Tobacco Stalk as Source of CMCase Enzyme Production of Actinomycetes Isolated from Rhizosphere of Tobacco (*Nicotiana tabacum* L.) by Submerged Fermentation

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Abstract. Tobacco stalk is tobacco plant waste which contains about 56% cellulose so that it is possible to use it as a substrate for the production of cellulase, namely CMCase. The study conducted in several stages, namely screening the cellulolytic activity of 71 actinomycetes isolates, optimizing CMCase production by three actinomycetes with the best CMCase activity index using tobacco stalk as substrate through submerged fermentation, and morphology identification actinomycetes with the highest CMCase activity. A total of 71 actinomycetes have been isolated from the rhizosphere of tobacco plant. In this study, the ability of actinomycetes to produce cellulase through submerged fermentation using tobacco stalk substrate powder. There were about 71 actinomycetes isolated and all of them have cellulolytic activities. Based on the screening of cellulolytic activity on CMC (carboxymethyl cellulose) agar plate, the enzyme activity index of ATG 38 isolate was higher than others, with 2.50 ± 0.26 respectively. The cellulase production using tobacco stalk as substrate through submerged fermentation was optimal showing that CMCase production of actinomycete ATG 38 isolates maximal after four days incubation with 0,40 mU/mL enzyme activity. Microscopic morphological observations showed that actinomycete ATG 38 isolate belongs to the genus *Streptomyces*.

Keywords: CMCase · Actinomycetes · Nicotiana tabacum L. · Submerged fermentation

1 Introduction

Tobacco (*Nicotiana tabacum* L.) is a plantation commodity that has high economic value [1]. Tobacco leaves are the main raw material for making cigarettes and leave tobacco stalks as waste. Handling of this waste is only done through combustion, while the smoke from the combustion still contains nicotine that causes carcinogenic in the respiratory tract [2, 3]. Therefore, an effort is needed to handle tobacco stalk waste.

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Tobacco stalks has a fairly high cellulose contain reaching 56.10%, so that it provides opportunities for its use as a source of cellulose. Cellulose degradation can be carried out by cellulase enzymes to produce glucose. Extracellular cellulases can be produced by microorganisms such as fungi, bacteria, and actinomycetes [4]. The molecular size of cellulase produced by bacteria is also relatively smaller than fungi, making it easier to diffuse into the cellulose tissue [5]. The morphology of actinomycetes in the form of filaments is easier to penetrate the crystalline cellulose chain [6]. The high cellulose content in tobacco stalks can be used as a substrate for cellulase production through submerged culture fermentation using actinomycetes [7]. This study aimed to determine the ability of the best actinomycetes isolates from the tobacco rhizosphere to produce cellulase through submerged culture fermentation using tobacco stalk powder as well as the level of purity of the cellulase through partial purification and identification of the best isolate's microscopic morphology.

2 Materials and Methods

2.1 Screening of Cellulase Activity

Screening of cellulase activity of 71 actinomycetes was carried out on solid CMC media. Each of 1 plug (8 mm diameter) from the fresh actinomycetes culture on ISP-4 media, was inoculated on CMC solid media (0.5 g CMC, 0.5 g yeast extract, 0.5 g peptone, 0.02 g $MgSO_4.7H_2O$, 0.1 g $K_2HPO_4.3H_2O$ and 2 g agar in 100 mL water) and incubated at room temperature for 4 days [8]. CMCase activity was detected using the Gram's Iodine method. The culture was flooded with the solution Gram's iodine (0.33% 2 g KI, 1 g iodine, in 300 mL water) for 3–5 min [9]. Screening of CMCase activity was carried out in three repetitions. The cellulolytic activity index was calculated based on the presence of a clearing zone formed using the following formula [10]:

$$\text{Index cellulolytic} = \frac{(\text{diameter of clearing zone - colony diameter})}{\text{colony diameter}} \quad (1)$$

2.2 Optimization of Cellulase Production by Selected Actinomycetes in Several Incubation Times

2.2.1 Preparation of Tobacco Stalk Powder

The tobacco stalks used in this study were the Kasturi variety from Jember Regency, Indonesia. The tobacco stalk powder used is 80 mesh in size [11]. The preparation of tobacco stalk powder as a production medium begins with the milling process. This process aims to increase the accessibility of the enzyme to the substrate [12].

2.2.2 Inoculum Preparation

Actinomycete that was used as inoculum in cellulase (CMCase) production was one actinomycete with enzyme activity index was higher than others. Inoculum preparation was carried out by inoculating the isolate on an ISP-4 medium for 7 days. Furthermore,

5 mL of 0.05% tween-80 solution was put into a tube of the culture and then scraped off. The 20 μl of the culture suspended was taken to calculate the spore density of each at intervals of one day using a hemocytometer tool. The calculation was to obtain a spore density of 10^8 cells/mL. The spore suspension with a density of 10^8 cells/mL was used as an inoculum in the optimization production process [13, 14]. The number of cells per milliliters in suspension spores was calculated by the following formula [15]:

$$S\ (\text{cells/mL}) = \frac{n}{L\ (0{,}04\ \text{mm}^2) \times h(0{,}1\ \text{mm})} \times \frac{1}{fp} \tag{2}$$

S: the number of spores (cells/mL).
n: the number of cells in the calculated field.
L: area of the medium square (0.04 mm2).
h: depth of field count (0.1 mm).
Fp: dilution factor.

2.2.3 Optimization of Incubation Time in CMCase Production of Selected Actinomycetes

Crude extract cellulase production was carried out through submerged culture fermentation using tobacco stalk powder as a substrate. The optimal incubation time for cellulase production was determined by measuring the CMCase activity of the crude extract cellulase. The composition of the culture media for submerged fermentation using tobacco stalk powder as substrate was 1 g of tobacco stalk powder, 0.5 g yeast extract, 0.5 g peptone, 0.02 g $MgSO_4.7H_2O$, 0.1 g $K_2HPO_4.3H_2O$, in 100 mL water at pH 7 [4, 11]. Furthermore, 1% inoculum as much as 1 mL of actinomycetes isolate suspension with a spore density of 10^8 cells/mL was inoculated in 99 mL of sterile tobacco stalk media and incubated with a 120-rpm shaker at room temperature for 10 days. The optimization production was carried out three times [16, 17]. Crude cellulase extract was prepared daily by centrifugation (Oregon LC-04S) at 12000 rpm at 4 °C for 20 min. The supernatant was a crude extract of cellulase which will then be assayed for CMCase activity using the DNS (Dinitro Salicylic Acid) method [18] and 1% (w/v) CMC was used as substrate [11].

2.3 Cellulase (CMCase) Activity Assay

2.3.1 Glucose Standard Curve

The standard curve for glucose preparation was started by making a glucose stock solution (0.01 gr of glucose dissolved in 10 mL of distilled water) so that the concentration was 10 g/mL. The serial dilutions of the glucose stock solution were directed to obtain the concentration of standard glucose were 0 g/mL, 0.2 g/mL, 0.1 g/mL, 0.08 g/mL, 0.06 g/mL, 0.05 g/mL, 0.05 g/mL, 0.044 g /mL, 0.04 g/mL. The glucose standard was determined by adding 1.5 mL of DNS reagent to 1 mL of glucose standard solution of each concentration, then heating it in boiling water for 10 min. At each glucose concentration, the absorbance was measured using a spectrophotometer of 540 nm [11].

2.3.2 CMCase Activity Assay

CMCase activity was measured using a modified method of Wood and Bhat (1998), namely by reacting 0.5 mL of crude cellulase extract with 0.5 mL of 1% (w/v) CMC in 100 mM phosphate buffer pH 6 and incubated at room temperature for 30 min. The reaction was stopped by adding 1.5 mL of 3,5-DNS reagent (Dinitro Salicylic Acid) and heating it in boiling water for 10 min. The absorbance of glucose products was measured using a spectrophotometer 540 nm [18].

The control was prepared using 0.5 mL of crude extract cellulase which was incubated at room temperature for 30 min. Then 1.5 mL of DNS reagent was added and each 1% (w/v) CMC in 100 mM phosphate buffer pH 6. Then heated in boiling water for 10 min and cooled. The blank contained 1 mL of 100 mM phosphate buffer pH 6 and 1.5 mL of DNS solution. The CMCase activity test was carried out two times [11, 19]. The value of enzyme activity is determined based on the following calculations [11]:

$$\text{Cellulase activity (U/mL)} = \frac{\mu \text{ mol sample reducing sugar} - \mu\text{mol control reducing sugar}}{\text{The volume of extract enzyme} \times \text{time of incubation}} \quad (3)$$

2.4 Identification of the Microscopic Morphology of Selected Isolate

The morphological identification of actinomycete was carried out through microscopic observation using the cover slide method. Sterile cover glass inserted in ISP-4 agar growth medium with an angle of 45° in the center of the media. Actinomycete were inoculated at the contact between the cover glass and the media and incubated for 7 days at room temperature. Furthermore, observation of the spore structure on the cover glass was carried out using a microscope which included cell shape and spore chain shapes [20, 21].

2.5 Data Analysis

This research was conducted using descriptive statistical analysis. The data is displayed in the form of tables and graphics. The determination of the best results of the enzyme activity index was based on the highest rank of values.

3 Results and Discussion

3.1 Cellulolytic Actinomycetes Screening

Cellulolytic enzyme activity was indicated by the formation of the clearing zone around the actinomycetes colonies when grown on the CMC medium after a certain incubation time. The clear zone formed on the media occurred because the Gram's Iodine reagent could not diffuse into the media around the colony (Fig. 1). This is because the substrate around the colony has been hydrolyzed by the cellulase enzyme into glucose (monosaccharide). Cellulose substrates that are not hydrolyzed by cellulase enzymes can bind to

Table 1. Average index enzyme activity of cellulase of 71 actinomycetes isolates

No	Isolate code	Cellulase index	No	Isolate code	Cellulase index
1	ATG 38	2,50 ± 0,26	37	ATG 36A	1,53 ± 0,04
2	ATG 34B	2,28 ± 0,19	38	ATG 46	1,52 ± 0,07
3	ATG 69	2,23 ± 0,12	39	ATG 40	1,51 ± 0,08
4	ATG 13	2,23 ± 0,10	40	ATG 68	1,51 ± 0,36
5	ATG 57A	2,17 ± 0,17	41	ATG 9B	1,49 ± 0,05
6	ATG 26	2,15 ± 0,22	42	ATG 33	1,46 ± 0,10
7	ATG 80	2,11 ± 0,12	43	ATG 35B	1,45 ± 0,19
8	ATG 16	2,11 ± 0,28	44	ATG 35A	1,45 ± 0,03
9	ATG 10	2,09 ± 0,13	45	ATG 61	1,43 ± 0,15
10	ATG 12	2,07 ± 0,33	46	ATG 59C	1,42 ± 0,18
11	ATG 79	2,06 ± 0,17	47	ATG 72	1,39 ± 0,14
12	ATG 51	2,04 ± 0,10	48	ATG 71	1,36 ± 0,01
13	ATG 77	2,03 ± 0,40	49	ATG 41	1,33 ± 0,28
14	ATG 58	1,97 ± 0,21	50	ATG 57C	1,32 ± 0,99
15	ATG 76	1,95 ± 0,21	51	ATG 8	1,30 ± 0,59
16	ATG 39B	1,92 ± 0,67	52	ATG 59A	1,27 ± 0,01
17	ATG 74	1,92 ± 0,13	53	ATG 27	1,27 ± 0,09
18	ATG 59B	1,90 ± 0,11	54	ATG 7	1,26 ± 0,30
19	ATG 34	1,89 ± 0,15	55	ATG 47	1,25 ± 0,13
20	ATG 67A	1,86 ± 0,35	56	ATG 54	1,24 ± 0,07
21	ATG 65	1,86 ± 0,06	57	ATG 50	1,23 ± 0,25
22	ATG 60	1,85 ± 0,16	58	ATG 5	1,22 ± 0,32
23	ATG 1	1,83 ± 0,15	59	ATG 53	1,21 ± 0,07
24	ATG 55	1,79 ± 0,24	60	ATG 4	1,21 ± 0,21
25	ATG 2	1,76 ± 0,24	61	ATG 37	1,21 ± 0,07
26	ATG 63	1,75 ± 0,67	62	ATG 15	1,20 ± 0,45
27	ATG 58B	1,74 ± 0,22	63	ATG 49	1,17 ± 0,16
28	ATG 3	1,73 ± 0,01	64	ATG 66	1,15 ± 0,21
29	ATG 64	1,73 ± 0,09	65	ATG 78	1,14 ± 0,04
30	ATG 67B	1,71 ± 0,05	66	ATG 52	1,12 ± 0,09
31	ATG 62	1,69 ± 0,05	67	ATG 36B	1,05 ± 0,28
32	ATG 70	1,68 ± 0,14	68	ATG 48	0,93 ± 0,25

(*continued*)

Table 1. (*continued*)

No	Isolate code	Cellulase index	No	Isolate code	Cellulase index
33	ATG 42	1,65 ± 0,07	69	ATG 75	0,83 ± 0,26
34	ATG 56	1,63 ± 0,036	70	ATG 39A	0,77 ± 0,224
35	ATG 11	1,59 ± 0,14	71	ATG 14	0,63 ± 0,27
36	ATG 9A	1,58 ± 0,08			

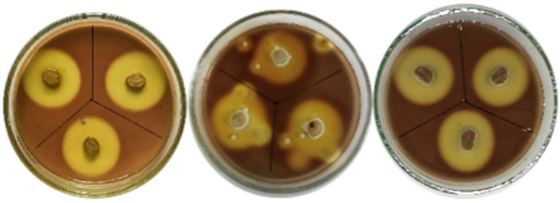

Fig. 1. Screening the cellulolytic activity of isolate ATG 38, 69, and 34B on CMC plate agar using Gram's Iodine reagent (a. ATG 38 b. ATG 69 c. ATG 34B).

Gram's Iodine reagent and form an iodine complex to produce a reddish-brown color [9, 22].

The screening of cellulolytic activities of 71 isolates showed that all actinomycetes could hydrolyze cellulose on CMC substrate as the sole carbon source (Table 1). All of the actinomycete isolates analyzed were cellulose-degrading cellulolytic microorganisms (CMCase). The formation of this clear zone indicates the cellulolytic activity of actinomycete isolates in the form of CMCase [23]. Dillon et al. [26] reported that each type of cellulase enzyme can hydrolyze and the pH range varies according to its ability. The highest CMCase activity index obtained was 2.50 ± 0.26 in ATG 38 isolate.

3.2 Cellulolytic Cellulase Production at Several Incubation Times

The best isolate based on the results of screening for CMCase activity, actinomycete ATG 38 was then tested for optimization production on tobacco stalk powder substrate with several incubation times using the submerged culture method. The density of spore that was used as inoculum on the cellulase production media from actinomycete ATG 38 was $2{,}4 \times 10^8$ cells/mL. This is by several research reports which state that the average amount of inoculum density used is 10^5–10^7 or 10^8 spore/mL [4, 24].

The results showed that the CMCase production of actinomycete ATG 38 isolates increased from day 1 which was 0.04 mU/mL until optimum on day 4 with CMCase activity of 0.40 mU/mL. Furthermore, the enzyme activity began to decrease on the 5th day of production with an activity of 0,34 mU/mL (Fig. 2).

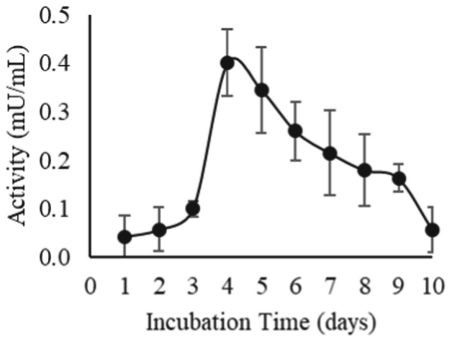

Fig. 2. Optimization test of isolate 38 CMCase activity at several incubation times.

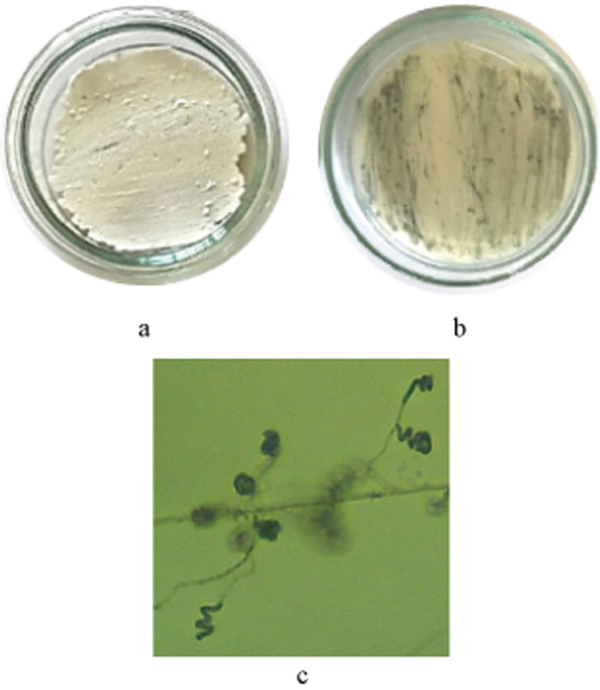

Fig. 3. Morphological colony (a and b) and structure of actinomycetes spore ATG 38 (c).

3.3 Identification of Microscopic Morphology of Actinomycete Isolates 38

Observations of spore arrangement and sporulation structure were based on Holt et al. [25]. The characteristics of the spores obtained based on observations using a microscope with a magnification of 400x are actinomycetes isolate 38 having conidia in the form of long chains of conidia with long spiral spore chains, streptococcal cell shape accompanied by the formation of air mycelium. These actinomycetes isolate has characteristics of white aerial mycelia and substrate mycelia that can produce a green insoluble

pigment. Based on the characteristics obtained, it was analyzed that the actinomycete ATG 38 belonged to the genus Streptomyces (Fig. 3).

A total of 71 actinomycetes isolates had cellulolytic activity. The actinomycete ATG 38 had a higher cellulolytic on the CMC agar plate than others. The production using tobacco stalk powder as substrate through submerged culture fermentation of actinomycete ATG 38 optimal was obtained in the 4^{th} day incubation time which the CMCase activity was 0.40 mU/mL. Microscopic morphological identification of actinomycete ATG 38 showed that the isolate belongs to the genus *Streptomyces.*

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References

1. B. P. Statistik, "Produksi Perkebunan Tembakau di Jawa Timur Tahun 2006-2015 (Ton)," 2020, [Online]. Available: https://jatim.bps.go.id/ statictable/2018/02/08/920/produksi-perkebunan-tembakau-di-jawa-timur-ton-2006-2015-.htmL
2. B. Palupi, I. Rahmawati, and M. F. Rizkiana, "Pemberdayaan Masyarakat Agribisnis Berbasis Pemanfaatan Sumber Daya Lokal Limbah Batang Tembakau sebagai Pewarna Alami Batik di Desa Tamansari," *War. Pengabdi.*, vol. 12, no. 4, p. 398, 2019, doi: https://doi.org/10.19184/wrtp.v12i4.9293.
3. W. A. Fransiska, Hardiansyah, O. K. Sari, D. Nugroho, and S. R. D. W. S. Riva, "Pengolahan Serat Batang Tembakau sebagai Soundproofing Material: Alternatif Penanggulangan Limbah Batang Tembakau," *Pros. Semin. Nas. PERTETA*, no. August 2015, pp. 1–6, 2015, [Online]. Available: https://www.researchgate.net/publication/291020107_Pengolahan_Serat_Batang_Tembakau_sebagai_Soundproofing_Material_Alternatif_Penanggulangan_Limbah_Batang_Tembakau
4. A. Pesrita, T. Marta Linda, S. Devi, and J. Biologi Fakultas Matematika Ilmu pengetahuan Alam Universitas Riau, "Seleksi dan Akivitas Enzim Selulase Aktinomisetes Lokal Riau pada Media Lignoselulosa Ampas Tebu," *J. Riau Biol.*, vol. 2, no. 1, pp. 8–13, 2018, [Online]. Available: http://jurnalunri.org/ojs/index.php/JRB
5. A. M. Dyah Ayu Saropah, Akyunul Jannah, "Kinetika reaksi enzimatis ekstrak kasar enzim selulase bakteri selulolitik hasil isolasi dari bekatul," *Alchemy*, vol. 2, no. 1, pp. 34–45, 2012.
6. E. A. Barka *et al.*, "Correction for Barka et al., Taxonomy, Physiology, and Natural Products of Actinobacteria," *Microbiol. Mol. Biol. Rev.*, vol. 80, no. 4, 2016, doi: https://doi.org/10.1128/mmbr.00044-16.
7. Y. Liu *et al.*, "Co-digestion of tobacco waste with different agricultural biomass feedstocks and the inhibition of tobacco viruses by anaerobic digestion," *Bioresour. Technol.*, vol. 189, pp. 210–216, 2015, doi: https://doi.org/10.1016/j.biortech.2015.04.003.
8. E. Utarti, A. Suwanto, M. T. Suhartono, and A. Meryandini, "Identifikasi Aktinomiset Selulolitik dan Xilanolitik Indigenous (Identification of Cellulolytic and Xylanolytic Indigenous Actinomycetes)," *Berk. Sainstek*, vol. VIII, no. 1, pp. 1–5, 2020.

9. R. C. Kasana, R. Salwan, H. Dhar, S. Dutt, and A. Gulati, "A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine," *Curr. Microbiol.*, vol. 57, no. 5, pp. 503–507, 2008, doi: https://doi.org/10.1007/s00284-008-9276-8.
10. A. Ferbiyanto, I. Rusmana, and R. Raffiudin, "Characterization and Identification of Cellulolytic Bacteria from gut of Worker Macrotermes gilvus," *HAYATI J. Biosci.*, vol. 22, no. 4, pp. 197–200, 2015, doi: https://doi.org/10.1016/j.hjb.2015.07.001.
11. E. Utarti, A. Meryandini, S. Suwanto, and T. Maggy, "Degradasi enzimatis lignoselulosa oleh aktinomiset Indigenous: potensi penggunaan lytic polysaccharide Monooxygenase dalam pra perlakuan limbah berlignoselulosa," *Disertasi*, vol. Bogor, p. Sekolah Pascasarjana Institut Pertanian Bogor, 2019.
12. A. R. Sari, E. Kusdoyantini, and M. I. Rukmi, "Produksi selulase oleh kapang Aspergillus sp. hasil isolasi dari limbah pengolahan sagu (*Metroxylon* sp.) dengan variasi konsentrasi inokulum pada fermentasi terendam statis," *J. Biol.*, vol. 6, no. 1, pp. 11–20, 2017.
13. P. Ruanpanun, N. Tangchitsomkid, K. D. Hyde, and S. Lumyong, "Actinomycetes and fungi isolated from plant-parasitic nematode infested soils: Screening of the effective biocontrol potential, indole-3-acetic acid, and siderophore production," *World J. Microbiol. Biotechnol.*, vol. 26, no. 9, pp. 1569–1578, 2010, doi: https://doi.org/10.1007/s11274-010-0332-8.
14. L. Fangohoi, A. Agustina, and L. Navitasari, "Penggunaan mixed culture jamur dan penambahan sumber N pada biodegradasi tandan kosong kelapa sawit dalam pengkomposan," *Buana Sains*, vol. 14, no. 2, pp. 105–111, 2014.
15. M. T. Madigan, S. B. Kelly, H. B. Daniel, W. S. Matthew, and A. S. David, *Brock biology of microorganisms*, 15th ed. United States: Pearson Education, 2018.
16. N. A. El-Sersy, H. Abd-Elnaby, G. M. Abou-Elela, H. A. H. Ibrahim, and N. M. K. El-Toukhy, "Optimization, economization and characterization of cellulase produced by marine Streptomyces ruber," *African J. Biotechnol.*, vol. 9, no. 38, pp. 6355–6364, 2010.
17. V. Ventorino *et al.*, "Lignocellulose-adapted endo-cellulase producing streptomyces strains for bioconversion of cellulose-based materials," *Front. Microbiol.*, vol. 7, no. DEC, pp. 1–15, 2016, doi: https://doi.org/10.3389/fmicb.2016.02061.
18. Miller Gail Lorenz, "Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar," *Anal. Chem.*, vol. 31, no. 3, pp. 426–428, 1959.
19. F. Razie, I. Anas, A. Sutandi, L. Gunarto, and S. Sugiyanta, "Aktivitas Enzim Selulase Mikroba Yang Diisolasi Dari Jerami Padi Di Persawahan Pasang Surut Di Kalimantan Selatan," *J. Ilmu Tanah dan Lingkung.*, vol. 13, no. 2, p. 43, 2011, doi: https://doi.org/10.29244/jitl.13.2.43-48.
20. E. Armaida and S. Khotimah, "Karakterisasi Actinomycetes yang Berasosiasi dengan Porifera (Axinella spp .) dari Perairan Pulau Lemukutan Kalimantan Barat," *Protobiont*, vol. 5, no. 1, pp. 68–73, 2016.
21. F. M. Salim, S. A. Sharmili, J. Anbumalarmathi, and K. Umamaheswari, "Isolation, molecular characterization and identification of antibiotic producing actinomycetes from soil samples," *J. Appl. Pharm. Sci.*, vol. 7, no. 9, pp. 69–75, 2017, doi: https://doi.org/10.7324/JAPS.2017.70909.
22. H. R. Johnsen and K. Krause, "Cellulase activity screening using pure carboxymethylcellulose: Application to soluble cellulolytic samples and to plant tissue prints," *Int. J. Mol. Sci.*, vol. 15, no. 1, pp. 830–838, 2014, doi: https://doi.org/10.3390/ijms15010830.
23. S. Nompo, A. Meryandini, and T. C. Sunarti, "Produksi Enzim Selulase Oleh Aktinomiset Menggunakan Frond Sagu," *J. Penelit. Pascapanen Pertan.*, vol. 16, no. 2, p. 80, 2019, doi: https://doi.org/10.21082/jpasca.v16n2.2019.80-89.
24. D. Payal, K. Prateek, K. Munendra, S. Renu, and K. K. Monisha, "Purification and molecular characterization of chitinases from soil actinomycetes," *African J. Microbiol. Res.*, vol. 11, no. 27, pp. 1086–1102, 2017, doi: https://doi.org/10.5897/ajmr2017.8612.

25. J. G. Holt, N. R. Krieg, P. H. A. Sneath, J. T. Stanley, and S. T. William, *Bergey's Manual of Determinative Bacteriology 9th edition*, 9th ed. USA: William and Wilkin, 2019.
26. G. S. Dhillon, S. Kaur, S. K. Brar, and M. Verma. "Potential of apple pomace as a solid substrate for fungal cellulase and hemicellulase bioproduction through solid-state fermentation," *Industrial Crops and Products*. vol. 38, no. 1, pp. 6-13, 2012.