellulose matrix as an immobilizer, it is crucial to minimize non-specific adsorption, as this greatly affects the stability of the biological molecules that are immobilized on the cellulose surface [31].

4 Conclusion

The papain enzyme immobilization is enhanced through the synthesis of an alginate-based hydrogel that is enriched with microfibrillated cellulose (MFC) obtained from solid residues produced by HCl hydrolysis. The study's findings indicated that the addition of 0.5% and 1% MFC resulted in enhanced immobilization characteristics of alginate hydrogels. However, it is worth noting that these improvements did not reach statistical significance. The research findings indicate that solid industrial agar leftovers containing cellulose can be effectively

converted into MFC (microfibrillated cellulose). This has significant implications for the application of biotechnology in this industry.

The authors express their gratitude to the Microbiology Laboratory, which is affiliated with the Agency of Standardization Forestry and Environment Instruments (ASEFI), Ministry of Environment and Forestry, Republic of Indonesia, for providing stereo-microscope images of the hydrogel.

References

1. I. Munifah, Limbah padat industri agar-agar (PT. Nasya Expanding Management, Pekalongan, 2021), in Indonesian
2. R.B.D. Sormin, D. Soukotta, Saiful, A. Risambessy, S. J. Ferdinandus, JPHPI **21**, 1 (2018)
3. W. Ramadhan, W. Trilaksani, JPHPI **20**, 1 (2017)
4. K. Qotimah, E.N. Dewi, L. Purnamayanti, JPHPI **23**, 1 (2020)
5. Sujuliyani, P. Pebriyanti, Y.H. Siputar, JPHPI **24**, 3 (2021)
6. A. Langford, S. Waldron, Sulfahri, H. Saleh, Mar. Policy **127** (2021)
7. Uju, J. Santoso, W. Ramadhan, M.F. Abrory, JPHPI **21**, 3 (2018)
8. Z. Peng Li, Q. Song Hu, J. Fang Chen, X. Liu, Y. Yan Sang, J. Wang, M. Jing Zheng, H. Ni, Q. Biao Li, Z.Dong Jiang, Y. Zhu, J. Clean. Prod **378,** 134631 (2022)
9. M.E. El-Hefnawy, S. Alhayyani, A. Ismail, M. El-Sherbiny, M. Al-Harbi, A. Abomohra, M. Sakran, N. Zidan, J. Clean. Prod **392**, 136286 (2023)
10. G. Suresh, H. Kopperi, S.V. Mohan, Bioresour, Technol **382,** 129063 (2023)
11. N. Ferrera-Lorenzo, E. Fuente, I. Suárez-Ruiz, B. Ruiz, Fuel Process. Technol **121** (2014)
12. M. Martínez-Sanz, V. Cebrián-Lloret, J. Mazarro-Ruiz, A. López-Rubio, Carbohydr. Polym **233** (2020)
13. D. Klemm, B. Heublein, H.P. Fink, A. Bohn, Angew, Chemie - Int. Ed **44** (2005)
14. G. Chinga-Carrasco, Nanoscale Res. Lett. **6**, 417 (2011)
15. G. Fortunato, T. Zimmermann, J. Lübben, N. Bordeanu, R. Hufenus, Macromol. Mater. Eng **297**, 6 (2012)
16. M. Larsson, J. Hjärtstam, A. Larsson, Carbohydr. Polym. **88**, 2 (2012)
17. K.Y. Goh, Y.C. Ching, M.H. Ng, C.H. Chuah, S.B.J. Julaihi, J. Drug Deliv. Sci. Technol **71** (2022)
18. M. Bouhoute, M. Nakajima, H. Isoda, Carbohydr. Polym **274**, 11862 (2021)
19. J. Zhang, M. Song, X. Wang, J. Wu, Z. Yang, J. Cao, Y. Chen, Q. Wei, J. Appl. Polym. Sci **133** (2016)
20. Y. Wang, Y. Lo Hsieh, J. Polym. Sci. Part A Polym. Chem **42,** 17 (2004)
21. D.R. Ningrum, W. Kosasih, S. Priatni, J. Kim. Terap. Indones. **19**, 2 (2018)
22. W. Sheng, Y.Xi, L. Zhang, T. Ye, X. Zhao, Int J Biol Macromol **114** (2018)
23. J. Ma, Y.Tang, M. Yaseen, L. Qin, X. Chen, S. Xiong, D. Liao, Z.Tong, Sep Purif Technol **322**, 124278 (2023)
24. N. Hastuti, L. Agustini, S.D. Hardiningtyas, W. Ramadhan, D.A. Indrawan, M. Ismayati, A. Tazkiatunnisa, *Microfibrillated cellulose made of agar waste in alginate-based hydrogels for papain enzyme immobilization*, in The 2nd international

conference of lignocellulose, ICONLIG, 24–25 November 2022, Bogor, Indonesia (2024)

25. N. Hastuti, K. Kanomata, T. Kitaoka, J. Polym. Environ **26** (2018)
26. K. Goh, C.Y. Ching, M.H. Ng, C.H. Chuah, S.B.J., Julaihi, J Drug Deliv Sci Tec. **71**, 103324 (2022)
27. N. Hastuti, R.F. Darmayanti, S.D. Hardiningtyas, K. Kanomata, M.Goto, T. Kitaoka, Bioresources **14**, 3 (2019)
28. S. Okubayashi, U. Griesser, T. Bechtold, Carbohydr. Polym **58**, 3 (2004)
29. S. Belbekhouche, J. Bras, G. Sigueira, C. Chappey, L. Lebrun, B. Khelifi, S. Marais, A. Dufresne, Carbohydr. Polym **83**, 4 (2011)
30. Y. Cheng, L. Lu, W. Zhang, J. Shi, Y. Cao, Carbohydr. Polym **88**, 3 (2012)
31. M.E. Culica, A.L. Chibac-Scutaru, T. Mohan, S. Coseri, Biosens. Bioelectron. **182**, 113170 (2021)