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To: Widyastuti Unila <widyastuti.unila@gmail.com>

Cc: John Hendri <john.hendri@fmipa.unila.ac.id>, Andi Setiawan <andi.setiawan@fmipa.unila.ac.id>

Dear Widyastuti

I hope you stay healthy and safe. Your paper entitled **Application SEM-EDX in Biodegradation of Seafood Wastes by Sponge-Derived Actinomycetes 19C38A1 in Solid Fermentation** with Manuscript ID **JESSD-179** is published on IOP: Earth and Environmental Science, please check this link: <https://iopscience.iop.org/issue/1755-1315/940/1>. We will inform you once your paper is listed in Google Scholar and Scopus.

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It's a pleasure for us to work with you, however I would like to apologize if during the process there are any inconveniences. I hope we can meet again in the 3rd JESSD Symposium which is estimated to be in August or September 2022. More info will be updated soon.

3

Sincerely,

Herdis Herdiansyah

2nd JESSD Symposium Chair

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ANDI SETIAWAN <andi.setiawan@fmipa.unila.ac.id>

Wed, Dec 15, 2021 at 9:26 PM

To: International Symposium of JESSD <symposium.jessd@ui.ac.id>

Dear Herdis Herdiansyah,

Thank you very much for your information.

Sincerely,
Andi Setiawan.

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Application SEM-EDX in Biodegradation of Seafood Wastes by Sponge-Derived Actinomycetes 19C38A1 in Solid Fermentation

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Application SEM-EDX in Biodegradation of Seafood Wastes by Sponge-Derived Actinomycetes 19C38A1 in Solid Fermentation

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Abstract. Biodegradation of chitin by microorganisms can produce derivative products that have economic value. This research aims to apply SEM-EDX analysis in observing the biodegradation process of seafood industrial waste by actinomycetes. Shrimp shells, cuttlefish bones and fish scales were obtained from the free market. In the early stages, the SEM-EDX spectrum analysis of the substrate showed almost the same carbon, oxygen, and mineral compositions. While the surface of each substrate is quite varied. On the second day of the fermentation, SEM image analysis showed that the growth rate of actinomycetes on each substrate was significantly different. The difference in growth was supported by SEM imaging data which showed damage to the surface of each substrate. Further analysis of the degradation products by HPLC on the second and third days showed the formation of glucosamine. It suspected that actinomycetes can break down shrimp shell waste into glucosamine. This information is very important as the basis for further research related to the optimization of the glucosamine and chitooligosaccharide (COS) production process using the solid fermentation method.

1. Introduction

The demand for seafood to date tends to increase [1]. This obviously has the consequence of increasing seafood product industrial waste. The low value of seafood product waste raises problems related to distribution, transportation, waste handling, and environmental degradation. Efforts to utilize the availability of seafood product industrial waste as a source of chitin and chitosan have been carried out. Seafood industry waste is chemically processed to extract chitin. Furthermore, chitin products can be converted into chitosan and its derivative products which have a higher economic value. This conventional method is quite effective but can reduce the quality of the surrounding environment [2].

In line with technological developments, solid waste treatment such as seafood industrial waste can be overcome by solid-state fermentation (SSF) methods. Currently, the application of the SSF method is a reliable alternative because the process is relatively cheaper when compared to other fermentation methods. In addition, the SSF process is more environmentally friendly and easy to perform. The



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success of SSF is strongly influenced by the type of microorganisms utilized, the selection of media, and environmental conditions such as pH, minerals, and humidity [3].

Actinomycetes are decomposing microorganisms that play a very important role in the ecosystem [4]. Actinomycetes have also been widely utilized in solid fermentation processes for agro-industrial waste treatment. However, in the development of conventional optimization techniques in the fermentation process, it takes time because of the many parameters that must be measured. Considering this, this research aims to utilize the Scanning Electron Microscope-Electron Diffraction-X (SEM-EDX) analysis method in the biodegradation research of seafood industrial waste by actinomycetes. The application of SEM-EDX has been widely utilized in the biomedical field, but it is still very limited for researches on the development of the solid fermentation field. EDX microanalytic information contains semi-qualitative and semi-quantitative information. Furthermore, SEM imaging data can also be performed quickly [5].

This research will describe the stages of actinomycetes ability to convert chitin in seafood waste into its derivative products. Supported by HPLC analysis for the resulting biodegradation products, the incorporation of the data obtained will accelerate optimal conditioning in the solid fermentation process. This research will certainly be very useful as a first step in the development of solid fermentation processes.

2. Method

2.1. Materials

2.1.1. Isolate Actinomycetes

In this research, the isolate actinomycetes 19C38A1 was selected from the deposit of ⁴Unit Pelayanan Teknis Laboratorium Terpadu dan Sentra Inovasi Teknologi (UPT LTSIT) University of Lampung, which was obtained from the isolation of the 19C38 sponge off the coast of Oluhuta, Gorontalo. The isolates were juvenile in ISP2 media and 1% chitin colloid.

2.1.2. Substrate for Solid State Fermentation

Shrimp shells, cuttlefish bones, and fish scales were obtained from free-market Lempasing, Bandar Lampung, while fish scales were obtained from the Pringsewu market. Fresh waste samples were washed with water to separate from impurities and then dried at 55°C overnight. Dry waste samples were stored in the refrigerator for further experiments.

2.2. Procedure

All work and equipment related to microorganisms was carried out under sterile conditions.

2.2.1. Microscopic analysis.

The characteristics of the isolate 19C38A1 were ²examined microscopically using the cover glass culture method [6]. Briefly, a sterile coverslip was inserted at an angle of 45° in the center for 2% chitin agar. After 4 days of incubation at 32°C, the cover slip was removed and placed on a clean slide. The coverslip was finally observed under a light microscope (100X), using a Zeiss Observer A1 microscope.

2.2.2. Solid state fermentation.

The SSF process was carried out using waste substrates of shrimp shells, cuttlefish bones and fish scales. Each substrate weighing 10 grams was placed in a petri dish. Then the media was inoculated with 1 ml of actinomycetes suspension and incubated statically at 32°C. Observation of actinomycetes growth was carried out every day and observed using SEM. While the results of biodegradation ⁵were analyzed using high performance liquid chromatography (HPLC).

2.2.3. Analysis SEM-EDX.

At the initial stage, the respective surface and composition were analyzed by SEM-EDX. The next stage, actinomycetes growth and substrate surface growth were observed using SEM. For analysis purposes, the gold coating process was carried out for 15 minutes. Once gold coated, the specimen could be examined with an electron microscope (SEM-EDX Zeiss EVO MA 10). Gold coated specimens on stub were observed by SEM at a high electron (EHT) voltage of 10 kV, using a secondary electron detector. The surface is scanned at low magnification until a growth line is clearly detected. The surface area with a clear and intact spore structure was then selected and examined at a higher magnification. Suitable surface in the preparation is ready to be photographed [7].

2.2.4. Analysis Glukosamine

The results of solid fermentation were analyzed using HPLC 20A Shimadzu based on the glucosamine formed. Fermentation of 10 grams of solid waste was extracted with 10 ml of distilled water, then the extraction results were centrifuged at 8000 rpm. After that, 1 mL of the filtrate was added with 4 mL of absolute ethanol (EtOH), then centrifuged at 8000 rpm to separate the precipitate formed. The precipitate is then redissolved with distilled water and ready for analysis. Glucosamine was analyzed chromatographically using a C18 Shimadzu column and acetonitrile (CH₃CN: H₂O) mobile phase flowed in a gradient (30% to 70%) for 10 minutes, the column temperature was adjusted with CTO 20A at 40°C, the results of the separation were detected with the PDA detector LC20AD.

3. Results and discussion

3.1. Isolate actinomycetes 19C38

Isolate 19C38A1 is one of the actinomycetes collections deposited at UPT LTSIT, Universitas Lampung. This isolate was obtained from the sponge coded 19C38 showed in Figure 1a. The sponge was collected from the waters off the coast of Oluhuta, Gorontalo in 2019. Macroscopic observations on isolate 19C38A1 appeared gray, and microscopic observations using a light microscope into Figure 1b showed nocardioform mycelium substrate characteristics (mycelium that decomposed into *bacilli* and *cocci*) [8]. The results of the chitinolytic test of isolate 19C38A1 in Figure 1c observed a clear zone on the outside around the isolated colony. This indicates the ability of isolate 19C38A1 to utilize chitin as a carbon source in its life cycle. Or it can be said that 19C38A1 isolate was able to degrade chitin biomaterial. The ability of actinomycetes to produce has been previously reported, but researches on the application of chitinase from actinomycetes are still very rare.

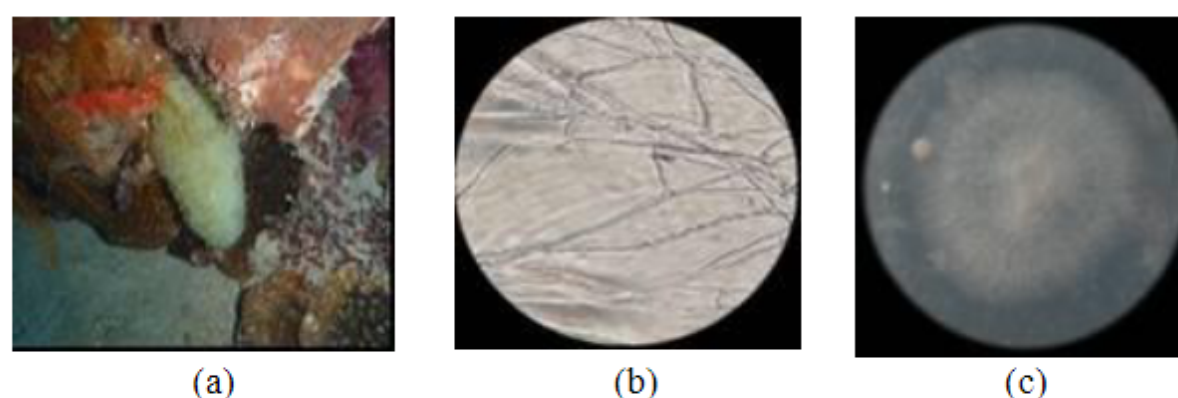


Figure 1. (a) Under water image sponge 19C38; (b) mikroskopis 19C38A1; (c) Chitinolytic clear zone.

3.2. Solid State Fermentation Substrate (SSF)

3.2.1. Substrate. In addition to the type of microorganism, another determining factor that supports the success of SSF application is the type of substrate to be selected. In this research, it focused on the utilization of substrates from seafood industry waste, including shrimp shells, cuttlefish bones, and

fish scales. The three types of waste are sources of chitin biopolymer. The ability of microorganisms to produce chitinase that can degrade chitin has been previously reported by Sneha Ja et al. [9]. Theoretically, substrates obviously containing chitin can be degraded by chitinase-producing microorganisms. In the degradation process, chitin can be converted into its derivative products such as chitosan and chitoligosaccharides. At the initial stage of characterization, the three types of substrates utilized were analyzed using SEM-EDX.

The results of observations using SEM-EDX are shown in Figures 2a, 2b, and 2c. The semi-qualitative and quantitative data of the EDX spectrum shown each substrate has a similar composition of Carbon (C), Oxygen (O) while the mineral content (Ca, Mg, Zn) was quite varied. This difference in composition will certainly affect the growth rate of microorganisms on the surface of the substrate. As it has been reported that the variation of actinomycetes media composition such as carbon, oxygen, nitrogen, pH, temperature and metal ions affect the production of chitinase.

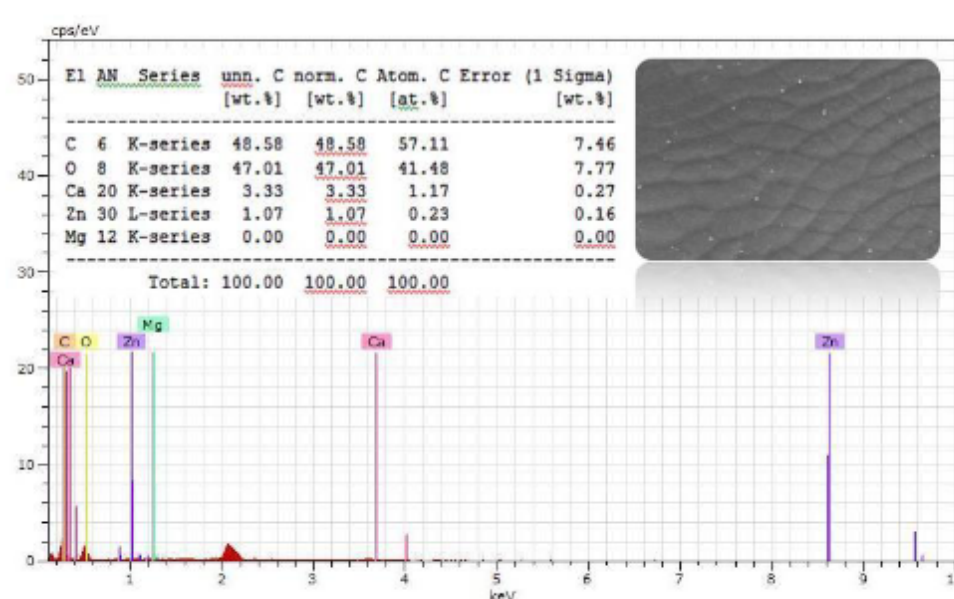


Figure 2a. SEM-EDX spectrum of shrimp shell substrate

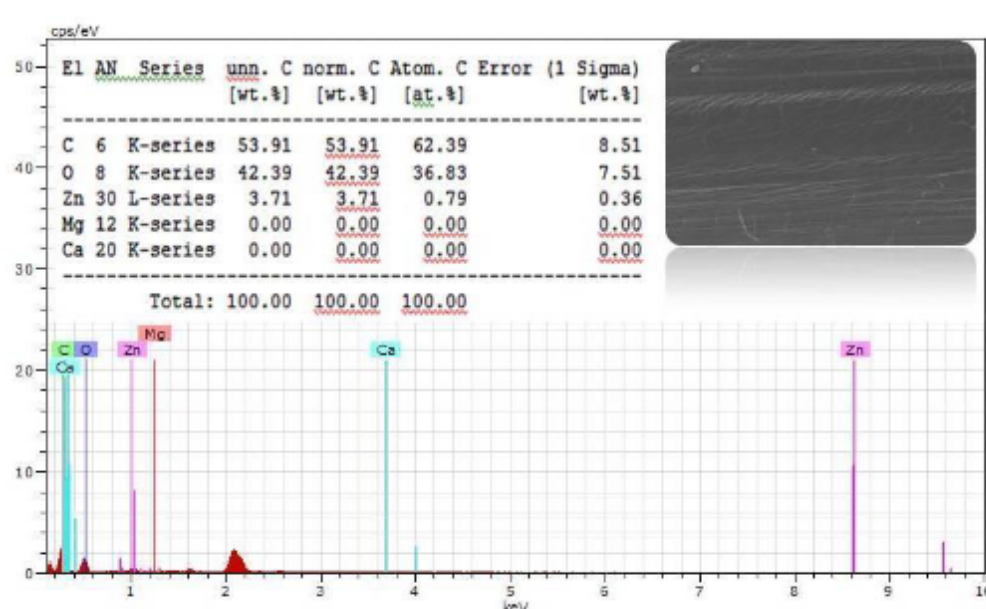


Figure 2b. SEM-EDX spectra of cuttlefish bone substrate

Further analysis using SEM imaging on each substrate showed that the surface of each substrate was very varied. This difference is obviously related to differences in mineral composition which consequent differences in the physical and chemical properties of the substrate.

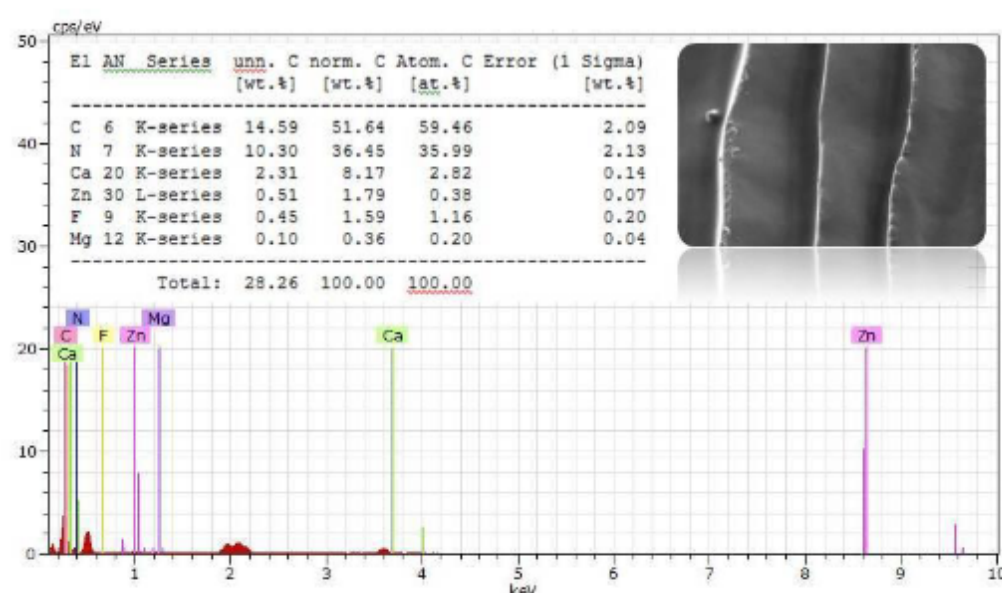


Figure 2c. SEM-EDX spectrum of fish scale substrate

3.2.2. Solid State Fermentation. The results of the fermentation process in a solid-state against 3 types of waste consisting of shrimp shells, strong bones, and fish scales using isolate 19C38 are shown in Figures 3, 4, and 5.

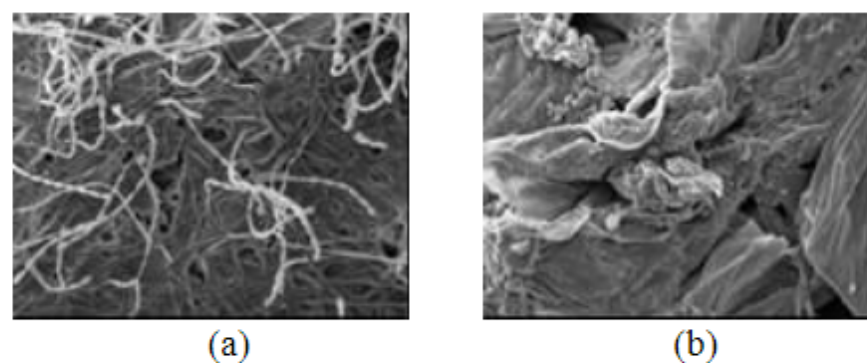


Figure 3. Isolate 19C38A1 on shrimp shell surface
(a) observation on 2nd day; (b) shrimp shell surface after washing

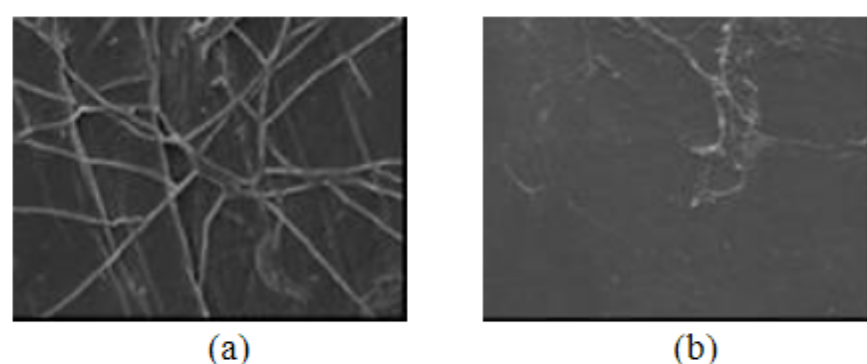


Figure 4. Isolate 19C38A1 on cuttlefish bone surface
(a) observation on 2nd day; (b) cuttlefish bone surface after washing

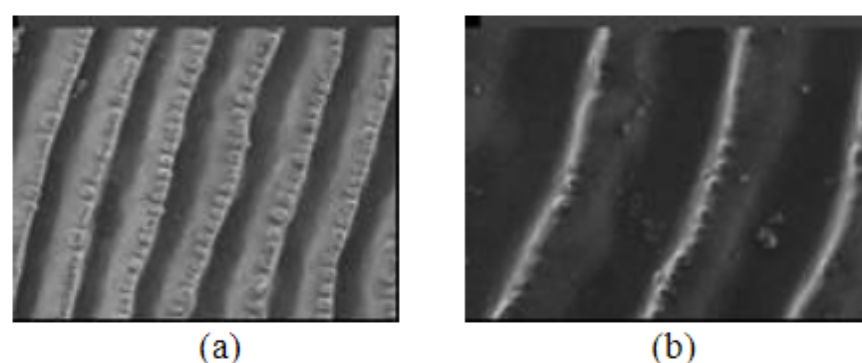
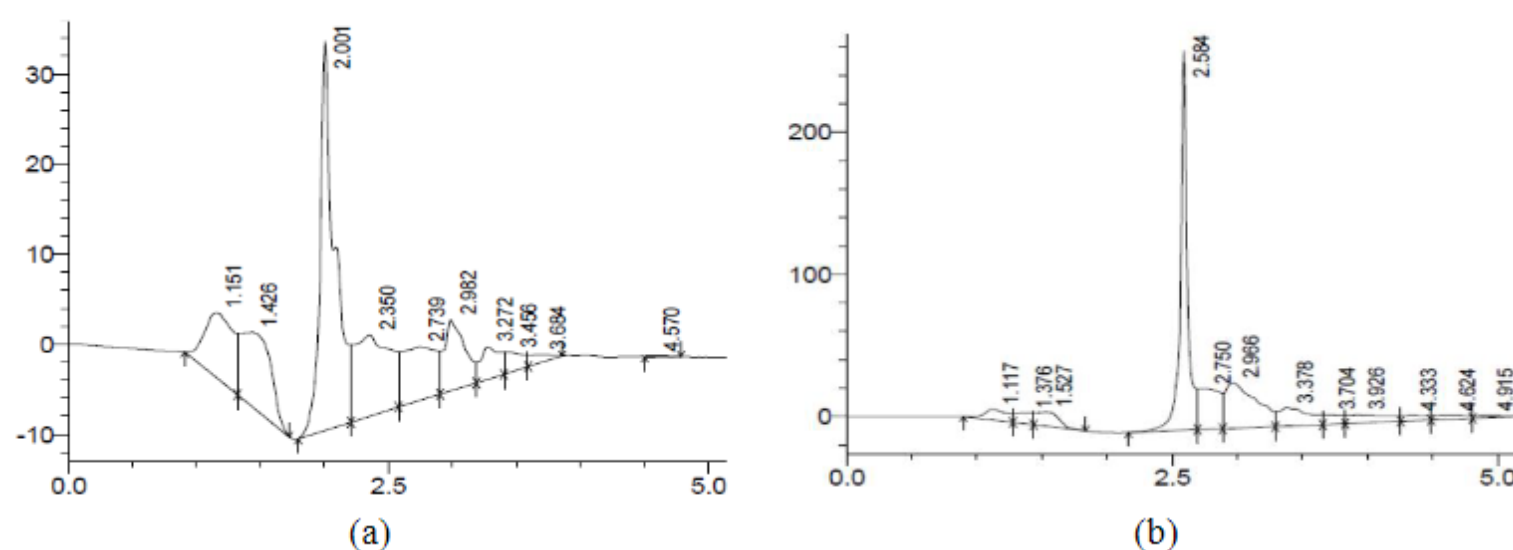


Figure 5. Isolate 19C38A1 on the surface of fish scales
(a) observations on 2nd day; (b) fish scale after washing

The results of SEM imaging on the second day, shown in Figure 3a, it was observed that actinomycetes produced spores and hyphae on the shrimp shell substrate, in Figure 4a observed the presence of hyphae on the cuttlefish bone substrate, whereas in Figure 5a there was no growth of actinomycetes. This indicated that the substrate affects the growth rate of actinomycetes. Further analysis of SEM imaging after each washing process observed damage to the surface of the shrimp shell substrate as depicted in Figure 3b and partial damage to the cuttlefish bone in Figure (4b) and no damage was observed on the surface of fish scales in Figure 5b. This indicated the growth rate of actinomycetes related to the ability of the mycelium to enter the substrate. To obtain more complete information from the results of the biotransformation process, the next experiment will focus on the production of chitin degradation analysis on shrimp shell media.

3.3. Glucosamine Analysis

The results of the analysis of waste biodegradation products observed on days 0, 2 and 3, are shown in Figure 6. The chromatogram at the initial conditions (day 0) showed that the substrate did not contain glucosamine (Figure 6a). The results of further observations were observed that the chromatogram peaks at retention time (rt) 2,593 minutes, and 2,567 minutes (Figures 6c and 6d) respectively on the second and third days. The peak that appeared was similar to the retention time with standard glucosamine (rt = 2,584) min (Fig. 6b). The interpretation of glucosamine peaks on day 2 and day 3 showed a continuous pattern of enzyme degradation. This can be seen from the decrease in the peak intensity of glucosamine on day 3 and the difference in the chromatogram pattern with increasing degradation products to form more polar compounds.



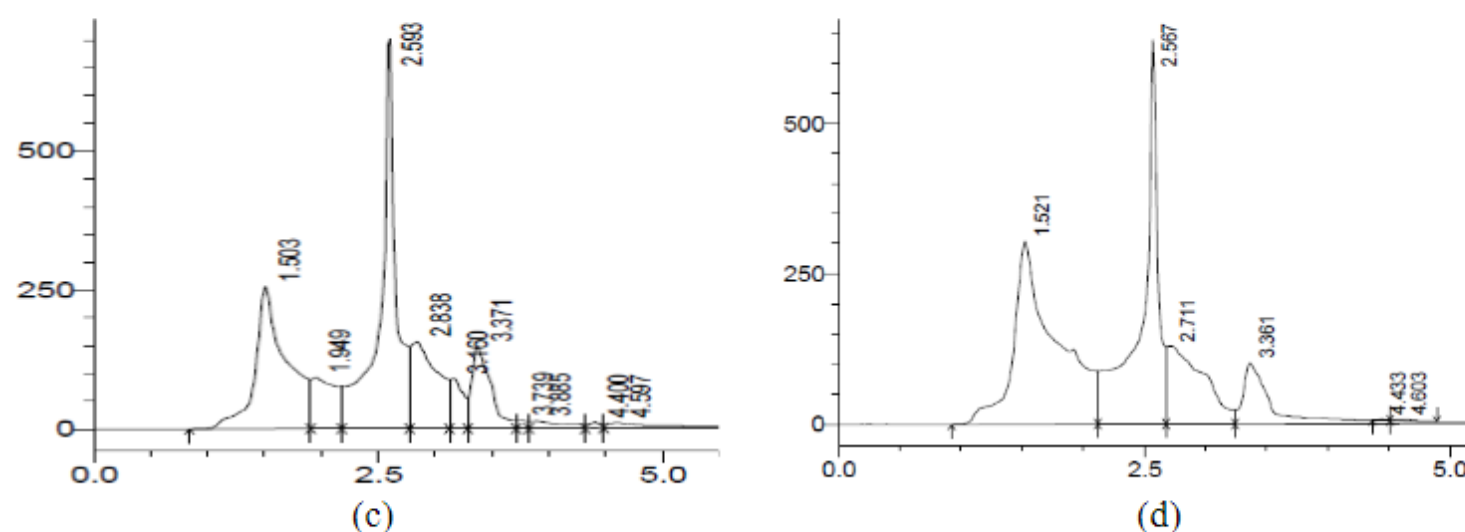


Figure 6. Glucosamine test results of SFF (a) control; (b) measurement Standard glucosamine; (c) the 2nd day measurement; (d) 3rd day measurement.

4. Conclusion

Based on the data obtained, it can be concluded that the application of SEM-EDX in the SSF process is very helpful to provide microanalysis data in a semi-micro-qualitative and semi-micro quantitative manner quickly. This information will be very important as the basis for developing the SSF research.

Acknowledgements

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