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Syncephalastrum racemosum AND *Rhizopus arrhizus* ISOLATED FROM OIL PALM TRUNKS PRODUCE CHITOSANS THAT INHIBIT THE GROWTH OF *Ganoderma boninense*

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ABSTRACT

Chitosan has been proposed as a treatment to suppress Ganoderma infection on oil palm. Syncephalastrum racemosum and Rhizopus arrhizus, growing on oil palm trunk, were examined on their ability to produce chitosan. Chitinous materials from both fungi were deproteinised and deacetylated using different concentrations of sodium hydroxide (NaOH). Chitosans from R. arrhizus (FuCsRa), S. racemosum (FuCsSr) were compared with crab shells' chitosan (CrCs) through the Fourier-transform infrared spectroscopy (FTIR) analysis. The deacetylation degrees of different chitosan sources were also determined. FTIR analysis showed that chitosans produced by these fungi and crab shell's chitosan had a similar pattern of FTIR spectrum, but differed in their deacetylation degrees. The next experiment examined the ability of these chitosans to suppress the growth of Ganoderma boninense (G. boninense) in vitro. The experiment consisted of 11 treatments: controls, media with CrCs (at 5000 ppm, 7500 ppm, 10 000 ppm), media with FuCsRa (at 5000 ppm, 7500 ppm, 10 000 ppm), media with FuCsSr (at 5000 ppm, 7500 ppm, 10 000 ppm), and antagonistic test (S. racemosum; R. arrhizus). The results showed that CrCS at 5000 ppm, 7500 ppm and 10 000 ppm enhanced the growth of G. boninense. A concentration of 10 000 ppm of CrCs nearly doubled the growth of Ganoderma compared to control. FuCsSc inhibited the growth of G. boninense at all concentrations from -28% to 31% compared to control. The in vitro antagonistic effect of S. racemosum showed a high effect at inhibiting the growth of G. boninense (-43% compared to control). This in vitro study demonstrated the ability of chitosan extracted from fungi growing on oil palm trunks to suppress Ganoderma growth.

Keywords: chitosan, crab shells, *Ganoderma boninense*, oil palm trunk fungus.

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INTRODUCTION

Ganoderma boninense (G. boninense) is a lethal disease that affects the growth of oil palm trees. It is a severe disease in oil palm plantations in North Sumatra, Indonesia, as *Ganoderma* attacks can be severe and harmful. When *Ganoderma* infects a young oil palm, its growth may be severely affected and may lead to its death. Oil palm farmers have tried various products to control the problem. Many commercial products were offered with the promise of controlling *Ganoderma*. However, there is no scientific study on the efficacy of such products. One belief among oil palm growers is that chitosan can suppress *Ganoderma* attacks.

Chitosan is a biopolymer that has a similar structure to cellulose and can form a membrane. Chitosan has a hydroxyl and amino reactive group which allows it to be chemically modified, such as membrane. Chitosan can be found in nature, at the exoskeleton of crustaceans, mollusks, annelids, coelenterates, and insects. Some fungi have

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the potential to produce chitosan. The fungus *Syncephalastrum racemosum* was found to produce the highest concentration of chitosan (152 mg g^{-1} mycelium) compared to nine other strains (Amorim *et al*., 2003). *Rhizopus arrhizus* SIS 30 can produce chitosan as much as 12.56 mg g^{-1} mycelium, which is below the production of chitosan from *Cunningamella elegans* SIS 41 (15.63 mg g^{-1} mycelium) (Berger *et al*., 2018). A study has found that the addition of chitosan to artificial media could inhibit the growth of phytopathogenic *Colletotrichum* species (Berger *et al*., 2018).

Sabrina *et al*. (2018) found that *S. racemosum* and *R. arrhizus* can be isolated from chipped oil palm trunks. This study aimed to measure the ability of these two fungi to produce chitosan and investigate if they can be used to inhibit the growth of *Ganoderma*.

MATERIALS AND METHODS

Experimental Site and Treatment Application

This study was conducted at the University of Sumatera Utara from March to November 2018. *Rhizopus arrhizus strains* ATCC 111450, and *Syncephalastrum racemosum* isolates VPCI 1857/11(identified using DNA marker) were isolated from pieces of oil palm trunk from Labuhan Batu district in North Sumatera Province, Indonesia. The trunk has C= 29.64%, N = 0.53% and C/N = 55.9 (Sabrina *et al*., 2018). Crab shells were obtained from a crab farm in Langkat district. *G. boninense* was from the plant protection laboratory collection of the Faculty of Agriculture, University of North Sumatra.

Extraction of Chitosan and Analysis

The preparation began with washing of the raw crab shell waste with water repeatedly. The shell was dried under the sun until it was completely dry. The dried shell was ground with an electric blender and passed through 50 mesh sieves. The crab shell powder that passed through the 50 mesh sieve was dried in an oven at 60ºC for 6 hr. Extraction of chitosan from the crab shells included deproteinisation with 4% (w/v) sodium hydroxide (NaOH) (1:10, 80ºC 1 hr), demineralisation with 150 ml 1M hydrochloric acid (HCl) (1:15), depigmentation with 4% (w/v) sodium chloride (NaCl), deacetylation 40% (w/v) NaOH (1:15, 80ºC 1 hr).

Isolated *R. arrhizus* and *S. racemosum* were cultivated on Patato Dextrose Agar (PDA) at 28ºC for seven days. A suspension of each strain was prepared and adjusted to contain 10^7 sporangioles ml^{-1} using a hemocytometer. A 1 ml suspension was inoculated to sterile petri dishes containing PDA and maintained for 24 hr at 28ºC. Cultures were sub-cultured on PDA plates, incubated at 28°C for seven days, then cultivated in liquid PDA separately. Mycelia from both fungi were harvested by filtration, washed with distilled water and freeze-dried. The dry weight obtained for *S. racemosum* was at 26 g 900 ml^{-1} and *R. arrhizus* at 28 g 900 ml^{-1} .

The chitosan from 3-10 g lyophilised mycelia was isolated following the procedures of Synowiecki and Al-Khateeb (1997). The overall process involved: deproteinisation with 2% w/v NaOH solution (30:lv/w; 90ºC, 2 hr), separation of the alkali-insoluble fraction (AIF) by centrifugation (4000 x g,15 min), extraction of chitosan from AIF under reflux (10% v/v acetic acid, 40: lv/w, 60ºC, 6 hr), and precipitation of chitosan from the extract at pH 9.0, adjusted with 4 M NaOH solution. Chitosan was washed on a coarse sintered glass funnel (G-4) with water, ethanol, and acetone and air-dried at 20°C.

The chitosans obtained from the crab shells and the fungi were characterised using FTIR a Shimadzu IR-Prestige 21. The materials were made into pellets with 1% KBr (Kaban, 2007).

The degree of deacetylation (DD) was calculated from FTIR spectra using the baseline method of Domszy and Rovert (Khan *et al*., 2002). The absorbance value was calculated using the formula:

$$A = \log (P_0/P)$$

P0:% transmittance at a minimum peak. P:% transmittance on the baseline. A comparison between absorbance at A = 1655 cm^{-1} (amide band absorption 1) with absorbance at A = 3450 cm^{-1} (hydroxyl group uptake) was calculated. The chitin N-deacetylation (100%) obtained from absorbance value at 1655 cm^{-1} was 1.33. The degree of N-deacetylation can be calculated by:

$$\%N - \text{deacetylation} = 1 - \frac{AA1655}{AA3450 \times 1.33} \times 100\%$$

A1655: Absorbance at a wavelength of 1588 cm^{-1} amide/acetamide for group absorption.
A3450: Absorbance at 3410 cm^{-1} hydroxyl (OH) wavelength.

The chitosans were also characterised in terms of colour, particle-size, odour, and pH. Colour was determined visually, particle-size was based on feel, while odour was based on smell. The pH was determined in a 1:1 soil- to-water suspension.

Inhibition test of growth of *G. boninense* by *S. racemosum's* chitosan (FuCsSr), *R. arrhizus's* chitosan (FuCsRa) and crab shell's chitosan and antagonistic test with *S. racemosum* and *R. arrhizus* fungi.

The inhibitory test had 11 treatments in a complete randomised design with three replications. The treatments were: Control (without treatment); media containing chitosan crab shell (CrCs) at 5000 ppm, 7500 ppm and 10 000 ppm; media containing chitosan *R. arrhizus* (FuCsRa) at 5000 ppm, 7500ppm,

10 000 ppm; media containing chitosan *S. rasemosum* (FuCsSr) at 5000 ppm, 7500 ppm, 10 000 ppm; and antagonist test with *S. racemosum* and *R. arrhizus*.

Dextrose agar media with FuCsSr, FuCsRa and CrCs were made by dissolving FuCs and CrCs. The FuCS solution was made in 1% (v/v) acetic acid and added to the PDA (pH adjusted to 5.6 with NaOH 30% (b/v) to obtain a final FuCsSr and FuCsRa concentration of 5000 ppm, 7500 ppm and 10 000 ppm (Berger *et al*., 2018).

Inhibitory tests were carried out by growing *G. boninense* on chitosan-treated media, and also on petri dishes with *S. racemosum* and *R. arrhizus* isolates. Isolated *S. racemosum* and *R. arrhizus* were placed at a distance of 1 cm from the edge of the petri dish, and the *G. boninense* colony was cultured on the opposite side with a distance of 1 cm from the edge of the Petri dish. All isolates were obtained from their respective cultures using cork borer with a diameter of 4.3 mm. The plates were incubated at room temperature. The antagonistic ability calculation used the inhibition zone formula. Observations were made at 1 day, 2 days, and 3 days after inoculation (DAI) on the inhibitory ability by measuring the areas of *Ganoderma* colonies using the formula $A = 2\pi r^2$, A = area; r = radius of *Ganoderma* colony.

RESULTS AND DISCUSSION

Chitosan Production from Crab Shells, *S. racemosum* and *R. arrhizus*

The characterisation of chitosan extracted from crab shells, *S. racemosum*, and *R. arrhizus* using FTIR is shown in *Figures 1, 2* and *3*. Most amide groups were converted into amine groups through the deacetylation process. The specific wavenumber at 1647.21 cm^{-1} in CrCs and both FuCs was the absorption of the C=O amide group left from chitin, indicating the presence of an acetyl group, which was reduced due to the process. The uptake of the methyl group ($-CH_3$) at wavenumber 1435.04 cm^{-1}

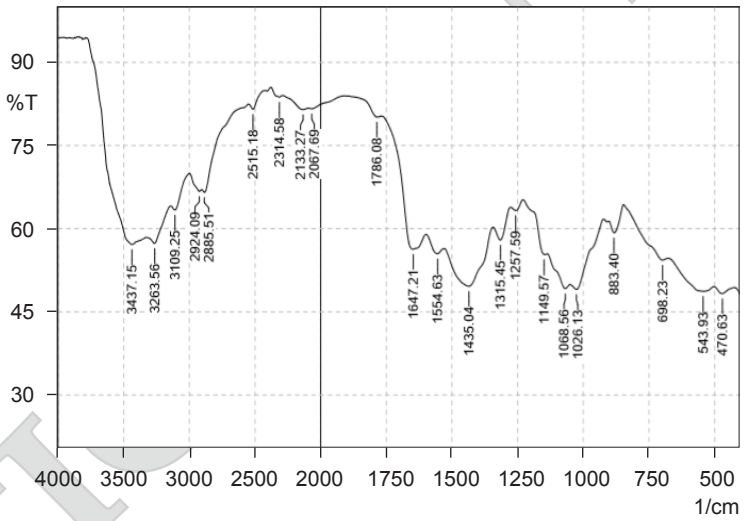

Figure 1. Chitosan spectrum origin of a crab shell.

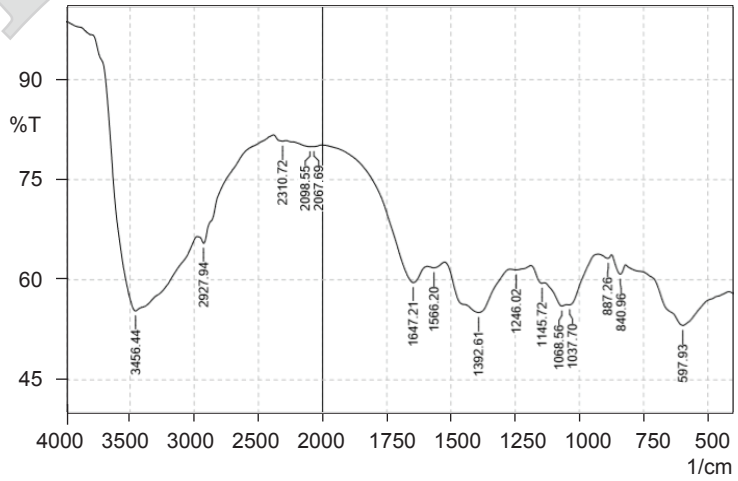

Figure 2. Chitosan spectrum origin of S. racemosum.

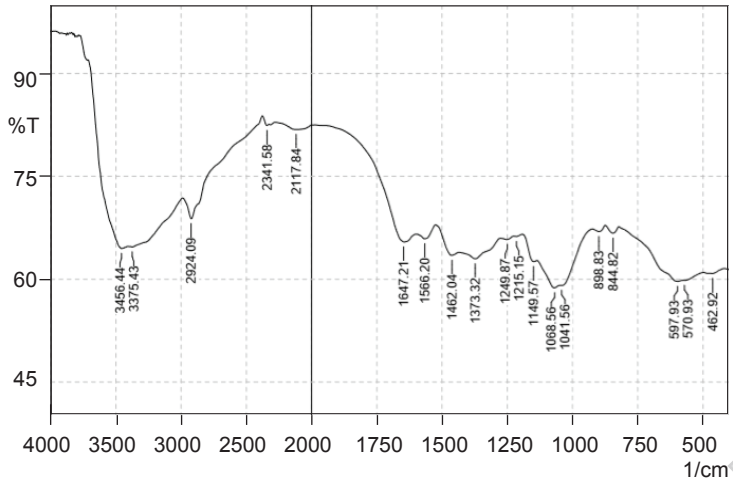

Figure 3. Chitosan spectrum origin of R. arrhizus.

confirmed the presence of an amide group from chitin (Puspawati and Simpen, 2010). Wavenumber 3437.15 cm^{-1} indicated that the absorption of O-H was overlapping with $–NH_2$ from amines. Flap $-NH_2$ absorption band and twist at 894.97 cm^{-1} confirmed that chitosan had been formed (*Figures 1, 2* and *3*).

The chitosan FTIR characterisation showed mixed results in all aspects of character testing. The chitosan was extracted from various sources showed diverse results. The best quality chitosan was extracted from *S. racemosum* with 52.50% deacetylation value, and the lowest was from *R. arrhizus* which was 29.10% (*Table 1*).

Characterisation of chitosan was carried out including colour, particle-size, aroma, pH, and deacetylation degree. The results showed that

TABLE 1. CHARACTERISATION OF CHITOSAN FTIR SPECTRUM OF CRAB SHELL, *S. rasemosum* AND *R. arrhizus*

No.	Vibration Modes	Wavelength, v cm^{-1}			
		Standard Chitosan*	Crab Shell	*S. rasemosum*	*R. arrhizus*
1	ν(NH2) assoc. in primary amines ν (OH) assoc. in pyranose ring	3 377.95	3 437.15	3 456.44	3 456.44
2	(vb) C-H Aliphatic	2 922.85	2 924.09	2 927.94	3 375.43
3	(vs) C-H Aliphatic	2 922.80	2 885.51	2 310.72	2 924.09
4	(vs) C-H Aromatic	2 361.41	2 314.58	2 098.56	2 431.58
5	(v) C=H Amide I band	1 660.55	1 647.21	1 647.21	1 647.21
6	(v) C=H) Amide II band amide II band	1 587.94	1 554.63	1 566.20	1 566.20
7	(v) C-H	1 422.73	1 435.04	1 392.61	1 462.04
8	(vs) C-O	1 259.54	1 257.59	1 246.02	1 249.87
9	(vs) C-O	1 154.64	1 149.57	1 145.72	1 149.57
10	v(C-O-C)	1 077.93	1 068.56	1 068.56	1 068.56
11	v(C-O-C)	1 026.63	1 026.13	1 037.70	1 041.56
12	ω β -1,4-glikosidik	897.41	883.40	887.26	898.83

Source: Dompeipen (2017).

chitosan with chitin deacetylation had a similar physical appearance. The differences were only apparent in the number of parameters. The extracted chitosans had a fine powder texture and coarse powder texture. The chitosan extracted from the crab shells gave out odour while chitosans extracted from the fungi were odourless. The pH level of all chitosans was alkaline with a value between 11.7 to 12.7 and had a degree of deacetylation of 29.10% up to 52.50% (*Table 2*).

Chitosans extracted from the crab shell, *S. racemosum,* and *R. arrhizus* had similar characteristics (Kumar, 2000). The readings using FTIR showed the results of different deacetylation degrees of each chitosan. The results showed that the chitosan from crustacean and chitosans from fungi were very different. The deacetylation process causes chitosan to contain amine and hydroxyl groups, which have high chemical reactivity (Adriana *et al*., 2001). Amine groups can be used immediately in chemical reactions to form salts and acids, for example, with calcium (Ca^{2+}), phosphate (PO_4^{3-}), sodium (Na^+) and several other elements (Sanford, 1989). Stretching vibration at wave number 1647.21 cm^{-1} is an absorption band of C=O bond group, which shows the presence of a secondary amine group, while the absorption band of 3437.15 cm^{-1} and 3456.44 cm^{-1} is a vibration of the OH and NH_2 groups. This absorption band pattern was the same as that of the standard chitosan spectrum, namely at wavenumbers 1660.55 cm^{-1} and 2922.85 cm^{-1}. One characteristic absorption for chitosan compounds is the presence of weak stretch absorption in the area of 1650 cm^{-1} which indicates the presence of a C=O group on the bond (-$NHCOCH_3$) (Silverstein *et al*., 1981) The absorption bands at 2885.51 cm^{-1}, 2927.94 cm^{-1} and 2924.09 cm^{-1} in the extracted chitosans and 2922.85 cm^{-1} in standard chitosan showed symmetrical stretching vibration of C-H bonds. The absorption bands at wavenumber 1554.63 cm^{-1}, 1566.20 cm^{-1}, and 1566.20 cm^{-1} showed that there was a stretching vibration of the C=O bond, which indicated the presence of carbonyl groups. The standard chitosan spectrum also shows absorption in the same wavenumber area of 1587.94 cm^{-1} (Dompeipen, 2017).

Based on the analysis of the infrared spectrum characteristics compared to standard chitosan, there was no significant difference between the two spectra. The presence of the main spectrum in certain wavelength regions indicates the presence of a major functional group, which indicates that the compound from the deacetylation reaction is chitosan (Dompeipen, 2017). The characteristic that strengthens the formation of chitosan was characterised by the appearance of wave absorption bands, which were formed at a vibration stretching wave between 3550 cm^{-1} and 1655 cm^{-1}. The length of absorption of the waveband indicated the presence of secondary amide groups and OH and NH groups. The loss of the methyl (CH_3) group bound to amide ($NHCOCH_3$) could be seen from the loss of absorption at wavenumber 2982.65 cm^{-1} and the loss of group C=O an amide (NHCO) known from the loss of absorption band at wavenumber 1670.35 cm^{-1}. The typical absorption of chitosan at wavenumber 1666.30 cm^{-1} shows the vibration stretching of N-H from amide (Silverstein *et al*., 1981).

TABLE 2. CHARACTERISATION OF CHITOSAN EXTRACTION RESULTS

Characteristics	Source of Chitosan		
	Crab shells	*R. arrhizus*	*S. racemosum*
Colour	White	black	Dark green
Texture	Smooth	Coarse powder	Coarse powder
Aroma	Odour	Odour less	Odour less
pH	12,7	11,7	11,7
DD	43.00%	29.10%	52.50%

Note: Description: DD - Degree of deacetylation.

Ganoderma Growth Inhibition Test

The *in vitro* inhibitory test on 1, 2, and 3 DAI (days after incubation) showed different results in the suppression of *Ganoderm*a growth. The incubation test at 1 and 2 DAI showed that *R. arrhizus* was the best treatment in suppressing the growth of *Ganoderma*. Incubation test in 3 DAI showed different results, namely, the treatment with *S. racemosum* inoculation was the best treatment. The incubation results in 3 DAI were not significantly different from the direct treatment of *R. arrhizus* inoculation (*Table 3*).

The addition of chitosan from crab shells increased the development of *G. boninense*. CrCs concentration of 10 000 ppm in the media resulted in increased growth of *G. boninense* 1.57 times compared to control. This result warrants careful consideration on the source of chitosan for use in *G. boninense* treatment.

Chitosan from *S. racemosum* suppressed *G. boninense* development, but there was no difference in the effect of different concentrations. Chitosan as an antifungal material produced by *R. arrhizus* and *S. racemosum* could affect the growth of *Ganoderma*. This is in line with the research of Berger *et al.* (2018), which stated that Mucolares fungi naturally produce chitin and FuCS on their cell walls during their growth through the action of chitin deacetylase enzymes, which catalyse chitin deacetylation to form chitosan.

Basically, *Ganoderma* does have slightly slower growth. But in this experiment, the growth of the treated *Ganoderma* had slower growth than control.

TABLE 3. TEST FOR INHIBITION OF THE DEVELOPMENT OF *G. boninense* AT 1 DAY, 2 DAYS AND 3 DAYS AFTER ISOLATION

Treatments	Development of *G. boninense* colony area					
	1 day		2 days		3 days	
	---------- mm^2 ----------					
Control	4.97	ab	10.20	ab	16.20	cd
CrCs 5 000 ppm	4.83	abc	9.63	bc	12.53	de
CrCs 7 500 ppm	5.03	a	10.57	ab	21.47	ab
CrCs 10 000 ppm	4.90	abc	13.13	a	25.47	a
FuCsRa 5 000 ppm	4.53	cd	7.70	bc	12.43	de
FuCsRa 7 500 ppm	4.43	d	10.07	b	16.17	cd
FuCsRa 10 000 ppm	4.63	bcd	9.53	bc	19.73	bc
FuCsSr 5 000 ppm	5.07	a	8.40	bc	11.50	de
FuCsSr 7 500 ppm	5.03	a	7.90	bc	11.13	de
FuCsSr 10 000 ppm	4.63	bcd	9.07	bc	11.40	de
Antagonist test *S. racemosum*	4.73	abcd	7.73	bc	9.23	e
Antagonist test *R. arrhizus*	4.43	d	6.37	c	10.13	e

Note: The number followed by the same letter in the same column is not significantly different according to the Duncan test at α 5% .

The results of secondary metabolism produced from the fungi *R. arrhizus* and *S. racemosum* affected the growth system of *Ganoderma*. El Ghaouth *et al.* (1997) reported that chitosan inhibits the growth of *in vitro* of most postharvest pathogens. Chitosan was also reported to inhibit the proliferation of *Botrytis cinerea*, causal fungus that damages and stimulates cellular changes in *Rhizopus stolonifer* and *B. cinerea*. The treatment of chitosan was also reported to cause pathogenic hyphae to undergo cellular disorganisation that ranges from the decomposition of cell walls to cytoplasmic disintegration.

CONCLUSION

Chitosan has a fine to a coarse powder particle-size with white, black, dark green colour with a pH ranging from 11.7 to 12.7. All types of chitosan from this experiment have a purity level (degree of deacetylation) of 29.10%-52.50%. Chitosan can be extracted from fungi found in chipped oil palm trunks.

Chitosan from *S. racemosum* at concentrations of 5000 ppm, 7500 ppm and 10 000 ppm were able to inhibit the growth of *Ganoderma* (-28% to -31%), similar to the ability of well-known antagonists *R. arrhizus* (-37%) and *S. racemosum* (-43%). The results of this study indicated that the application of FuCsSr and *S. racemosum* had a significant effect in inhibiting the growth of *Ganoderma in vitro*, therefore it is recommended to carry out a test in the field.

REFERENCES

Adriana, A; Mudjiajati, E; Selvy, V and Setijawati (2001). Adsorbs Cr (IV) with Chitosan Adsorbent. *Environmental Chemistry, 3*: 31-36.

Amorim, R V S; Melo, E S; Carneiro-da-Cunha, M G; Ledingham, W M and Campos-Takaki, G M (2003). Chitosan from *Syncephalastrum racemosum* used as a film support for lipase immobilization. *Bioresource Technology, 89(1)*: 35-39.

Berger, L R R; Stamford,T C M; de Oleveira, K A R; Pessoa, A M P; de Lima, M A B; Pintado, M M E; Camara M P S; Franco L O; Camara, M P S; Franco, L O; Magnani, M and de Souza, E L (2018). Chitosan produced from Mucorales fungi using agroindustrial by-products and its efficacy to inhibit *Colletotrichum species*. *Int. J. Biological Macromolecules, 108*: 635-641.

Dompeipen, E J (2017). Isolation and identification of chitin and chitosan from shrimp skin (*Penaeus monodon*) with infrared spectroscopy. Industrial Research and Standardization Center Ambon. *J. Ministry of Industry.* e-ISSN: 2548-4842 p-ISSN: 0215-1464.

El Ghaouth, A; Arul, J; Wilson, C and Benhamou, N (1997). Biochemical and cytochemical aspects of the interactions of chitosan and *Botrytis cinerea* in bell pepper fruit. *Postharvest Biol. Technol*., *12(2)*: 183-194.

Kaban, J (2007). *Characteristic Study and Application of Alginate-Chitosan Alkali Metal Coating*. Ph.D thesis. Universitas Sumatera Utara Medan. 271 pp.

Khan, T A; Peh, K K and Chang, H S (2002). Reporting degree of deacetylation values of chitosan; the influence of analytical methods. *J. Pharm. Sci. Vol. 5(3)*: 205-212.

Kumar, M N V R (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers, 46(1)*: 1-27.

Puspawati and Simpen (2010). Optimization of chitin deacetylation from shrimp shells and crab shells of seafood restaurant waste became chitosan through variations in NaOH concentration. *Chemical Journal, 4(1)*: 79-90.

Sabrina, T; Poltak and Hanafiah, A S (2018). Five fungus originated from oil palm trunk: Type and potency. Proc. of the National Seminar, University Mataram Lombok. 27 January 2018.

Sanford, P A (1989). Chitosan: commercial uses and potential application. *Chitin and Chitosan*. p. 51-59.

Silverstein, R M; Francis, X W and David, J K (1981). *Spectrometric identification of organic compound*. John Wiley & Sons. 550 pp.

Synowiecki, J and Al-Khateeb, N A A Q (1997). Mycelia of *Mucor rouxii* as a source of chitin and chitosan. *Food Chem*., *60(4)*: 605-610.