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Research Paper

Novel *Micrococcus unila* to Produce Glucosamine by Solid-state Fermentation of Shrimp Shell Waste

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Abstract
This study aimed to assess glucosamine production through enzymatic activity, utilizing actinomycetes sourced from shrimp shell waste (SSW) in a solid-state fermentation (SSF) process. A total of 16 actinomycetes underwent chitinase activity screening, and the strain exhibiting the highest chitinolytic index was chosen for subsequent morphological and phylogenetic analyses. High Performance Liquid Chromatography (HPLC) was employed to analyze glucosamine produced from the bioconversion of SSW via SSF. Optimal conditions for glucosamine production were determined by varying time, pH, and temperature. Isolate 18D36-A2 showed the highest chitinolytic index of 1.02 in the 32-mm clean zone. Phylogenetic analysis revealed 97% similarity to the genus Micrococcus, identifying it as a novel *Micrococcus unila* strain 18D36-A2 and deposited in GenBank. This isolate effectively converted shrimp shells. The findings showcase the bioconversion of SSW to glucosamine through SSF using the *Micrococcus unila* 18D36-A2. Furthermore, this study establishes a foundation for future research on environmentally friendly and sustainable designs for glucosamine production.

Keywords
Actinomycetes, Chitinase, Glucosamine, Solid-State Fermentation, Shrimp Shell Waste

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1. INTRODUCTION

Osteoarthritis (OA) ranks 10th among the leading causes of global disability-adjusted life years. Reports indicate that over 300 million people are affected by arthritis. OA, a prevalent global disease, is a chronic joint condition often aggravated by multiple comorbidities, predominantly associated with aging. This condition can lead to functional impairment, pain, and disability (Hunter and Bierma-Zeinstra, 2019). Glucosamine is currently recommended for treating OA in patients with concurrent diabetes, cardiovascular disease, and metabolic syndrome (Conrozier and Lohse, 2022). In industrial settings, glucosamine is chemically produced through demineralization, deproteinization, and hydrolysis (Bertuzzi et al., 2018). However, the chemical production of glucosamine generates waste products that may harm the surrounding ecosystem (Topić Popović and Lorencin, 2022). Hence, there is a pressing need to explore more environmentally friendly methods for glucosamine production (Montiel-Montoya et al., 2019). Recent studies have highlighted the capability of marine actinomycetes to produce the enzyme chitinase (Laila et al., 2023). However, the chitinase-producing ability of actinomycetes varies significantly based on their specific type and habitat (Setiawan et al., 2021). Consequently, there is a need to identify novel actinomycetes capable of bioconverting shrimp shells into glucosamine.

Conversely, global shrimp production for 2022 was 9.4 million tons (Food and Agriculture Organization, 2023). Typically, an exported segment of shrimp consists of shell-separated meat, while shrimp shell waste (SSW) is commonly repurposed as animal feed. All components of the shrimp shell contain various chemical compounds, including Chitin (18.1%), protein (38.6%), minerals including $CaCO_3$ (33.3%), and lipids (Kaur

and Dhillon, 2015; Hu et al., 2020), which can be utilized. Chitin can be further processed in solid-state fermentation (SSF) to yield valuable compounds like N-acetyl glucosamine and glucosamine (Setiawan et al., 2021). The economic aspect of SSF is currently a crucial consideration. Despite its relatively low energy consumption and environmental friendliness, the bioconversion yield is low (Yafetto, 2022). Nevertheless, optimization is achievable through bioprocess technology (Yafetto, 2022) and by selecting suitable actinomycete strains (Raziq et al., 2020).

This study focused on utilizing the chitinase enzyme from marine actinomycetes, isolated from marine biota in Buleleng, Bali, to bioconvert chitin into glucosamine compounds. Verifying shrimp shells appropriateness as a nutrient source for marine actinomycetes in order to increase the value of products made with SSF was the main goal. The novel marine actinomycetes isolates were analyzed through morphology and phylogenetic examination.

2. EXPERIMENTAL SECTION

2.1 Materials

2.1.1 Tunicate Collection

In August 2018, samples from marine organisms were collected near the shore of Singaraja, Buleleng, Bali, Indonesia, in order to isolate actinomycetes. The collection process was conducted through SCUBA diving.

2.1.2 Isolation of Actinomycetes

A tiny amount of tunic was homogenized and washed in sterile seawater. The resulting suspension underwent serial dilution and was spread onto colloidal chitin agar plates consisting of colloidal chitin (Kotb et al., 2023). Artificial seawater (ASW) 50%, cycloheximide 25 μg/mL, and nalidixic acid 25 μg/mL were used to make these plates. The culture was maintained for 14 days at 28°C. Actinomycetes were then separated and refined using a shrimp shell medium that included 1% chitin in an agar/seawater solution at 50% (v/v). The single isolate was streaked on colloidal chitin agar slants 1% (w/v) at 4°C and then preserved in glycerol suspension 20% (v/v) at -20°C.

2.2 Methods

2.2.1 Screening of Chitinolytic Activity

Isolate screening was performed on colloidal chitin agar media comprising 1% w/v colloidal chitin and 2% w/v agar powder dissolved in artificial seawater (Krithika and Chellaram, 2016) and International Streptomyces Project 2 (ISP 2) medium (Nord et al., 2019). The isolates were inoculated at the center of the media, incubated for 7–14 days (Wang et al., 2022), and utilized to determine the chitinolytic index of the colony.

2.2.2 Morphological Study of Actinomycetes

With the use of the coverslip culture technique, the organization of the spore ornament structure was investigated (Kurtböke, 2022). A sterile coverslip was placed at a 45° angle on a colloidal chitin agar plate 1% and incubated for 7 days. After being incubated, the coverslip was gently taken out with tweezers from the agar plate and placed faceup on a sanitized glass slide. A 400× magnification Zeiss Axio Imager microscope was used to examine the coverslip.

An examination of the isolated actinomycetes' mycelium and spore arrangement was done using scanning electron microscopy (SEM). The chosen actinomycetes were cultivated in artificial seawater using a 1% colloidal chitin liquid media and were incubated in a static environment. Little bits of shrimp shell were put in a Petri plate with the inoculum and left to sit for 7 days. The culture was then incubated at room temperature under static conditions for up to 14 days, with observations made every 2 days over 2 weeks. After incubation, a Kenko L-500 disposable slee blade microtome (Bekasi, Indonesia) was used to cut a portion of the shrimp shell. The prepared sample was affixed to aluminum stubs using carbon adhesive tabs and gold plated for 20 minutes. SEM EVO, electron accelerating voltage 10 kV (EVO MA 10 Carl Zeiss, Germany), was used to examine the gold-plated metal stub.

2.2.3 Phylogenetic Analysis

Genomic DNA extraction followed the genomic DNEasy Plant Mini KIT protocol (cat. No. 69104, Qiagen, Hilden, Germany). To amplify 16S rDNA sequences, a thermocycler (Sensoquest Sensodirect, Germany) was utilized. A reverse primer, 5'-CGG TAC TCC CCA GGC GGG G-3' and forward primer, 5'-AGA GTT TGA TCM TGG CTC AG-3' were used in the PCR (Setiawan et al., 2021), amplifying a fragment of 858 bp. 12.5 μL of 2g Fast ReadyMix (cat. No. KK5102, Merck, Germany), 1.5 μL of RNAse-free water, 0.5 μL of the forward primer, and 0.5 μL reverse primer were added to a total volume 25 μL, which was used for the PCR reaction. The amplification process involved 35 cycles, encompassing denaturation at 95°C for 1 minute, primer annealing at 54°C 1 minute, and polymerization at 72°C for 1 minute. As directed by the manufacturer, the amplicon was identified using the Qiaxcel Advanced (Qiagen, Germany), and the Sanger method was used for sequencing. The sequencing data were subjected to phylogenetic analysis using MEGA version 11 software (Tamura et al., 2021).

2.2.4 Solid-State Fermentation

The fermenting medium was fresh SSW that was purchased from Bandar Lampung's Gudang Lelang market. The SSW was carefully cleaned twice using running water with a ratio of 1:10 (w/v), and unwanted elements including dirt, and sand particles were removed with the use of a filter. The cleaned SSW was then dried in a drying oven for the entire night at 60°C. Using an electric blender, the dried SSW was subsequently turned into shrimp shell powder, which was used as a nutrient source for SSF without demineralization or deproteinization processes. Into a 2000 mL Erlenmeyer flask, 500 mL of 1% colloidal chitin medium and 10 mL of inoculum were added. The suspension was cultivated at room temperature for 7 days under static conditions.

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The fermentation media employed in this study are categorized as solid media, commonly known as SSF. This stage follows the published method with several modifications (Widyastuti et al., 2022). A total of 10 g of dried shrimp shell powder was put in a 500-mL Erlenmeyer and sterilized. Subsequently, the medium was inoculated with 10 mL inoculum and incubated under various conditions (time, pH, temperature). After incubation, the fermentation medium was combined with 40 mL of ASW, shaken at 100 rpm for 1 h at room temperature, and then centrifuged for 15 minutes at 7000 rpm. The resulting filtrate was utilized for subsequent tests. Kinetic studies were conducted under diverse fermentation conditions (time: 2–14 days; pH: 5, 6, 7, and 8; temperature: 30°C, 40°C, 50°C, and 60°C). This stage refers to a published procedure Gupta et al. (2017)) with some modifications.

2.2.5 Glucosamine Analysis

The glucosamine concentration was determined using the dinitrosalicylic acid (DNS) method (Atalla et al., 2020; Lv et al., 2021) with some modifications. In each sample, the filtrate of 1 mL was mixed with DNS reagent, boiled for 10 minutes, and then cooled. The value of absorbance was observed at 540 nm. This procedure was repeated three times for each sample. A reference standard solution of glucosamine-HCl was prepared at various concentrations and employed for comparison. Glucosamine was analyzed with Liquid Chromatography Mass Spectrometry/ Mass Spectrometry (LC-MS/MS) from Waters, Beverly, MA, USA.

2.2.6 Chitinase Enzyme Activity Analysis

Chitinase enzyme activity was assessed using the specific reagent DNS with some modifications (Atalla et al., 2020; Lv et al., 2021). Briefly, cultivation filtrate 1 mL was mixed with colloidal chitin 1% in ASW, homogenized, and incubated in a water bath at 40°C for 30 minutes. Subsequently, the reducing sugar reagent (DNS) 1 mL was added to the solutions, boiled for 10 minutes, and allowed to cool. The absorbance was observed at 540 nm. This process was repeated three times for each sample. The activity of chitinase was measured by defining one unit as the amount of enzyme required to release one mol of glucosamine in one minute.

2.2.7 Determination of Glucosamine from SSF

The fermentation product analysis HPLC system (Shimadzu, Japan) used a C18 column (reverse phase) with acetonitrile: water (70:30) as the mobile phase at a flow rate of 1 mL/minute. Detection was conducted at a wavelength of 210 nm using a diode array detector (detector SPD-M20A), and the system was equipped with an LC-20AD pump. The fermented extract obtained was washed with ethanol and then dissolved in distilled water. Subsequently, the sample was passed through a membrane filter 0.2-μm (Whatman), and the filtrate of sample 12 μL was injected into the injector with a loop of 10 μm. HPLC standards, specifically N-acetyl-D-glucosamine and glucosamine-HCl (WAKO, Japan), were employed as reference compounds. Glucosamine concentration is calculated via Equation (1).

$$\text{Glucosamine Concentration} = \frac{\text{Peak Area of Glucosamine}}{\text{Peak Area of Glucosamine Standard}} \times [\text{Glucosamine Standard}] \quad (1)$$

The glucosamine percentage is calculated based on the weight of the shrimp shell sample using Equation (2).

$$\%\ \text{Glucosamine} = \frac{\text{Glucosamine Concentration}}{\text{Shrimp Shells Weight}} \times 100\% \quad (2)$$

3. RESULTS AND DISCUSSION

3.1 Actinomycetes Isolation

In this study, actinomycetes were obtained from microorganisms collected in Buleleng, Bali, Indonesia. At a depth of 5-20 meters, a marine organism sampling was carried out at coordinates (8° 07' 20.9" S 114° 34' 03.8" E). Actinomycetes were then separated from marine organisms by employing selective colloid media at a concentration of 0.1% on agar media that was artificially salted. The screening results for each isolate, based on the chitinolytic index value, are presented in Table 1.

6 sponges (18A13, 18B18, 18D33, 18D35, 18E41, and 18F47) and 2 tunicates (18D38) yielded 14 actinomycete isolates (Figure 1). It has been previously reported that the isolation of actinobacteria from various marine organisms reveals their potential as producers of bioactive metabolites (Sun et al., 2015; Abdelmohsen et al., 2014). Despite the demonstrated presence of actinobacteria in many marine organisms such as tunicates and sponges, their use is uncommon (Topić Popović and Lorencin, 2022).

3.2 Morphology of Selected Actinomycetes

Actinomycetes grown in the media revealed that whereas aerial filaments grew vertically at the media-air interface, mycelium penetrated the media. Under a microscope, all of the 16 samples showed the characteristic size of mycelia with a wide 2 μm and an average diameter of 6.1 μm (Acharyabhatta et al., 2013). This size is smaller than that of many fungi, where the hyphae itself even typically can grow up to 30 μm (Islam et al., 2017). Actinomycetes are anaerobic microorganisms that grow on solid substrates in filamentous and branching patterns that resemble the mycelia of fungi. Their colonies expand widely, much like mycelium, and many actinomycetes genera have been shown to have aerial hyphae. As shown in Figure 2, these characteristics confirm the classification of isolate 18D36-A2 as actinomycetes.

3.3 Phylogenetic Analysis of the 18D36-A2 Isolate

The 16S rDNA gene sequence showed the genus Micrococcus. With a similarity ratio of 97.32%, *Micrococcus unila* was

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Table 1. Screening Chitinolytic Activity

Isolate Code	Colony Color	Colony Shape	Colony (Ø mm)	Clear Zone (mm)	Chitinolytic Index
18A13A1	White to cream	Filamentous	9	-	-
18B18A1	White to cream	Filamentous, Irregular	8	-	-
18B18A2	White	Rhizoid	6	-	-
18B18A3	White	Round	1	-	-
18B21A1	White	Round	1	-	-
18B21A2	White	Filamentous	5	-	-
18D32A2	White	Rhizoid	9	-	-
18D33A1	White	Filamentous	7	-	-
18D33A2	White	Spots	1	-	-
18D35A2	White to cream	Filamentous	5	-	-
18D35A3	White to cream	Filamentous, Irregular	6	-	
18D36A1	White to cream	Filamentous, Irregular	34	35	1.02
18D36-A2	White to cream	Filamentous, Irregular	27	30	1.10
18E41A2	White to cream	Filamentous, Irregular	10	-	
18E45A1	White	Filamentous	6	-	-
18E45A2	White to cream	Filamentous, Irregular	10	-	-

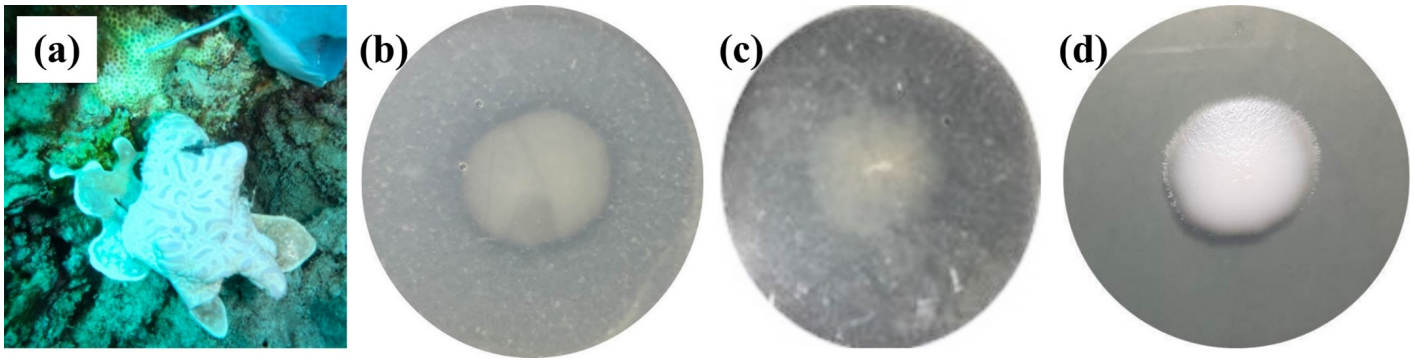

Figure 1. (a) Tunicate 18D36 as The Source for Actinomycetes 18D36-A2, (b) Isolate 18D36-A2 and (c) Isolate 18E45A2 in Colloidal Chitin Medium, (d) Isolate 18D36-A2 in ISP 2 Medium

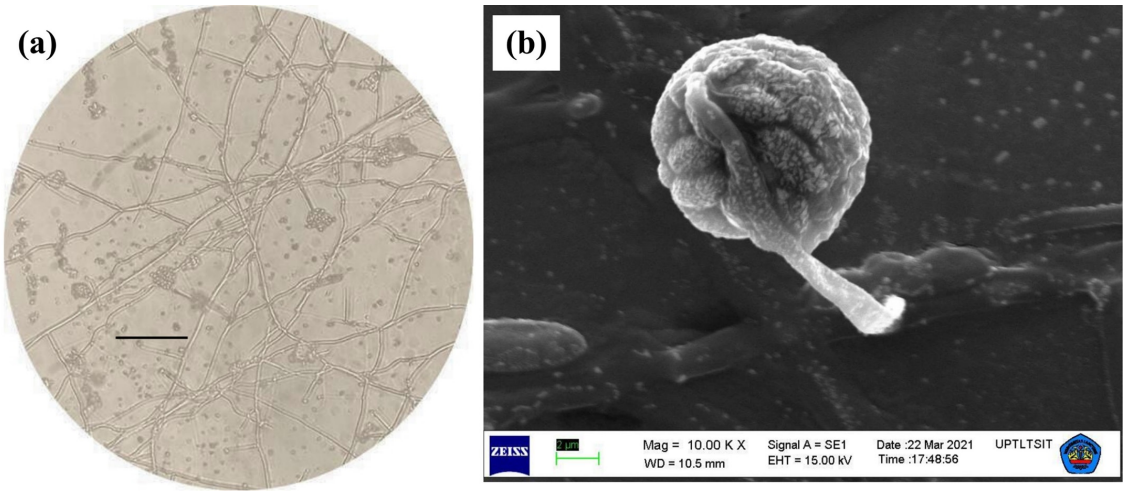

Figure 2. (a). Visualization of 18D36-A2 Seen at 400× Magnification using A Light Microscope (The Bar Stands for 20 μm), (b) Scanning Electron Microscope (SEM) Image of The Aerial Hyphae of Isolate 18D36-A2 (The Bar Represents 4 μm)

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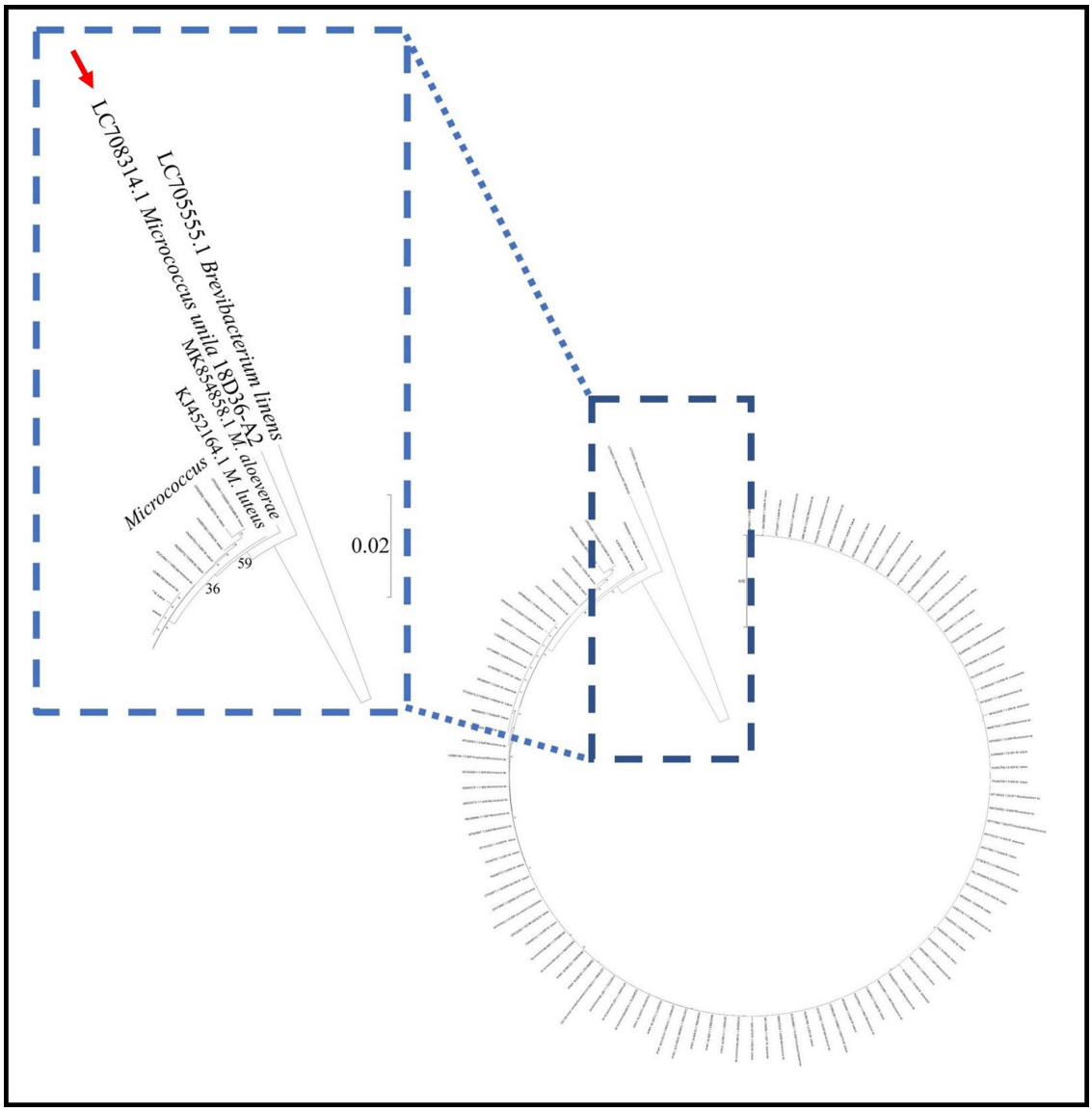

Figure 3. A Phylogenetic Tree was Constructed using The Neighbor-Joining Method and The Tamura 3-Parameter Model, Incorporating 108 Representatives of Micrococcus. *Brevibacterium linens* (Acc. No. LC705555) was Included as An Outgroup (Kirchberger et al., 2014). Bootstrap Values, Obtained from 5000 Resamples, are Presented s Percentages at The Tree Nodes. The Tree Incorporates A Representation of Isolate 18D36-A2

discovered through sequencing and subsequently registered in GenBank under the accession number LC708314, tentatively named Micrococcus sp. 18D36-A2. Phylogenetic analysis confirms that this isolate is a novel species within the Micrococcus genus, as shown in Figure 3.

This isolate is currently undergoing registration for a new species name in the NCBI taxonomic database. Additionally, Micrococcaceae's prevalence is widespread in terrestrial and marine environments. Based on previous studies, Micrococcus from seawater has been reported to exhibit chitinase activity (Annamalai et al., 2010; Rozirwan et al., 2020).

3.4 Solid-State Fermentation

Chitin hydrolysis is the prevailing method for industrial-scale glucosamine production owing to its simplicity and scalability. This process can yield substantial quantities of glucosamine. Nevertheless, this process generates byproducts, including ammonia and sulfur dioxide, posing potential environmental risks. In contrast, microbial fermentation is a more environmentally friendly approach to glucosamine production. However, it is a more complex and less established process than the chitin hydrolysis method.

As shown in Figure 4, the SSF process begins with the growth of actinomycetes responsible for glucosamine produc-

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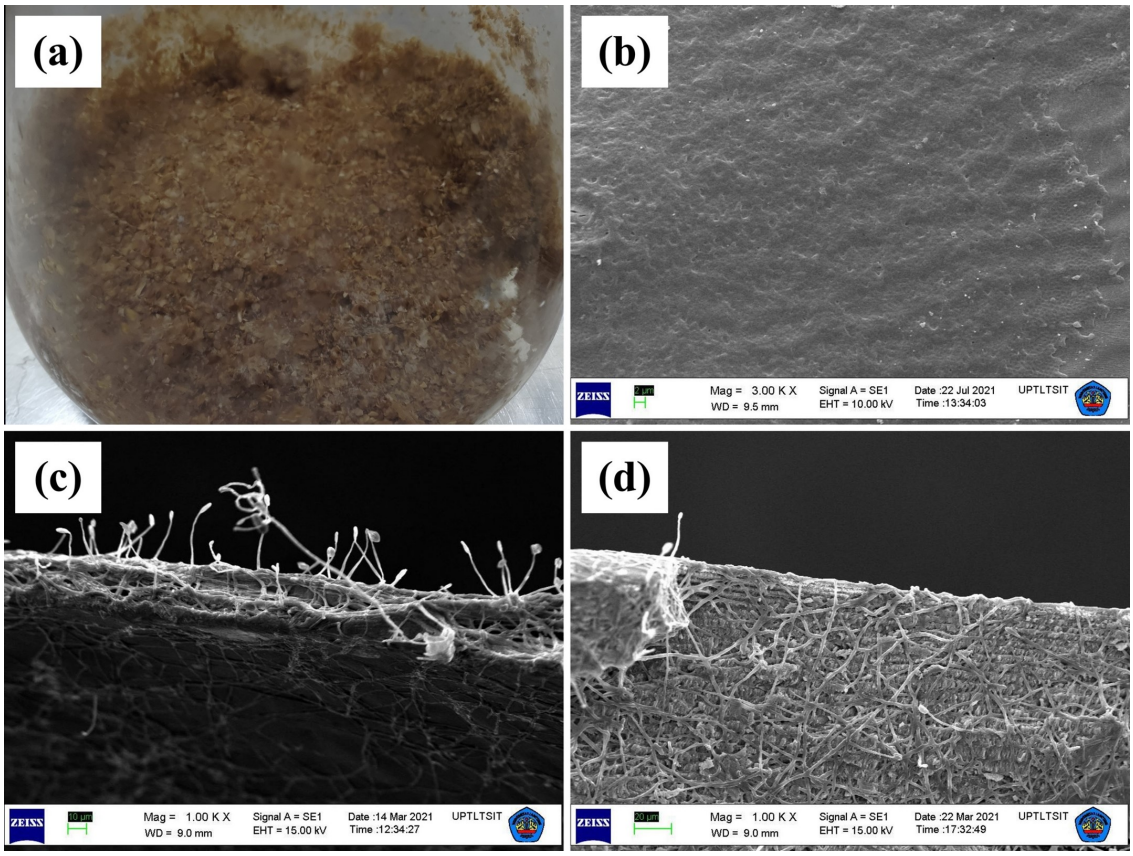

Figure 4. (a) Solid-State Fermentation of *Micrococcus unila* on Shrimp Shell Media, (b) SEM Photograph of The Shrimp Shell Surface, (c) 6 Days Later (d), and 14 Days Later

tion. Isolate 18D36-A2 was cultivated in fermentation tanks (Figure 4a) filled with nutrient-rich SSW (Figure 4b). Subsequently, the isolate was allowed to grow over several days (Figures 4c and d). Throughout fermentation, bacteria produce glucosamine as a byproduct. Glucosamine can then be extracted from the fermentation broth and purified. The SSF method is more environmentally friendly than the chemical hydrolysis process for producing glucosamine. However, it is a more complex and less established process.

3.5 Glucosamine Production

Typically, the fermentation duration must be sufficiently long to enable microorganism growth and substantial glucosamine production. However, it should not be too long owing to possible glucosamine degradation or the generation of undesirable compounds. As shown in Figure 5, the maximum time variation was on day 6, as evidenced by glucosamine concentration and chitinase activity of 0.6401 mg/mL and 0.0766 U/mL, respectively.

The optimal fermentation time to produce glucosamine using SSF depends on several factors, including the specific microorganisms, the substrate under fermentation, and the targeted glucosamine concentration. Typically, fermentation lasts 3–7 days. A shorter duration may not provide sufficient time for microorganism growth and substantial glucosamine production. Conversely, an extended fermentation time may result in glucosamine degradation or undesired compounds.

pH plays a crucial role in SSF because it affects the activity of the microorganisms involved in the fermentation process. The optimal pH for fermentation varies based on the microorganisms employed and the targeted product. Generally, most microorganisms prefer a neutral pH (pH 7). As depicted in Figure 6, the maximum pH observed for the isolate was pH 6, with glucosamine concentration and chitinase activity of 0.4851 mg/mL and 0.0559 U/mL, respectively.

An excessively high or low pH can inhibit the growth and activity of Micrococcus spp., leading to reduced glucosamine production. For instance, an overly high pH may denature essential enzymes involved in glucosamine production. In contrast, an excessively low pH may harm the cell membranes of Micrococcus spp., potentially killing the bacteria. Consequently, meticulous pH control in the SSF process is crucial to ensure optimal glucosamine production. This can be achieved by adjusting the substrate pH before or during fermentation.

Figure 7 shows a graph illustrating the fermentation results under varying temperature conditions. The optimum temperature during the fermentation process was 30°C ± 2°C, as evidenced by concentration values of 0.5482 mg/mL for

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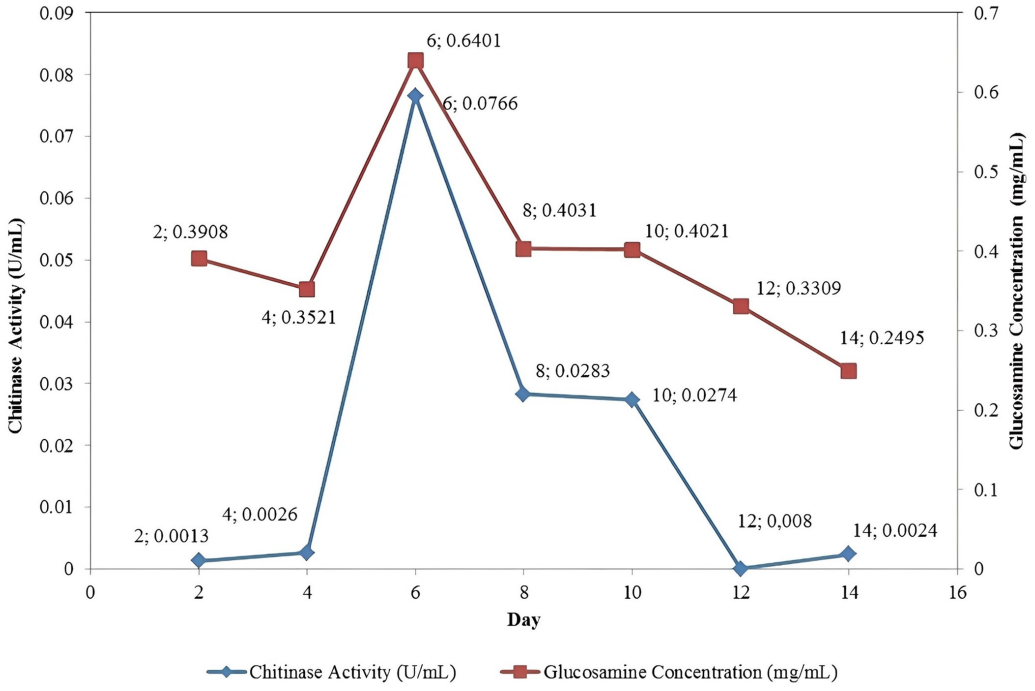

Figure 5. Fermentation Results of Different Days

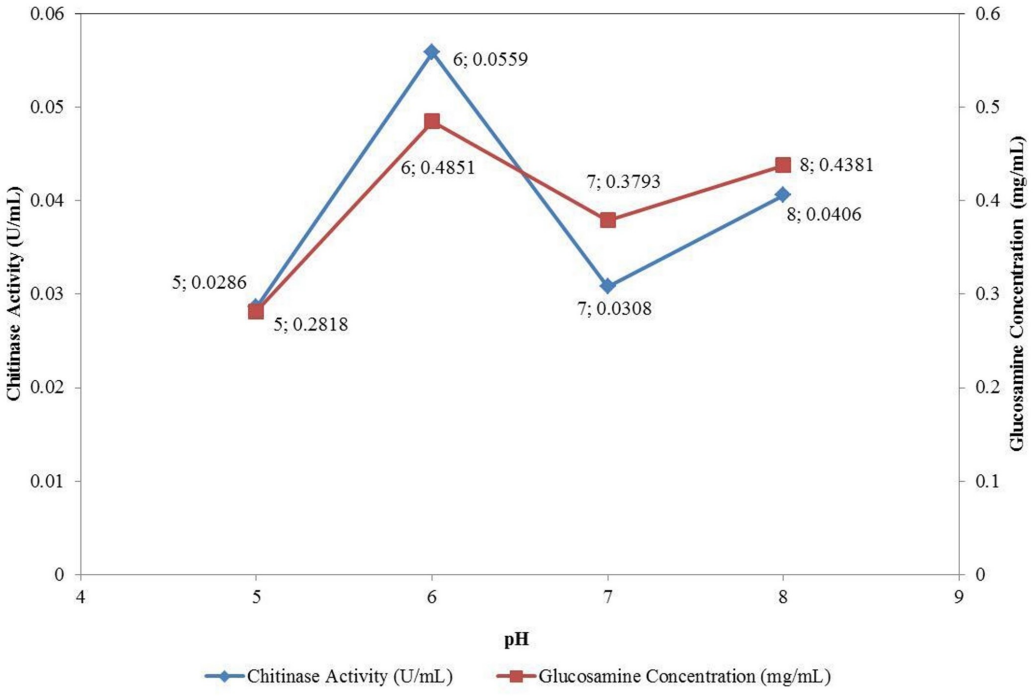

Figure 6. Fermentation Results at Different pH Values

glucosamine and 0.0145 U/mL for chitinase activity.

Fermentation was conducted on liquid chitin colloidal media for comparison, yielding comparable results. Specifically, glucosamine and optimal chitinase activity were obtained on fermentation day 7, at pH 6, and 30°C, with the values of 0.1782 mg/mL and 0.000868 U/mL, respectively. The assessment of glucosamine in the fermented products was conducted under optimal conditions of time, pH, and temperature, as illus-

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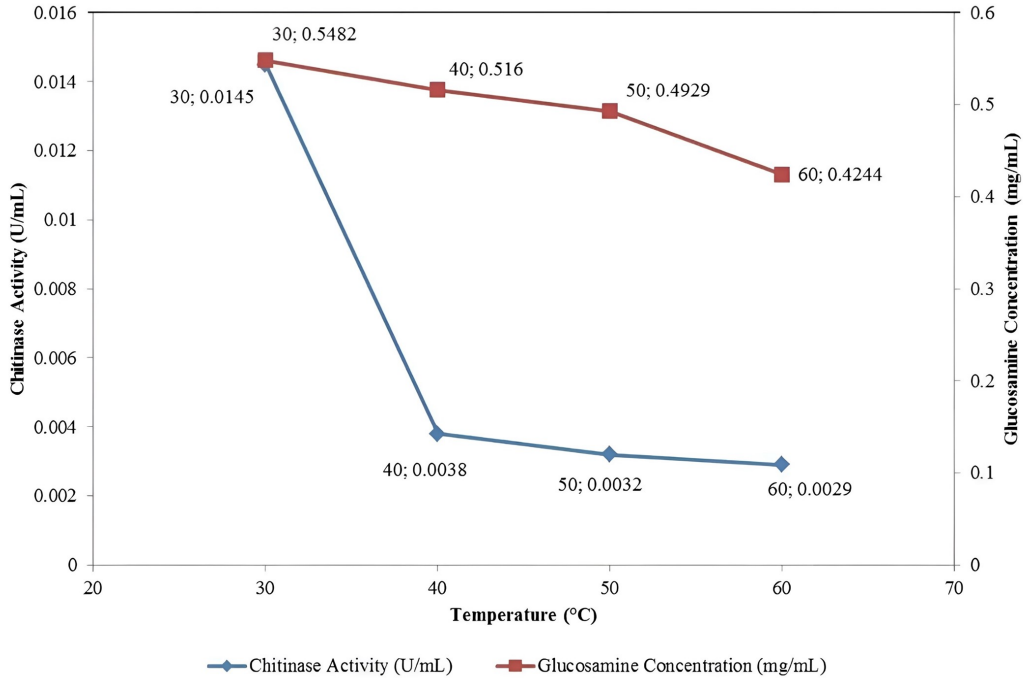

Figure 7. Fermentation Results under Varying Temperature Conditions

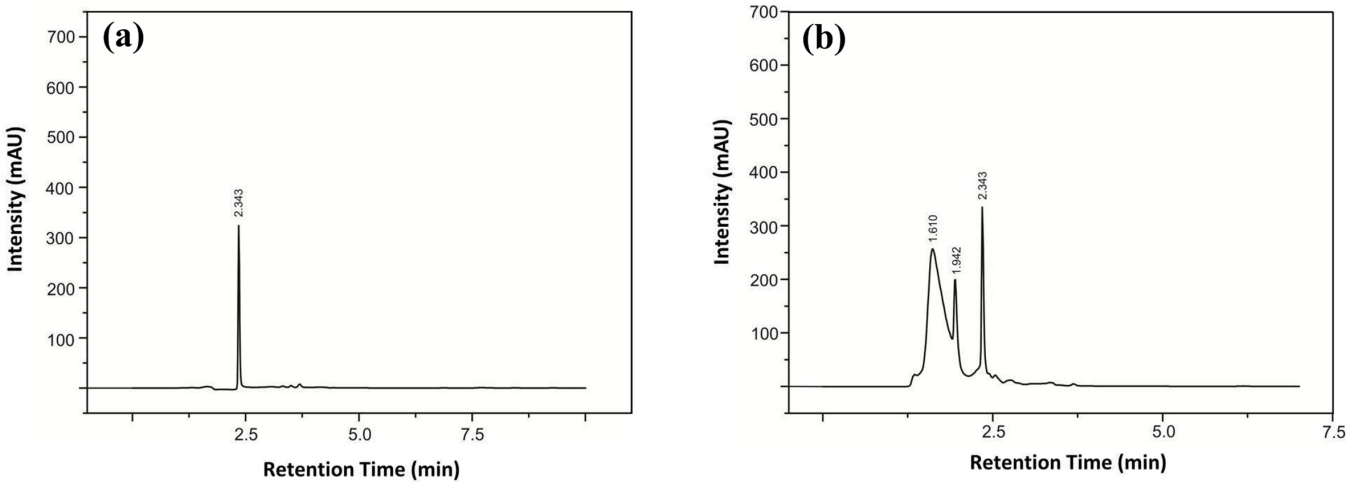

Figure 8. HPLC Chromatogram of (a) 500-ppm Glucosamine Standard and (b) H6 Extract Obtained at pH 6 and 30°C

trated in Figure 8. HPLC analysis was carried out on the 6th-day fermented extract at pH 6 and a temperature of 30°C ± 2°C. Through comparisons with standard glucosamine-HCl with Rt 2.343 (Figure 8a), the degradation results of isolate 18D36-A2 also showed the same HPLC peak as the glucosamine standard at Rt 2.343 (Figure 8b), indicating the success of the bioconversion process of shrimp shells into glucosamine by 18D36-A2. The shrimp shell substrate used was 10 g. The SSF results were extracted with seawater until a volume of 50 mL was obtained. Furthermore, the glucosamine concentration of 0.86 mg/mL of glucosamine was determined in the fermented extract with a yield concentration was 0.43%.

To confirm the glucosamine compound, the MS analysis in positive mode obtained a spectrum indicating the presence of a glucosamine compound component at m/z 180 (Figure 9). After fragmentation at m/z 164, it is a glucosamine component with the loss of OH molecules which are shot with energy in the MS process (Figure 10). The pattern that characterizes glucosamine fragmentation from m/z 180 to m/z 72 is in accordance with research by Zhou et al. (2011) and Nurfikari and de Boer (2021).

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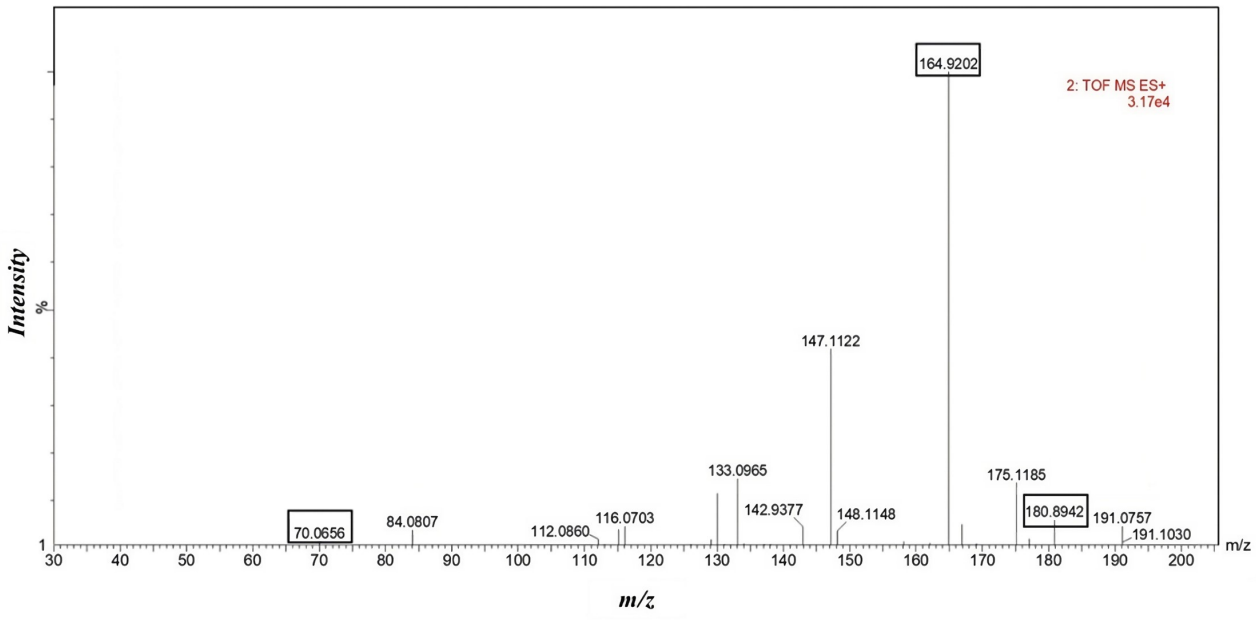

Figure 9. LC-MS/MS Analysis of Glucosamine

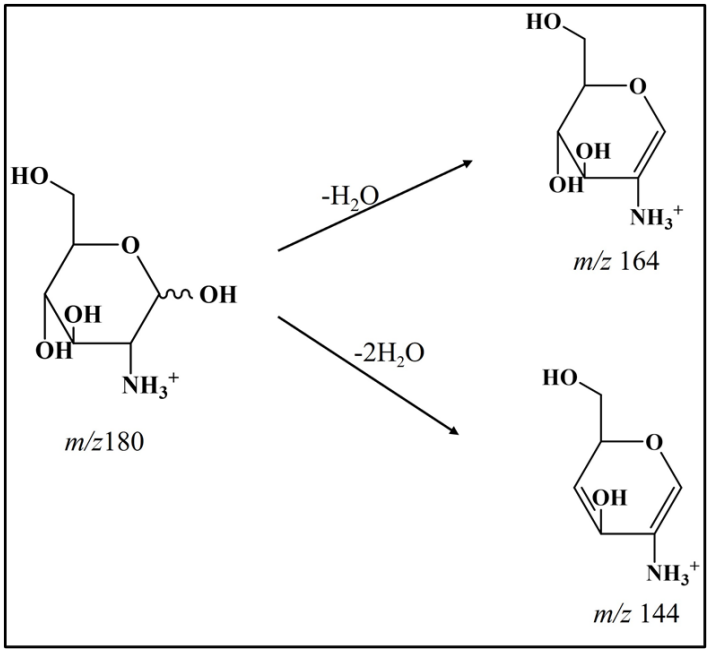

Figure 10. Illustration of the Glucosamine Fragmentation Pattern Model

4. CONCLUSIONS

The SSF of the novel marine tunicate-associated *Micrococcus unila* 18D36-A2 on shrimp shell medium has never been reported before. Our knowledge of the ability of microorganisms to manufacture enzymes capable of chitin breakdown from SSW into derivative chemicals through SSF is improved by this work. As indicated by this study, actinomycetes derived from tunicates exhibit robust growth on selective media comprised of SSW during the fermentation process, leading to glucosamine production. This information is vital for future research on developing medicinal preparations from natural waste products.

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