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Analysis of Protein Profile and Functional Groups in Biofilms in the Hypersaline Environment

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Abstract: Halophilic microorganisms include extremophiles that can live and develop in a hypersaline environment. Biofilm, as microecology, consists of a collection of microorganisms attached to a surface, forming an Extracellular Polymeric Substance (EPS) matrix. This study aims to analyze the protein profile and functional groups of biofilms that grow in hypersaline environments. The method used in this study is the SDS PAGE test to diagnose protein profiling and FTIR to analyze biofilm functional groups. The samples used to grow the biofilm came from salt ponds with different NaCl levels, namely 2%, 25%, and 40%. The results showed that each NaCl treatment had different functional groups in the biofilm samples. In the 2% NaCl treatment, there were peaks with solid intensity at the wavenumber 1100 $cm^{-1,}$ indicating the carboxylic acid functional group. In the 25% NaCl treatment, peaks with solid intensity at wavenumber 2250 cm^{-1} indicate the isocyanate functional group. In the 40% NaCl treatment, there were peaks with solid intensity at wavenumber 860 $cm^{-1,}$ indicating the alkene functional group. The protein analysis for each treatment shows the presence of pyruvate kinase protein in the biofilm with 2% NaCl. The 25% NaCl biofilm sample contained avidin protein, and the 40% NaCl biofilm sample contained tyrosine protein.

Keywords: Biofilms; halophilic; hypersaline; microbial ecology; protein.

Introduction

A hypersaline environment is an environment where there are not many organisms because it has a high salt content. One example of a hypersaline environment is a salt pond. This environment is a natural habitat for halophilic and halotolerant microorganisms (Hakim et al., 2020). Halophilic microorganisms include extremophile organisms capable of living and developing in a hypersaline environment (Kajale et al., 2020). The existence of halophilic microorganisms in hypersaline environments is often affected by self-acclimation and lack of competition with other phototrophic organisms (Musa et al., 2018).

Many microecological explorations are carried out in a study to find new things that benefit the environment. Biofilms, as microecology, consist of a collection of microorganisms that attach to a surface and form an Extracellular Polymeric Substance (EPS) matrix, causing biofilms to resist antimicrobial agents naturally (Astuti, 2020).

According to Wang et al. (2022), forming biofilms begins with cell communication called quorum sensing. This process is initiated by the production and secretion of signaling molecules that can be influenced by environmental factors such as nutrition, which trigger pathways necessary for biofilm formation. In addition, according to Yolazenia et al. (2018), there are five stages of the biofilm life cycle namely; 1) Reversible attachment, namely random contact of the biofilm with the surface. At this stage, no biofilm has yet been formed. So it is still easily separated. 2) Irreversible attachment is the production of the extracellular matrix, which is an

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adhesin to attach the bacterial complex to the surface. 3) Aggregation, the formation of microcolonies. At this stage, active replication/aggregation of organisms attached to the surface occurs so that the density and complexity of the entire biofilm increase. 4) Maturation, the interaction between bacterial colonies and the extracellular substances they produce, results in the maturation of biofilm forms and the redistribution of organisms away from the substratum. 5) Detachment occurs when the biofilm reaches a critical mass determined by some environmental conditions that release free-living bacteria to different colonies to maintain the bacterial cycle (Yolazenia et al., 2018). Biofilms develop when bacteria grow in favorable temperatures and nutrient conditions in ecosystems (Kurniawan & Yamamoto, 2019). In this case, the possibility of biofilm formation in extreme environments such as hypersaline can occur because this environment is an environment that contains water and nutrients.

Biofilms play a role in purifying water by decomposing harmful compounds (Kurniawan & Yamamoto, 2019). In addition, in her research, Tarczewska et al. (2022) also explained that the protein content contained in biofilms reached 19.05%. Protein is an essential element that plays a role in fish growth and generates energy (Lin et al., 2022). Thus, protein profile analysis can be the first step in conducting biofilm exploration. Therefore, this study aims to analyze biofilms' protein profiles and functional groups that grow in hypersaline environments.

Method

Experiment Design

Water samples came from the South Coast salt ponds in Malang, Indonesia. The brine samples were taken based on the different NaCl content according to the stages of the salt-producing process. This study used three types of saltwater samples, namely:

A = Brine with 2% NaCl
B = Brine with 25% NaCl
C = Brine with 40% NaCl

The biofilm growth process was carried out by incorporating 5 liters of salt water into a 10-liter aquarium. The media used for the growth of biofilms uses High-density polyethylene (HDPE) with a size of 100 x 150 mm and a thickness of 5 mm. HDPE is put into each aquarium containing salt water with different NaCl content.

The biofilm collection process was carried out by brushing the HDPE with a soft toothbrush in one direction and slowly placing it in a sample bottle containing 40 ml of distilled water. The biofilm and distilled water samples were homogenized, then divided into two. Some are used for protein profile analysis, and some for functional group analysis.

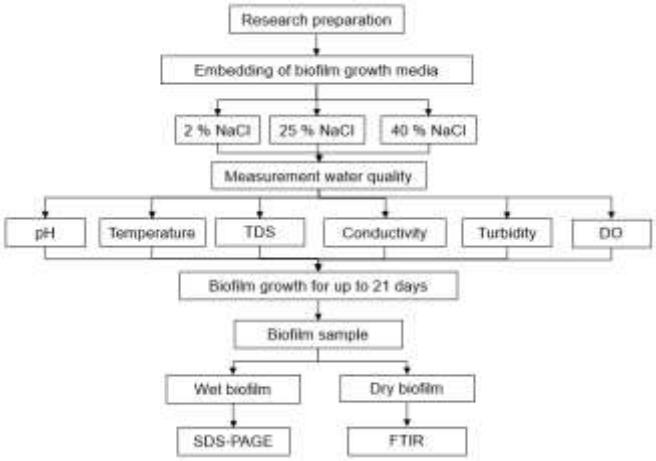

Figure 1. Image of design operasional research

Protein Profiling Analysis

Biofilm protein profiling was analyzed using the Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) method. Preparation for SDS PAGE testing was carried out in several stages (Salarian et al., 2019), the first of which was to prepare biofilm samples that had been brushed. Furthermore, the following treatment is the process of enzymatic hydrolysis with acetate buffer pH 4.5. Samples that had been hydrolyzed were added to a sample buffer with a ratio of 1:1, then heated at 85 °C for 5 minutes. Then the sample is put into the acrylamide gel column, and the electrodes are installed according to the poles. Electrophoresis was run at 200 V, 15 mA/gel for 60 minutes. After this, the gel was stained by staining 0.05% (w/v) in 15% (v/v) methanol and 5% acetic acid (v/v), heated in the microwave for 15 seconds, and incubated for 60 minutes. The gel was rinsed in a mixture of 30% methanol and 10% acetic acid and incubated in a water bath for 2-3 hours. Data analysis was performed by calculating each protein's molecular weight (BM) based on the marker used. The SDS-PAGE test in this study was conducted at the Animal Health Research and Diagnostic Laboratory, Malang, Indonesia.

Functional Group Analysis

Functional groups inside the biofilms were analyzed using Fourier Transform Infrared Spectroscopy (FTIR). Preparation for FTIR testing was carried out by centrifuging the biofilm suspension to collect the pellets. The biofilm pellets were then dried using an incubator at 60°C for 24 hours. The biofilm pellet is mixed with KBr (Potassium bromide) at a ratio of 1:50 mg. Then an infrared scan is carried out with a

4000-450 cm-1 wave range. FTIR analysis is used to analyze the functional groups formed in the biofilm samples seen from the shape of the presented spectra and the position of the absorption bands expressed as wave numbers (wavenumber) in units of cm-1. Functional group analysis with FTIR has the advantage of not damaging the sample's chemical structure (Toffolo et al., 2019). FTIR testing in this study was carried out at the Laboratory of Minerals and Materials at Advanced Central, State University of Malang, Indonesia.

Water Quality Measurement

The water quality parameters used in this study were pH, dissolved oxygen, temperature, turbidity, and conductivity, which were measured using a Water Quality Checker (WQC-22A, DKK TOA Corporation, Japan). Total Dissolved Solid (TDS) measurement using a TDS meter (Lutron YK22CTA, USA).

Result and Discussion

Profiling Protein

Protein profiling uses SDS PAGE to identify proteins based on their molecular weight. This measure of the molecular weight of a protein is helpful in protein mapping. The protein profile is a depiction of the genome content of the bacteria. Protein profiles can be used to classify, identify, and compare bacteria. The results of the SDS PAGE analysis of hypersaline biofilms are shown in Figure 1.

Protein profiling testing on 2% NaCl is detectable at 58 kDa, indicating pyruvate kinase protein. Pyruvate kinase is an apoenzyme that catalyzes the last step of the cellular process of glucose degradation (glycolysis) (Suhartono et al., 2021). Its function is to accelerate the transfer of phosphate groups from phosphoenol pyruvate to adenosine diphosphate, producing one pyruvate molecule and one ATP. The presence of this protein is supported by the FTIR results, which contain a carboxylic acid functional group with solid intensity. The carboxylic anion of pyruvic acid is called pyruvate, so this glycolysis process involves a biochemical process between pyruvate kinase and carboxylic acid.

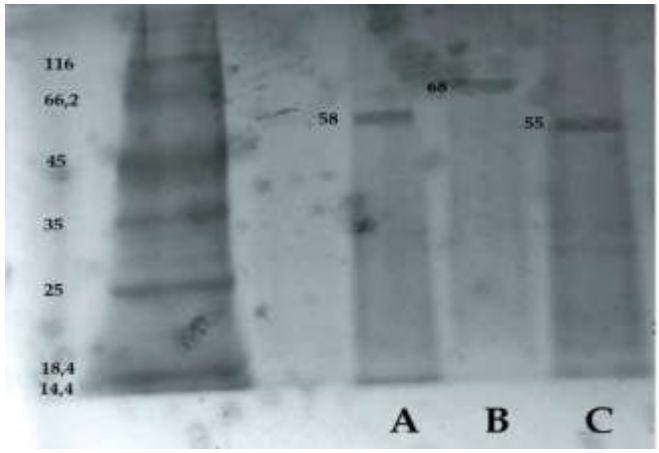

Figure 2. Image of protein bands of the biofilms

Protein profiling testing on 25% NaCl is detectable at 68 kDa as the avidin protein (Lee et al., 2021). Avidin is a protein that can bind to biotin with high selectivity and specificity (Lian et al., 2020). The avidin-biotin interaction is considered the strongest non-covalent bond in nature with a dissociation constant at Kda 10−15 M. It is frequently used in biochemical assays, diagnostics, and tissue engineering. Moreover, these protein affinity systems have been exploited for many applications in chemistry, biosciences, and tissue engineering. Several avidin proteins are found in Alphaproteobacteria class microbes (Salarian et al., 2019). Avidin is a glycoprotein molecule (a combination of carbohydrates and proteins).

Protein profiling testing on 40% NaCl detected 55 kDa, namely tyrosine (Cazalé et al., 2020). Tyrosine is a non-essential amino acid that is neutral, polar (hydrophilic), and uncharged. The results of the FTIR test indicated the presence of the C-H functional group in the aromatic ring. This presence makes tyrosine have biochemical reactions that are glycogenic and ketogenic, and its chemical structure is cyclic, which is aromatic (Sayed & Ahmed, 2022). Tyrosine is an amino acid with a phenol group and weak acid (Lalopua et al., 2022).

Analysis of Functional Groups

The functional groups inside the biofilms forming in hypersaline environments were analyzed using the FTIR test. The results of the FTIR test for each treatment can be seen in Figure 3.

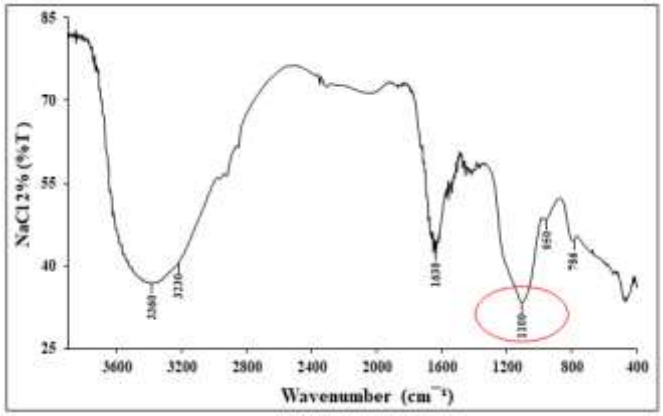

Figure 3. FTIR spectrum of biofilm formed in 2% of NaCl

The results of the halophilic biofilm FTIR test showed that there were several compositions of the same functional groups between treatments. Each treatment has a functional group that has a vigorous intensity. Several peaks are read, including 786 cm-1 functional group C-H Alkene, 950 cm^{-1} functional group C-H aromatic ring, 1100 cm^{-1} functional group C-O Acidcarboxylates, 1630 cm^{-1} functional group C=C Alkene, 3230 cm^{-1} functional group O-H phenol, 3360 cm^{-1} functional group N-H Amine. In the 2% NaCl treatment, there was one functional group that was not present in the other treatments, namely acid carboxylates with a vigorous intensity with a wavenumber of 1100 $cm^{-1,}$ indicating the acidic C-O functional group carboxylates found in bacterial cellulose (Surya et al., 2022).

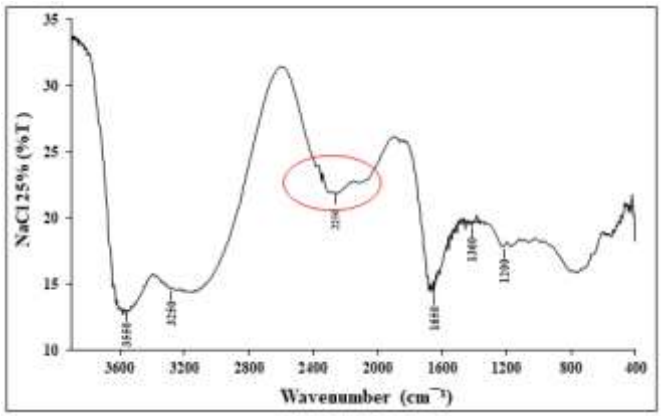

Figure 4. FTIR spectrum of biofilm formed in 25% of NaCl

The results of the FTIR test of halophilic biofilms in the 25% NaCl treatment showed several peaks that were read, including 1200 cm^{-1} functional group C-O Carboxylic acid, 1360 cm^{-1} functional group C-N Amine, 1650 cm^{-1} functional group C=C Alkene, 2250 cm^{-1}1 functional group N=C=O Isocyanate, 3250 cm^{-1} functional group O-H phenol, 3550 cm^{-1} functional group Carboxylic acid monomer. In the 25% NaCl treatment, an isocyanate functional group N=C=O at wavenumber 2250 cm^{-1}. Isocyanates are organic fiber polymers with a sticky shape or character (Birniwa et al., 2023).

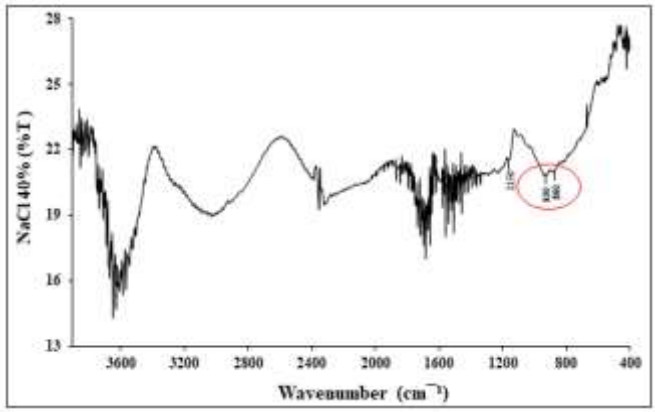

Figure 5. FTIR spectrum of biofilm formed in 40% of NaCl

The results of the FTIR test for halophilic biofilms in the 40% NaCl treatment showed several peaks that were read, including 860 cm^{-1} indicating the C-H Alkene functional group, 930 cm^{-1} indicating the C-H functional group Aromatic ring, 1150 cm^{-1} indicating the C-O functional group Carboxylic acid. Functional groups at high salinity levels are less readable. This condition is thought to result in the least biofilm forming on the medium, so FTIR readings are limited because there are more KBr than biofilm pellets. According to (Toffolo et al., 2019), FTIR readings are not optimal if the number of sample pellets being analyzed is minimal. In the end, only the KBr is read by infrared.

Water Quality Parameters

pH

The results of biofilm pH measurements in treatment A (25% NaCl) obtained the highest value, meaning the pH condition is alkaline. In comparison, the lowest pH value was found in treatment B (25% NaCl), meaning the pH condition is acidic (Figure 6). It seems that halophilic bacteria can reproduce in environmental conditions with high salt content, so it is assumed that if the treatment with the lowest NaCl addition, bacteria's abundance, and metabolic activity is not too high, bacterial metabolism can lower the pH (Van Thuoc et al., 2021). On the other hand, in treatment C, with the highest salt content (NaCl 40%), the pH value was obtained between treatments A and B. This result is assumed to be due to factors that affect pH, not only the high abundance of bacteria which also causes the metabolism to increase and the pH to fall. (acids), but also because there are inputs of other organic materials from the environment (Irawan et al., 2021).

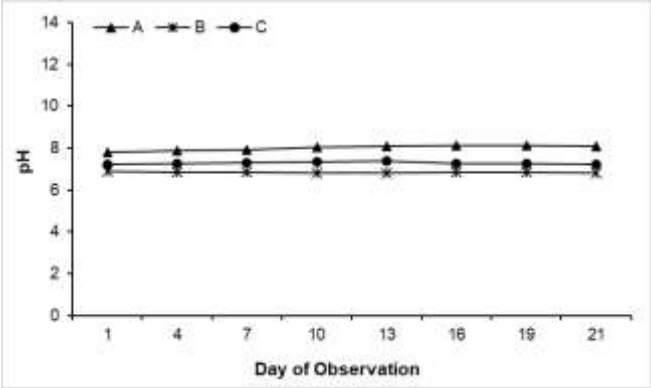

Figure 6. pH values in a hypersaline environment

Dissolved Oxygen (DO)

The highest biofilm DO measurement results were in treatment A (2% NaCl) and the lowest in treatment B (25% NaCl). The DO graph is the same as the pH graph, where treatment C (40% NaCl) has a value between treatments A and B (Figure 7). This result is because if the pH is low (acidic), DO is also low (Daroini & Arisandi, 2020). Apart from the low DO level in the metabolic process, other factors are also due to environmental pollutants (Wahyuningsih et al., 2020).

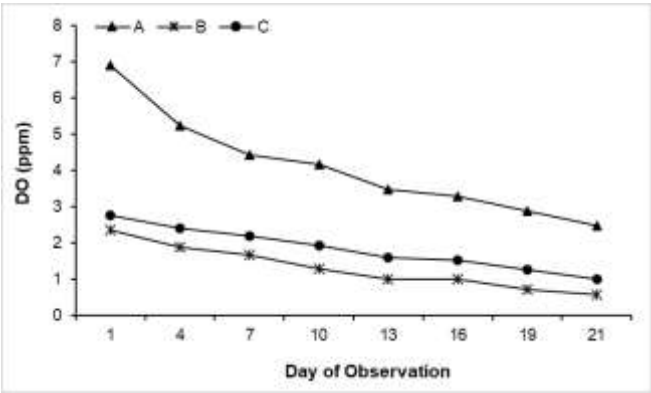

Figure 7. DO values in a hypersaline environment

Temperature

The results of measuring the temperature of the biofilm samples in all treatments (2%, 25%, or 40% NaCl addition) were relatively the same (Figure 8). This condition is because the amount of NaCl content does not affect the temperature of the sample water. Following the opinion of (Van Thuoc et al., 2021), the factors that significantly affect water temperature are external factors, namely sunlight, weather, and wind.

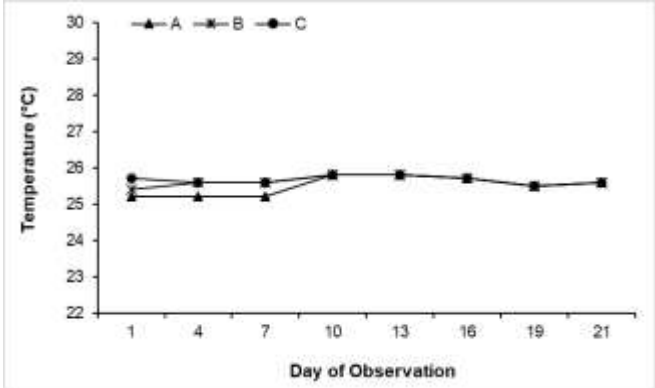

Figure 8. Temperature values in a hypersaline environment

Total Dissolved Solid (TDS)

The results of TDS measurement of biofilm samples were highest in treatment B (NaCl 25%) and lowest in treatment A (NaCl 2%) (Figure 9). The TDS rate in waters is affected not only by the NaCl content but also by tidal activity, seasonal variations, and the water depth at the time of sampling (Fahimah et al., 2021).

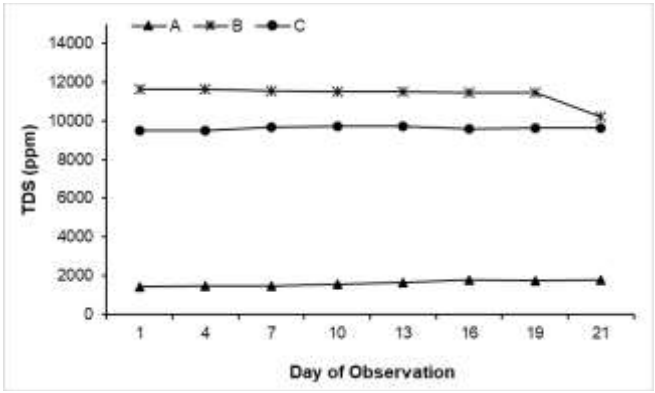

Figure 9. TDS values in a hypersaline environment

Turbidity

The highest turbidity measurement result was in treatment C (NaCl 40%), and the lowest was almost worthless in treatment A (NaCl 2%) (Figure 10). According to (Abada et al., 2022), water turbidity is influenced by organic and inorganic materials, suspended and dissolved, such as mud, sand, and organic matter (plankton or other microorganisms). The NaCl content dissolved in the waters can also increase the turbidity of the waters, so the higher the NaCl level, the turbidity of the waters also increases.

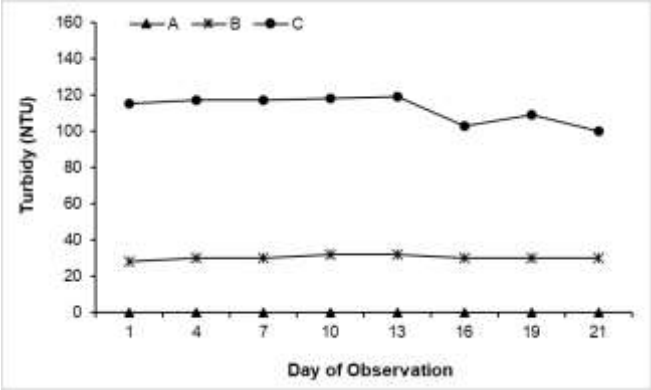

Figure 10. Turbidity values in a hypersaline environment

Conductivity

The results of the conductivity measurements were ordered from the highest to samples B (25% NaCl), C (40% NaCl), and the lowest is A (2% NaCl) (Figure 11). According to (Jiao et al., 2021), conductivity (electrical conductivity) is a numerical description of the ability of water to transmit electricity. Therefore, the more dissolved salts can be ionized, the higher the DHL value (Prihatno et al., 2021). However, in the graph, treatment C (NaCl 40%), with the highest salt content, has a lower conductivity value than treatment B (NaCl 25%), with a lower salt content. This condition is because other factors affect the conductivity value, such as organic matter (Abbas et al., 2022).

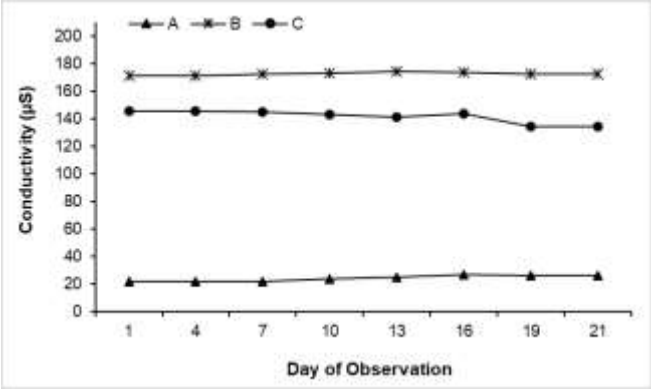

Figure 11. Conductivity values in a hypersaline environment

Conclusion

The results of the research on protein profiling showed that the 2% NaCl biofilm sample contained pyruvate kinase with a molecular weight of 58 kDa. The 25% NaCl biofilm sample has an avidin protein with a molecular weight of 68 kDa. The 40% NaCl biofilm sample has a tyrosine protein with a molecular weight of 55 kDa. The results of functional group analysis in the 2% NaCl biofilm sample contained functional groups including 786 cm-1 functional group C-H Alkene, 950 cm-1 functional group C-H aromatic ring, 1100 cm-1 functional group C-O Sourcarboxylates, 1630 cm-1 functional group C=C Alkene, 3230 cm-1 functional group O-H phenol, 3360 cm-1 functional group N-H Amine. Then on the 25% NaCl biofilm, several peaks were read, including 1200 cm-1 functional group C-O Sourcarboxylates, 1360 cm-1 functional group C-N Amine, 1650 cm-1 functional group C=C Alkene, 2250 cm-1 functional group N=C=O Isocyanate, 3250 cm-1 functional group O-H phenol, 3550 cm-1 functional group Acidcarboxylates monomer. There are functional groups in 40% NaCl biofilm, including 860 cm-1 functional group C-H Alkene, 930 cm-1 functional group C-H Aromatic ring, and 1150 cm-1 functional group C-O Sourcarboxylates.

Author Contribution

All authors have contributed to the final manuscript. The contribution of each author as follow:

AK : Conceptualization, Investigation, Data curation, Formal analysis, Methodology, Writing – review & editing, Funding acquisition, Project administration, Resources

IMAZ : Investigation, Data curation, Formal analysis, Methodology.

YN : Investigation, Data curation, Formal analysis, Methodology.

RI : Investigation, Data curation, Formal analysis, Methodology.

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Conflicts of Interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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