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Antifungal Agent Chitooligosaccharides Derived from Solid-State Fermentation of Shrimp Shell Waste by *Pseudonocardia antitumoralis* 18D36-A1

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Abstract: Shrimp shell waste is a potential source of the biopolymer chitin. Through fermentation, chitin can be converted into its derivative products. This study aimed to isolate and characterize the products of the biodegradation of chitin from shrimp shell waste through a solid-state fermentation process using actinomycetes. Actinomycete isolates were obtained from tunicate marine biota collected from the waters of Buleleng, Bali, using a dilution technique on 1% chitin colloid agar medium. The isolated actinomycetes were cultivated on a shrimp shell waste medium for 7 days, and then the products of the biodegradation of the oligomers were extracted using water. The extracts of the biodegradation products of the shrimp shells were isolated through several chromatographic steps and analyzed using LC–MS–MS, and the bioactivity of the biodegradation products against fungi was tested. The morphological observations and phylogenetic analysis showed that the isolate 18D36-A1 was a rare actinomycete with the proposed name *Pseudonocardia antitumoralis* 18D36-A1. The results of the analysis using TLC showed that the solid-state fermented water isolate 18D36-A1 produced several oligomeric components. These results indicate that the isolate 18D36-A1 was able to convert chitin into chitooligosaccharides. Further isolation of the extract produced the active fraction D36A1C38, which can inhibit the growth of fungi by 74% at a concentration of 1 mg/mL. This initial information is very important for further studies related to the development of a solid-state fermentation process for obtaining bioactive compounds from shrimp shell waste.

Keywords: actinomycetes; biodegradation; chitooligosaccharides; fungicide; shrimp shell waste; solid-state fermentation

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1. Introduction

Shrimp shell waste from the seafood industry is a potential source of chitin, which can be converted into its derivative products [1]. The amount of shrimp shell waste is increasing, along with the increasing demand for seafood. Indonesia is one of the countries that rely on oil and gas as their main export commodities [2]. The shrimp-processing businesses in Indonesia have a production capacity of around 500,000 tons per year [3]; 90% of the total shrimp production is exported in the form of frozen shrimp without heads and shells. The volume of waste produced globally can reach 15 million tons per year [4].

However, the use of this waste as a source of chitin in an environmentally friendly and sustainable manner has not been studied, and most of this waste causes environmental pollution. Therefore, it is necessary to process chitin in an effort to utilize shrimp shell waste, in order to obtain products that have high added value [5].

Chitin has recently come to be considered an important renewable marine resource due to its abundance as a waste product from the seafood-processing industry. The availability of chitin is estimated to be more than 100 billion tons per year [6]. Chitin has biocompatible properties but has the disadvantage that it is not soluble in water, which limits its uses [7]. Chitin-derived products in their oligomeric form have been reported to have varied applications. The conversion of chitin into oligosaccharide products is currently dominated by the use of strong acids/bases to break the β-1,4-glycosidic linkages [8]. In addition to using chemicals, there is potential for the synthesis of oligomeric compounds with specific structures, using enzymatic synthesis methods. However, the enzyme method requires relatively expensive enzyme biomaterials, and large-scale synthesis is not economically feasible [9]. Therefore, the development of environmentally friendly techniques for utilizing chitin derivatives is urgent. The biotransformation of shrimp shell waste by microorganisms is one viable alternative that should be further developed.

The solid-state fermentation method is an efficient, inexpensive, and environmentally friendly technique for converting organic waste into its derivatives [10]. To date, this technique has been widely used when combining agricultural waste raw materials with microorganisms such as fungi [11], bacteria [12], and actinomycetes [13]. The utilization of actinomycetes from the sea as a decomposer of shrimp shell waste into oligomer products is still very limited. Actinomycetes are known to decompose organic matter because they are able to produce various kinds of enzymes; one is chitinase, which is very suitable for breaking down biopolymers in nature into simple forms [14]. Several chitinase-producing isolates are *Thermobifida fusca* [15], *Saccharothrix yanglingensis* [16], and *Streptomyces alfalfae* [17].

As described above, this study aimed to evaluate the conversion of shrimp shell waste into chitooligosaccharides by actinomycetes using solid-state fermentation techniques. This study investigated the inhibitory effects of the bioactive products from the biotransformation of shrimp shell waste on the growth of the fungus *M. globosa*. The data obtained from this study are very important as initial information for the development of organic waste-fermentation techniques for the production of bioactive compounds with high added value based on shrimp shell waste.

2. Materials and Methods

2.1. Preparation of Shrimp Shell Waste and Colloidal Chitin

A total of 10 kg of fresh shrimp shell waste was obtained from the auction warehouse, Lempasing Bandar Lampung. Shrimp shells were washed under running tap water and then dried in an oven at 65 °C overnight. The stock of dry shrimp shells was stored in plastic bags in a refrigerator for further experiments. For the preparation of colloidal chitin, 10 g of dried shrimp shells were dissolved in concentrated HCl (65 mL) and stirred for 2 h. The resulting clear solution was poured into distilled water (400 mL) and stirred rapidly until colloidal chitin was formed. The colloidal chitin was separated by centrifugation at 7000 rpm for 10 min at 4 °C. The precipitate was washed with distilled water several times until a pH of 2.0–3.0 was reached. The colloidal chitin precipitate was neutralized with 1 N NaOH; the suspension was centrifuged at 7000 rpm (10 min) and then washed 3 times with distilled water. The resultant colloidal chitin samples were stored at 4 °C for further experiments [13].

2.2. Isolated Actinomycetes

Actinomycetes were isolated by serial dilution and the spread plate technique. Briefly, samples of marine organisms (sponges and tunicates) were collected from the Buleleng waters of Bali in 2018 at coordinates 8°07′20.9″ S 114°34′03.8″ E by SCUBA divers at

sea depths of 5−25 m. Small pieces of several items of marine life were ground with a sterile mortar; then the liquid was diluted (10^{-3}−10^{-6}) and enriched in a Petri dish using 1% chitin colloid as a selective medium. Incubation was carried out for 14 days at 30 °C. The actinomycetes observed were taken and then enriched again until pure isolates were obtained. The single isolates were deposited in the Technical Service Unit, Integrated Laboratory of Innovation and Technology Center (UPT LTSIT), Lampung University, and maintained in 1% colloidal chitin medium in artificial seawater.

2.3. Solid-State Fermentation

The solid-state fermentation was carried out referring to the method of Setiawan et al. [13]. Briefly, actinomycetes were inoculated on 25 mL of 1% liquid chitin colloid medium and incubated for 7 days. Next, the inoculum was poured into 50 g of sterile shrimp shell waste in a 500 mL Erlenmeyer flask and incubated for 6 days. The degraded shrimp shells were extracted using 100 mL of distilled water twice and then concentrated using a rotary evaporator at a pressure of 23 mbar, at 40 °C. Then, absolute EtOH was added in a ratio of 1:3 to precipitate the chitooligosaccharides (COS). The precipitate was then separated using a centrifuge at 7000 rpm for 10 min at 4 °C. The precipitate, as a product of degradation, was stored in a freezer. The components in the extract were analyzed using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

2.4. Extraction and Antifungal Activity

The initial stages of the components of the degraded extract were analyzed using TLC with a glucosamine standard as a reference. The TLC was carried out with the stationary phase in the form of a SiO_2 plate and the mobile phase in the form of isopropyl alcohol:H_2O:25% NH_3 with a ratio of 7:2:1; the components were visualized and identified using Ninhydrin reagent. The chitooligosaccharides were isolated using an open SiO_2 column method with the same solvent under isocratic TLC conditions. Then, the results of the isolation were tested for antifungal activity against *Malassezia globosa* using the microtiter 96-well plate method using the Hospitex Plate Reader, referring to the modifications of Leong et al. [18]. The antifungal test results are expressed as the percent inhibition according to the following equation:

$$\% \text{ inhibition} = GC - GT/GC \times 100\%$$

where GC is the growth control and GT is the growth treatment with the sample.

2.4.1. Microscopic Analysis of Fungicidal Activity

To verify the bioactivity of the degradation product of the 18D36-A1 isolate, *M. globosa* was observed using an Apotome Microscopy Zeiss Axio Imager Z2 and SEM EVO MA 10 (Carl Zeiss, Oberkochen, Germany). In the early stages, the *M. globosa* population was observed for spore germination by transferring 20 µL of the 48 h incubation products onto a glass slide. The sample was observed at 400× magnification (M). Conidia were considered germinated if the length of the germ tube was wider than the conidium and the active component resulted in a reduction in the population of *M. globosa*. Further analysis used SEM to visualize the surface morphology of the hyphae of *M. globosa* and to determine the possible mechanism between the active fraction and *M. globosa* cells. For microscopic analysis, the 96-well plates used were modified with carbon tape. The wells contained a mixture of 100 µL of extract and 100 µL of *M. globosa* (10^5 spores/mL). Wells containing *M. globosa* (10^5 spores/mL) were used as a control. At the edge of the well, sterile carbon tape was attached. The plate was then incubated for 48 h at 30 °C. Prior to SEM analysis, the carbon bands with adhering hyphae were carefully removed and washed three times with 0.1 M phosphate-buffered saline (PBS). The samples were fixed with 10% (*v/v*) formaldehyde overnight at room temperature [19], then rinsed three times with 0.1 M PBS, and dehydrated in graded ethanol starting from 30% for 10 min, 50% for 10 min, 70% for 10 min, 90% for 10 min, and 100% for 1 h. The samples were air-dried, mounted

on aluminium stubs using double-sided carbon tape, coated with gold, and visualized using SEM.

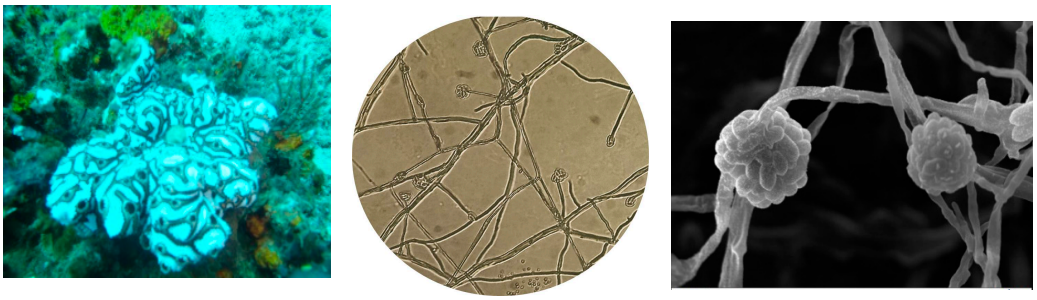
2.4.2. Liquid Chromatography–Mass Spectrophotometry

The oligomer compounds produced by the isolate 18D36-A1's fermentation of shrimp shell waste were analyzed using LC–MS–MS in positive mode. The active fraction was dissolved in water and subjected to LC–MS–MS analysis, for which the ACQUITY UPLC® H-Class System (Waters, Beverly, MA, USA), ACQUITY UPLC® HSS C_{18} column (1.8 µm, 2.1 mm × 100 mm) (Waters, Beverly, MA, USA), and Xevo G2-S Qtof Mass Spectro (Waters, Beverly, MA, USA) were used.

3. Results and Discussion

3.1. Isolation of Actinomycetes

The actinomycete 18D36-A1 was isolated from an unidentified tunicate, as shown in Figure 1a. Observations using light microscopy showed that the isolated strain 18D36-A1 had both the aerial mycelium and the substrate mycelium, as shown in Figure 1b. The morphological analysis of isolate 18D36-A1 (Figure 1c) showed the spore form of sporangia to be spherical, with many bent and coiled substrate mycelia, which are a special feature of the Pseudonocardiaceae family [20].



(**a**) (**b**) (**c**)

Figure 1. (**a**) Unidentified tunicate 18D36; (**b**) isolated actinomycete 18D36-A1 under the microscope imager at 400× magnification; (**c**) scanning electron micrograph of aerial mycelia and spore chains of strain 18D36-A1 with 2000× magnification after incubation for 14 days in 1% chitin agar medium at 29 °C.

For further analysis, the 16S rDNA sequencing results for 18D36-A1 were exported to the database and checked for homologous alignment. Based on the alignment results, strain 18D36-A1 was found to be *Pseudonocardia antitumoralis*, which showed 99.93% similarity. The strain was named *Pseudonocardia antitumoralis* 18D36-A1. The partial 16S rDNA sequence was deposited in GenBank under the accession number LC578481. Actinomycetes have been known to show antagonistic effects on various pathogens due to their ability to produce bioactive compounds. Actinomycetes also produce chitinase [21] and can break down chitin waste into its derivatives, with various bioactivities. However, there is currently no research on rare actinomycetes such as *Pseudonocardia antitumoralis* 18D36-A1, which decomposes shrimp shell waste into chitin derivatives, especially chitooligosaccharides, which are antifungal agents.

3.2. Solid-State Fermentation

The process of the degradation of shrimp shells by *P. antitumoralis* 18D36-A1 was observed by SEM, and the chitin degradation products were analyzed by HPLC as shown in Figure 2. The observations of the shrimp shells in the initial state showed a smooth texture and no damage (Figure 2a). Furthermore, as shown in Figure 2b, *P. antitumoralis* 18D36-A1 was observed to be growing on the surfaces of the shrimp shells with abundant substrate mycelium, and spores were produced on Day 6. Large pores indicated the

presence of chitinase enzymes that degrade shrimp shell waste, as shown in Figure 2c [22]. The HPLC analysis, shown in Figure 2d, used a blank of non-fermented actinomycetes, which produced no degradation products. To determine the pattern of the degradation of shrimp shells by *P. antitumoralis* 18D36-A1, a glucosamine standard was used, as shown in Figure 2e, and a sharp chromatogram peak was seen at a retention time of 2.5 min. Furthermore, the results of the analysis of the extract from the degradation of shrimp shell waste by *P. antitumoralis* 18D36-A1 on Day 6 in Figure 2f showed a chromatograph peak at retention times of 2.6, 3.1, and 3.4 min, indicating the presence of an oligomeric component in the degraded shrimp shell.

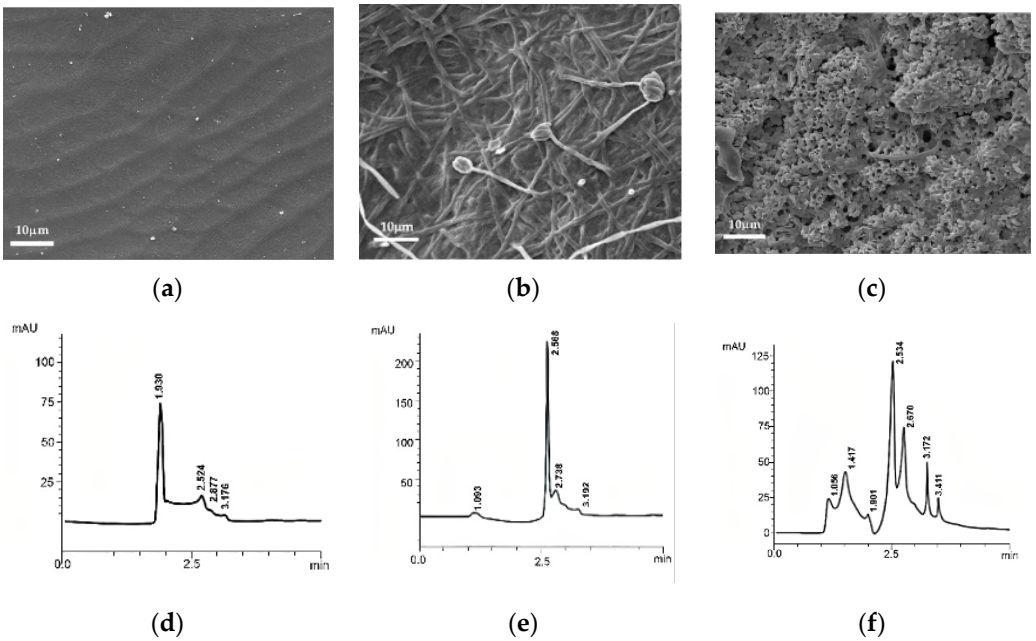


Figure 2. (**a**) Morphology of shrimp shell waste; (**b**) actinomycete growth on shrimp shell substrate; (**c**) shrimp shell degradation; (**d**) shrimp shell waste as blank; (**e**) GlcN standard; (**f**) chromatogram of products of shrimp shell waste degradation.

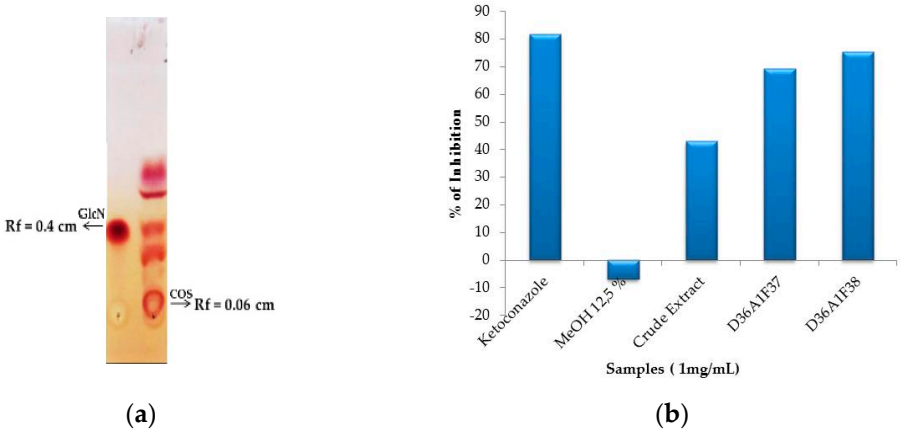
It is known that actinomycetes are decomposer microorganisms [23]. Actinomycetes are able to break down chitin into its derivative products such as monomers and oligomers, because the majority of actinomycetes can excrete GH18-family chitinases [24]. Furthermore, Lu et al. [25] stated that actinomycetes can degrade chitin into products such as GlcN, GlcNAc, and their oligomers.

3.3. Extraction and Antifungal Activity

The products of shrimp shell waste fermented using the isolate *P. antitumoralis* 18D36-A1 were extracted using distilled water to obtain a crude extract of D36A1 (3.2 g). The TLC test results for the crude extract D36A1, which were compared with the standard GlcN, showed that the standard glucosamine had an Rf value of 0.4 cm, while the D36A1 extract showed an oligomeric component with an Rf value of 0.06 cm, as shown in Figure 3a. Then, the crude extract D36A1 was fractionated through several chromatographic steps to obtain the fractions D36A1C37 (7 mg) and D36A1C38 (46 mg). The results of the activity test at a concentration of 1 mg/mL showed that the crude extract of D36A1 inhibited the growth of *M. globosa* by 43%, while the fractions D36A1C37 and D36A1C38 were able to inhibit growth by up to 69% and 74%, respectively, as shown in Figure 3b.

Based on De Oliveira et al.'s [26] general classification of antimicrobial agents by potency, 50% inhibitory concentrations in the range 50−500 µg/mL are considered to represent strong activity, while those in the range 600−1500 µg/mL indicate moderate activity. Meanwhile, according to the results of previous reports, chitosan and its derivatives, at a concentration of 1 mg/mL, were able to completely inhibit *Usilago maydis* [27]. Furthermore,

it was reported by da Silva et al. [28] that the MICs of COS were 1−2 mg/mL for the yeasts *C. albicans* and *C. tropicalalis*. However, the ability of COS to inhibit the growth of *M. globosa* has not been reported. To verify the inhibitory ability of the active fraction D36A1C38 against *M. globosa*, it was visualized using a microscopic method.



(**a**) (**b**)

Figure 3. (**a**) TLC identification of glucosamine (GlcN) and oligomer products (COS); (**b**) antifungal activity of D36A1, D36A1C37, and D36A1C38 against *M. globosa*.

3.4. Characterization

3.4.1. Analysis of Fungicidal Activity

Observations using apotome microscopy showed population differences between the growth controls (Figure 4a) and the effect of adding 1 mg/mL of D36A1C38 (Figure 4b). It can be seen that, in the growth control, there are branching spores and hyphae in Figure 4a, whereas in Figure 4b, there is no hyphal branching. Furthermore, observations using SEM showed that the growth of *M. globosa* cells was normal, with a smooth hyphal consistency and hyphal elongation being observed (Figure 4c). However, in Figure 4d, wrinkled hyphae can be observed, indicating cell damage as a result of the addition of the active fraction D36A1C38. This activity is in accordance with COS mechanism as an antifungal agent, causing the fungal mycelia to wrinkle and the cells to become cracked. COS also increases cellular permeability, leading to significant leakage of cellular components, resulting in membrane damage. These characteristics indicate that COS has an inhibitory effect on fungi by acting on cell walls and membranes, and changes cell metabolism [29].

This study reports, for the first time, the antifungal activity of the products of the degradation of shrimp shell waste using *Pseudonocardia antitumoralis* 18D36A1. The results of the microscopic study also reveal that the mycelium of *Malassezia globosa* exposed to chitooligosaccharides appeared dead and deformed. The presence of COS in the growth medium impaired the integrity of the cell membrane, and was also able to induce fungal death to various degrees in a concentration-dependent manner [30].

3.4.2. Analysis of FTIR and Liquid Chromatography–Mass Spectrophotometry

The results of the FTIR analysis of the D36A1C38 oligomer showed the presence of an acetylation group indicated by absorption at 1640 and 1558 cm^{-1} due to the tensile vibrations of the C=O (amide I) and C-N (amide II) groups, respectively, and the main characteristic of the presence of acetylation in COS was also shown by absorption at 1371 cm^{-1} (amide III). There is also a specific absorption peak of 1028 cm^{-1} according to the polysaccharide structure and the β-1,4-glycoside bond in the COS structure according to Vasilieva et al. [31], as shown in Figure 5.

In the LC–MS–MS analysis, the oligomer products resulted in major MS–MS spectra indicated by ion peaks at *m/z* 341 for $[(GlcN)_2+H]^+$, *m/z* 522.5 for $[(GlcN)_2\text{-}(GlcNAc)\text{-}H_2O\text{-}NH_3]^+$, and m/z 726 for $[(GlcN)2\text{-}(GlcNAc)_2\text{-}H_2O]^+$, and an ion peak at *m/z* 1060 with the pattern $[(GlcN)_4\text{-}(GlcNAc)_2\text{-}3H_2O\text{-}NH_3]^+$. A sequential termination pattern between the oligomers formed by breaking one GlcN unit from the parent ion produces a B-ion-type

pattern. As shown, the MS–MS spectrum indicates the loss of NH_3 and H_2O molecules, or it could represent a loss of the combination of the two functional groups. Both IR and ESI-TOF–MS data confirmed the presence of acetylation in the chitohexaose chain obtained, namely, chitohexaose. In addition, the ionic fraction at *m/z* 1060 with a base peak of *m/z* 726 is the main feature of (GlcN)4-(GlcNAc)2 (D4A2) di-N-acetylchitohexaose (Figure 6). Based on the interpretation of the IR and LC–MS–MS spectra, a chitooligosaccharide structure with DP 6 was proposed, as shown in Figure 7, in agreement with Li et al. [32] and Kang et al. [33].

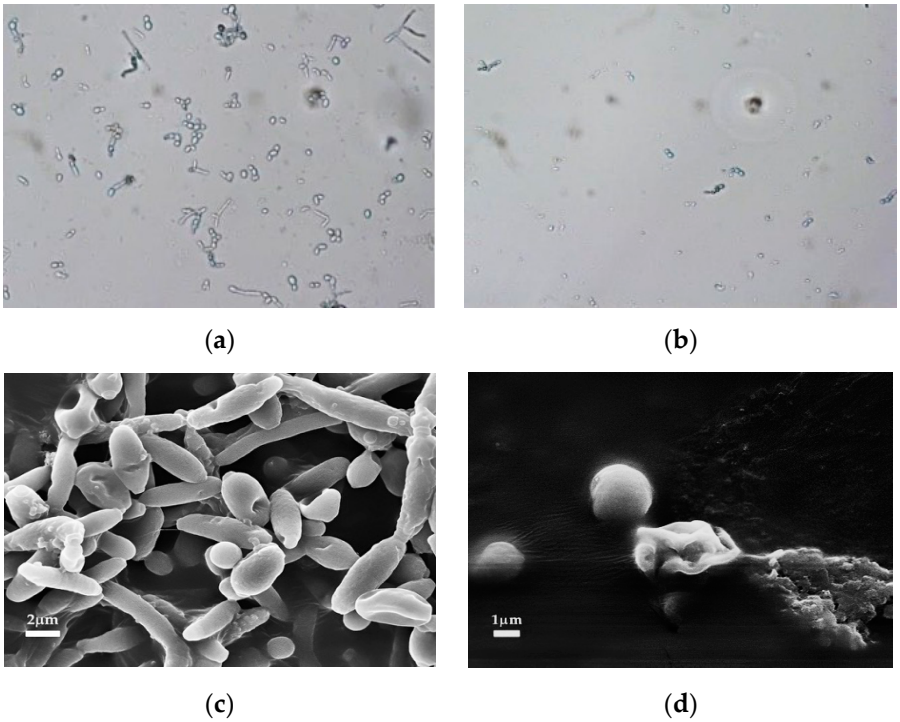


Figure 4. (**a**) *M. globosa* growth control, 400×; (**b**) effect of COS on the growth of *M. globosa*, 400×; (**c**) morphology of *M. globosa*, 15,000×; (**d**) morphological damage of *M. globosa*, 15,000×.

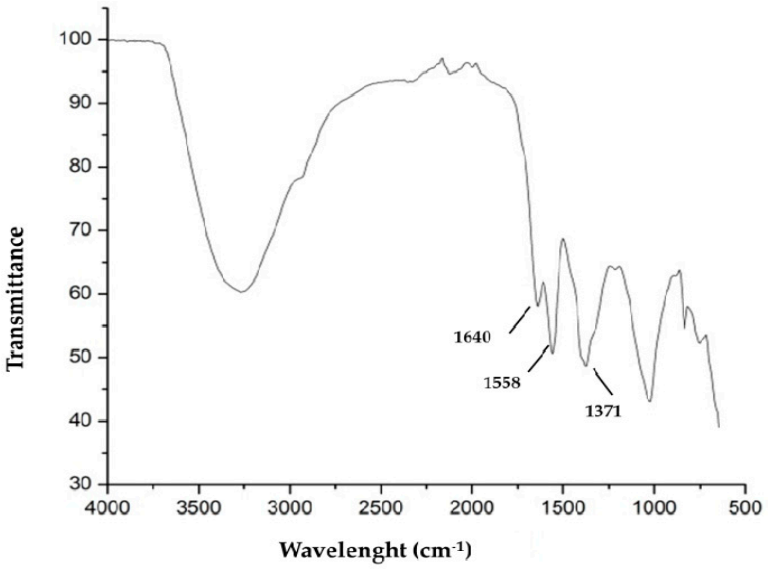


Figure 5. FT-IR spectrum of the chitooligosaccharides of D36A1C38.

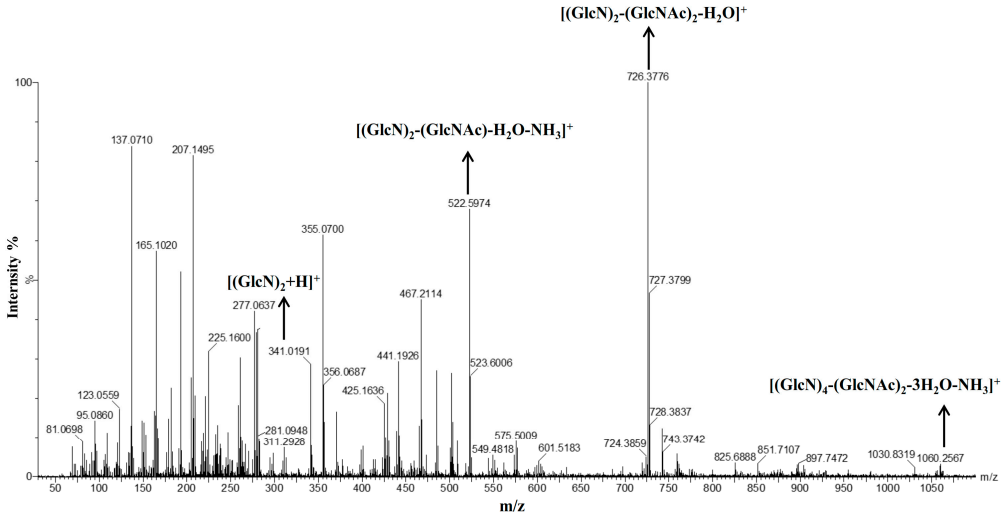


Figure 6. MS–MS analysis of the chitooligosaccharides of D36A1C38.

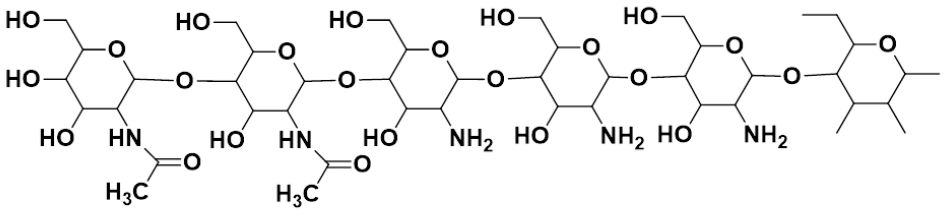


Figure 7. Structure of COS with DP 6.

In the MS data, it can be observed that there is a split pattern with OH and NH_2 ions, and, specifically, the C ion, which splits D4A2 into DD and AADD. This report is consistent with Li et al. [32], who successfully identified COS DP 6 by utilizing the chitinase enzyme from *Paenibacillus pabuli*, with DP 4 predominance in the MS results. This is in accordance with Tokuya et al. [34]; based on the pattern of the dimeric peak ion of glucosamine at *m/z* 341, it can be stated that glucosamine at the reducing end prefers to be acetylated. Based on this pattern, this type of actinomycete is indicated to have the -N-acetylhexosaminidase (EC 3.2.1.52) type of enzyme, which is exo-acting and cleaves chitin oligomers, and is the only enzyme that can cleave diacetylchitobiose at the non-reducing end; repeated hydrolysis occurs [35]. This indicates that the isolate 18D36A1 had the characteristics of chitinase A.

The antifungal activity observed in this study proves that the chitohexaose mechanism occurs because the -NH_2 group of COS can be transformed into an ammonium group upon absorbing H^+. It is then attracted to the negative charge on the fungal cell wall, resulting in cell damage that causes lysis. It is also capable of binding to the negatively charged carboxyl groups of the cell membrane components of microorganisms. In addition to antifungal activity, chitohexaose was also reported to have the strongest inhibitory effect on the proliferation of A549 human lung cancer cells. Chitohexaose was also found to be an immunomodulatory agent.

4. Conclusions

To our knowledge, this is the first report on the biotransformation of shrimp shell waste using actinomycetes to produce COS that has antifungal activity against *M. globosa*. This study provides an understanding of the potential to convert shrimp shell waste into a high-value-added antifungal agent. Moreover, this initial information will be very important for further research related to the development of high-potential products from food waste through solid-state fermentation.

Author Contributions: Conceptualization, A.S., M.A. and J.H.; methodology, W.W., F.S. and A.S.; formal analysis, W.W., W.A.S., A.I. and C.A.A.; investigation, W.W., W.A.S. and F.S.; writing—original draft preparation, W.W., F.S. and A.S.; writing—review and editing, M.A., J.H. and N.L.G.R.J.; supervision, N.L.G.R.J. and A.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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