Sains Malaysiana 51(12)(2022): 3923-3935
http://doi.org/10.17576/jsm-2022-5112-05

Antibacterial Properties of Chitosan Isolated from the Black Soldier Fly, *Hermetia illucens*

(Sifat Antibakteria Kitosan Pencilan daripada Lalat Askar Hitam, *Hermetia illucens*)

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Received: 22 December 2021/Accepted: 22 August 2022

ABSTRACT

Insects are receiving wide attention as alternative food and feed resources, and for the production of useful by-products such as chitin, which can be converted into chitosan, a natural antibacterial agent. The larvae of *Hermetia illucens*, commonly known as Black Soldier Fly (BSF), can be reared on organic waste substrates and can be produced on a large scale. In this study, we focused on the antibacterial activity of chitosan obtained from BSF. Chitin from different growth phases of BSF was isolated using chemical treatments, characterized, and further synthesized into chitosan by deacetylation. The identities and structures of all isolated and synthesized compounds were verified using Fourier-transform infrared spectroscopy (FTIR). The antibacterial effect of BSF chitosan compounds against pathogenic bacteria were assessed with the determination of a minimum inhibitory concentration (MIC). Results showed that the chitin content increased gradually during the transition from larvae to adult BSF, with the highest amount obtained in the pupal stage. In the antibacterial susceptibility assay, *Staphylococcus aureus* was the most resistant to the action of BSF chitosan, with no significant effect exerted on its growth. For other species of bacteria, BSF chitosan could only restrict bacterial growth at concentrations of 0.25% or 0.5%, with the two most susceptible species being identified as *Pseudomonas aeruginosa* and *Serratia marcescens*. In conclusion, BSF chitosan exhibited antibacterial activity against different bacteria with varying sensitivities, in which the chitosan concentration was demonstrated to play an essential role.

Keywords: Antibacterial activity; Black Soldier Fly; chitin; chitosan; *Hermetia illucens*

ABSTRAK

Serangga mendapat perhatian meluas sebagai punca makanan dan makanan alternatif, dan untuk penghasilan produk sampingan yang berguna seperti kitin, yang boleh ditukar menjadi kitosan, agen antibakteria semula jadi. Larva *Hermetia illucens*, biasanya dikenali sebagai Lalat Askar Hitam (BSF), boleh diternak pada substrat sisa organik dan boleh dihasilkan secara besar-besaran. Dalam kajian ini, tumpuan diberikan kepada aktiviti antibakteria kitosan yang diperoleh daripada BSF. Kitin daripada fasa pertumbuhan BSF yang berbeza telah diasingkan menggunakan rawatan kimia, dicirikan dan selanjutnya disintesis menjadi kitosan melalui penyahetilasi. Identiti dan struktur semua sebatian terpencil dan tersintesis telah disahkan menggunakan spektroskopi inframerah transformasi Fourier (FTIR). Kesan antibakteria sebatian kitosan BSF terhadap bakteria patogen telah dinilai dengan penentuan kepekatan perencatan minimum (MIC). Keputusan menunjukkan bahawa kandungan kitin meningkat secara beransur-ansur semasa peralihan daripada larva kepada BSF dewasa dengan jumlah tertinggi diperoleh pada peringkat pupa. Dalam asai kerentanan antibakteria, *Staphylococcus aureus* adalah yang paling tahan terhadap tindakan kitosan BSF, tanpa kesan ketara terhadap pertumbuhannya. Bagi spesies bakteria lain, kitosan BSF hanya boleh menyekat pertumbuhan bakteria pada kepekatan 0.25% atau 0.5% dengan dua spesies yang paling rentan dikenal pasti sebagai *Pseudomonas aeruginosa* dan *Serratia marcescens*. Kesimpulannya, kepekatan kitosan memainkan peranan penting kerana kitosan BSF mempamerkan aktiviti antibakteria terhadap bakteria yang berlainan dengan sensitiviti yang berbeza-beza.

Kata kunci: Aktiviti anti bakteria; kitin; kitosan; *Hermetia illucens*; Lalat Askar Hitam

Introduction

Aquaculture requires a consistent supply of fishmeal, which is a major component in commercial fish feeds (Stankus 2021). To keep up with the amount of fishmeal needed for thriving aquaculture, researchers are turning to insects as a source of animal feed (Makkar et al. 2014). Insects have a small carbon footprint compared to other protein sources and produce useful by-products such as oil for biodiesel and biopolymers like chitin. Chitin is of particular interest because it can be converted into chitosan, a potent antibacterial polymer (Liu et al. 2001). There are about two million insect species known worldwide and they represent almost 95% of the animal kingdom (Kaya et al. 2014). Isolation and characterization of chitin has been done on many insects from the order Lepidoptera, Coleoptera, Orthoptera, Hymenoptera, Diptera, Hemiptera, Dictyoptera, and Odonata (Mohan et al. 2020).

The black soldier (BSF) fly (*Hermetia illucens*) can be found in warm regions worldwide and is capable of surviving in demanding conditions like food scarcity, oxygen deficiency and drought (Diener et al. 2011). BSF larvae can feed on all sorts of organic waste, and rearing BSF has been proposed as an effective way to dispose organic waste since the 1990s (Ravi et al. 2020). Besides, they can be converted into fat-rich and protein-rich biomass, and serve as feed for different livestock species (Makkar et al. 2014). The life cycle of BSF consists of several stages, including eggs, larvae, pupae, and adults (Lagat et al. 2021). Despite having a great potential in various industrial areas, BSF is rarely considered as the best choice for chitin isolation due to the presence of impurities with melanin being found after the bleaching progress was carried out, and the current available literature offers only limited reports on chitin and chitosan isolated from the different life stages of BSF (Khayrova, Lopatin & Varlamov 2021). Thus, this study serves to examine the chitin content of the different life stages of BSF, with the aim of transforming this organism into an outstanding alternative source of chitin and chitosan, thereby further expanding its industrial utilization.

Both chitin and its N-deacetylated derivative, chitosan, have received much research interest as they possess numerous desirable biological and functional properties. In the last few years, scientists have been focusing on the discovery of new antimicrobial agents due to the emergence of antibiotic-resistant microorganisms (Jackson, Czaplewski & Piddock 2018). Some focused on the antimicrobial properties of chitosan as it has a track record for inherent antimicrobial activities against a broad spectrum of microorganisms (Ai et al. 2012; Benhabiles et al. 2012; Goy, Morais & Assis 2016). The promising antibacterial activity of chitosan has made it a crucial compound in the biomedical and pharmaceutical fields (Aranaz et al. 2009). However, since the 1980s, the source of chitin has always been obtained from crustaceans (Kumirska et al. 2010; Shimahara & Takiguchi 1988).

As previous studies focused on the antibacterial properties of chitosan obtained from one stage of the BSF's life cycle (Khayrova, Lopatin & Varlamov 2019; Jayanegara et al. 2020; Lagat et al. 2021; Xia, Ge & Yao 2021), the antibacterial activities of chitosan produced from the different life stages of BSF were investigated in this study. The objectives of this study were two-fold: to isolate chitin and chitosan from different growth stages of BSF (late larvae, prepupae, pupal exuviae, and imagoes) and report the findings of their relative content; and to determine the antibacterial properties of the prepared chitosan samples against different pathogenic bacteria by evaluating the minimum inhibitory concentration (MIC) values generated using the broth microdilution antibacterial assay.

Materials and Methods

Chitin and chitosan preparations

Samples of black soldier flies (BSF) (late larvae, prepupae, pupal exuviae, and imagoes) were obtained from insectarium units set up at the field research institute of the Crops for the Future (CFF) Organisation, Semenyih, Selangor, Malaysia. All BSF samples were fed with 100% coconut husk substrate. They were collected in plastic bags and stored in a -20 °C freezer (Panasonic, PQS 201). Isolation of chitin was performed according to Ai et al. (2008) and Waśko et al. (2016) with modifications comprising of demineralization, deproteinization and decolorization processes, which served to remove mineral salts, proteins and natural pigments associated with chitin molecules found on the BSF samples. Samples were separated into four main groups, with two replicates each: Group 1: late larvae; Group 2: prepupae; Group 3: pupal exuviae; and Group 4, imagoes. All the samples were first cleaned and dried at 65 °C to remove flesh residues. The dried materials were then ground into powder form using a blender (Sharp, EM110), and the initial weights of the samples were determined using an analytical balance (Mettler Toledo, AB204-S).

The demineralization process was performed by treating the powder with 1 M HCl solution in a ratio of

1:10 (w/v) for 2 h at room temperature under constant stirring with a magnetic stirrer, followed by filtration and rinsing with distilled water until a neutral pH (pH 7.0) was achieved. Demineralized samples were dried at 60 °C overnight. Next, to remove protein, the powders were treated with 1 M NaOH aqueous solution at 80 °C (with a ratio of 1 g/10 mL) for 6 h, and the residues were filtered, washed, and dried. The extracts were decolored using a 1% potassium permanganate ($KMnO_4$) solution in a 1:30 w/v ratio at room temperature for 4 h. Excess $KMnO_4$ was removed by 4% (w/v) citric acid for 3 h, and the final chitin products were neutralized with distilled water. The weights of the chitin produced from different BSF samples were determined, and their percentage yields were calculated from the weight of chitin produced as a percentage of the starting dry raw materials. Based on a modified protocol by Benhabiles et al. (2012), chitin was deacetylated by adding 50% (w/v) NaOH solution (in a 1:50 ratio) and under constant stirring for 4 h at 95 °C. After filtration, the residues were neutralized with distilled water. The derived chitosans were oven-dried at 60 °C overnight and stored at 4 °C until further usage. The percentage yields of different chitosan samples were calculated from the weight of chitosan obtained as a percentage of the chitin weight before the deacetylation process. In this study, a commercial chitosan (medium molecular weight, 75-85% deacetylated, product code: 101652913), procured from Sigma Aldrich, would also be used to serve as a comparison for the chitosans obtained from BSF samples, in terms of their characterization and antibacterial activities. Test of significance was carried out for the content of chitin and chitosan obtained from four growth stages of BSF using SPSS (PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.).

FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY ANALYSIS

FTIR analysis was carried out for the identification and characterization of chitin and chitosan compounds produced by different BSF developmental stages. Infrared spectra of the chitin and chitosan were recorded using a Nicolet iS5 FTIR spectrometer (Thermo Scientific, USA) equipped with iD5 Diamond Attenuated Total Reflectance (ATR) in the region 4000 – 650 cm^{-1}. The analysis was performed using the OMNIC statistical software package (Thermo Electron Scientific Instruments LLC, Madison, Wisconsin).

ANTIBACTERIAL ACTIVITIES OF CHITOSAN

Mueller-Hinton (MH) broth medium (Oxoid, UK) was prepared and adjusted to pH 5.9. The medium was autoclaved at 121 °C for 15 min. According to CLSI recommendation, the MH broth required supplementation with the divalent cations after sterilization for quantitative susceptibility testing of rapidly growing aerobic and facultative anaerobic bacteria with the antimicrobial agent (CLSI, 2012). The cation levels recommended were as follows: Mg^{2+}, 10-12.5 mg/L; Ca^{2+}, 20-25 mg/L. Six bacterial strains (*Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella enterica*, and *Serratia marcescens*) were used in this study, in which all were acquired from UCSI University, Kuala Lumpur, Malaysia. Bacterial stock cultures were sub-cultured onto nutrient agar plates and incubated for 24 h at 37 °C. Next, three to four well-isolated bacterial colonies from the respective agar plates were inoculated into 5 mL of sterile 0.9% (w/v) saline solutions. The turbidity was adjusted to be equivalent to a 0.5 McFarland standard. 200 µL of the bacterial suspensions were further diluted into 10 mL of cation-adjusted Mueller-Hinton broth (CAMHB). A cell density of 3×10^6 cfu/mL was achieved, which was then being used as the final inoculum. Chitosan solutions were prepared by dissolving 1% (w/v) chitosan in 0.1% (v/v) acetic acid. After stirring properly, the chitosan solutions were sterilized using an autoclave. The stock solutions were stored at 4 °C for further usage.

ANTIBACTERIAL SUSCEPTIBILITY TEST AND DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The broth microdilution method was adopted to demonstrate the antibacterial effects of chitosan produced from different lifecycle stages of BSF against pathogenic bacterial strains. The test was conducted according to Jiang et al. (2013) and Li, Wu and Zhao (2016) with some modifications. A volume of 100 µL of 1% (w/v) chitosan solution was dispensed into the first column well of a 96-well microtiter plate (Jet Biofil, China), and 50 µL of CAMHB (pH 5.9) into the remaining wells. Two-fold serial dilution of chitosan solutions (1:2 to 1:32) was made by pipetting 50 µL of first column wells chitosan solution into the second column and then moving on to the next column. After that, aliquots (50 µL) of the bacterial suspension (3×10^6 cfu/mL) were inoculated into the respective wells of the microtiter plate to achieve a concentration of 1.5×10^6 cfu/mL. In the end, a volume of 100 µL in each well of the plate was obtained. The final concentration of chitosan solution

was now one-half of the original concentration in each well, which were 0.5000%, 0.2500%, 0.1250%, 0.0625%, 0.0313%, and 0.0156%, respectively. The last two horizontal rows of each plate consisted of different sets of control, including antibiotic control, bacterial growth control, chitosan solution sterility control (chitosan stock solution in CAMHB without any bacterial suspension), and broth sterility control (CAMHB only). The penicillin-streptomycin antibiotic (10,000 U/mL) (Gibco, USA) was included in the study as the positive control, while the bacterial growth control with only CAMHB and bacterial suspension served as the negative control, to validate and corroborate the feasibility of this assay.

The same procedures were performed for different chitosan samples prepared and commercial chitosan, as well as for the various bacteria species. All the plates were incubated at 37 °C (Memmert, BE 500) for a duration of 24 h and observed for visible signs of bacterial growth or turbidity. The antibacterial effects of chitosans and their respective minimum inhibitory concentration (MIC) values were estimated by measuring the optical density (OD) of the cultured medium at 600 nm using a FLUOstar Omega microplate reader (BMG LABTECH, Germany). The chitosan's MICs were determined as the lowest concentration of chitosans in which no visible growth or turbidity was observed in the 96-well microtiter plates (Jiang et al. 2013). Test of significance was carried out for MIC values from four growth stages of BSF with the observed MIC value from commercial chitosan using SPSS (PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.).

Results and Discussion

Chitin and chitosan preparation

Table 1 shows the chitin and chitosan contents from the different growth stages of BSF. The pupal exuviae had the highest chitin content (18.8%), while late larvae had the lowest amount of chitin in their exoskeleton, which was only 3.025%. Imagoes contained a higher chitin content than prepupae, which were reported at 11.8% and 5.371%, respectively. There was no significant difference between chitin and different types of BSF samples ($p > 0.05$), while there was a significant difference between chitosan and different types of BSF samples ($p < 0.05$). The chitin content increased gradually during the transition from larvae to adult black soldier flies, as predicted, with the pupal stage having the highest chitin content, contributing to the hardening process of the insect cuticle.

TABLE 1. Chitin and chitosan contents in different growth stages of *Hermetia illucens*

Types of BSF samples	Chitin content (%)	Percentage yield of chitosan (%)
Late larvae	3.025	81.034
Prepupae	5.371	73.656
Pupal exuviae	18.800	79.701
Imagoes	11.846	63.158

In comparison, chitin extracted from other sources showed that the percentage of chitin was about 23% to 32% in *Apis mellifera* (western honey bees) (Nemtsev et al. 2004), 15% in *Holotrichia parallela* (beetle) (Liu et al. 2012), and an average of 20% in silkworm chrysalides (Paulino et al. 2006). Kaya et al. (2016) found that the chitin contents in larvae, pupa, and adults of *Vespa crabro* (wasp) were 2.2%, 6.2%, and 10.3%, respectively. Previous studies have reported the dry weight of the chitin content of crustacean shells to vary between 7% and 40%, depending on the species involved (Tolaimate et al. 2003). For instance, Cortizo, Berghoff and Alessandrini (2008) reported the chitin composition of *Illex argentines* (squid pen) to be 31%, while Abdou, Nagy and Elsabee (2008) reported chitin yields of 16.73%, 20.60%, and 23.72% for crab shells, crayfish, and pink shrimp, respectively.

In the present study, the dry weights of chitin content of BSF samples were observed to be lower than those of other insect and crustacean species. It was suggested that the lower chitin content obtained may be due to the rough conditions employed during the extraction process in this experiment, involving the usage of strong reagents that might have caused depolymerization or degradation of the native chitin compounds. Different methods used and the parameters adopted (e.g., chemical concentration, temperature, treatment duration, pH) could also contribute to such variations. Apart from that, the species or origin of the chitin compounds, as well as the physiological stage of the organisms, could have resulted in significant qualitative and quantitative changes in the chitin compounds (Fernandez-Kim 2004).

Next, it was found that the chitosan productivity corresponding to the isolated chitin was the highest for the late larvae (81.034%), while the least amount of chitosan had been produced from the imagoes (63.158%). Prepupae and pupal exuviae had similar chitosan yields resulting from the deacetylation process, of which about 73% and 79%, as shown in Table 1. Previous literature also reported chitosan yields of 67% and 72% from the adult and larvae chitins of *Leptinotarsa decemlineata* (Colorado potato beetle) (Kaya et al. 2014). Kaya et al. (2015a) also found the yield of chitosan to be 74% - 76% in the Orthoptera order of insects, *Calliptamus barbarous* (Eurasian Pincer Grasshopper) and *Oedaleus decorus* (Handsome Cross Grasshopper), with a degree of deacetylation of 70% - 75%. The variations in the chitosan yields in regards to the relative chitin contents of different BSF samples were mostly due to the random process of chemical deacetylation along the chitin chains. Besides, the intrinsic characteristics of the chitin compounds could also greatly affect the resulting chitosan yields and properties. To confirm the identities of the biopolymers, the products obtained from different growth stages of BSF and the commercial chitosan were characterized and compared by Fourier Transform Infrared (FTIR) spectroscopy.

FTIR SPECTROSCOPIC ANALYSIS OF CHITIN

The FTIR spectra of chitin samples isolated from adult black soldier flies (BSF) and their late larvae, prepupae, and pupal exuviae are presented in Figure 1. According to Waśko et al. (2016), the IR spectra of chitins isolated from BSF were characterized by three significant amide bands at 1650 cm^{-1} (C=O secondary amide stretch; amide I), 1620 cm^{-1} (C=O secondary amide stretch; amide I) and 1550 cm^{-1} (N-H bend, C-N stretch; amide II). The spilt in amide I indicates the chitin from all four stages is confirmed as α-chitin (Smets et al. 2020). The amide I band of the α-chitin split into two parts; 1651 cm^{-1} and 1620 cm^{-1} for late larvae, 1648 cm^{-1} and 1620 cm^{-1} for prepupae, 1650 cm^{-1} and 1619 cm^{-1} for pupal exuviae, and 1651 cm^{-1} and 1620 cm^{-1} for adult BSF. Similarly, as seen in Smets et al. (2020) and Purkayastha and Sarkar (2020), the asymmetric C-H peaks at 2920 and 2851 cm^{-1} (late larvae) are seen to deplete as the BSF matures, leaving one peak in the adult BSF at 2879 cm^{-1}. The remaining bands are compiled in Table 2.

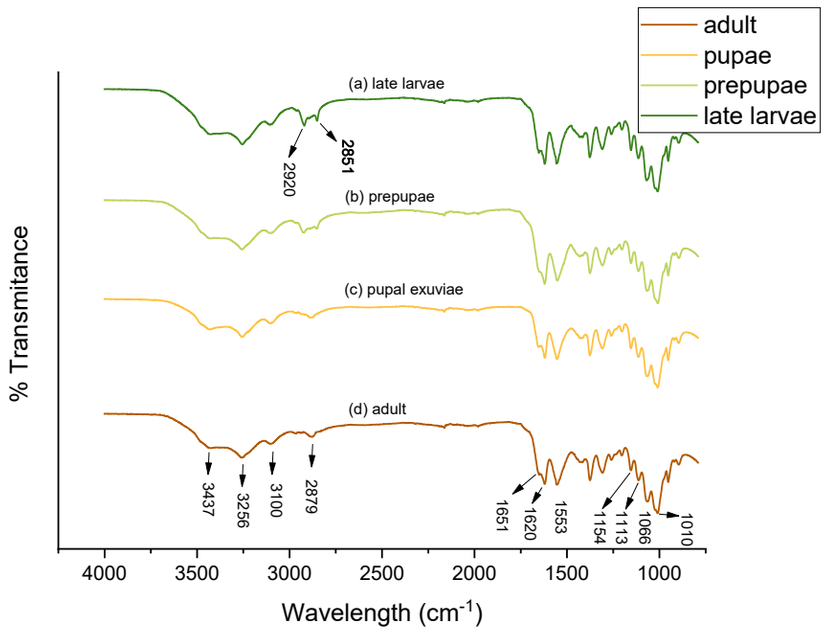


FIGURE 1. FTIR absorption spectra of the chitin isolated from (a) late larvae, (b) prepupae, (c) pupal exuviae, and (d) adult BSF

TABLE 2. FTIR frequency range and attribution according to BSF life stage

Attribution	Wavenumber (cm^{-1})	BSF life stage
C=O secondary amide stretch	1620.34	Late larvae
	1620.42	Prepupae
	1619.67	Pupal exuviae
	1620.60	Adult
N-H bend, C-N stretch	1556.53	Late larvae
	1551.75	Prepupae
	1552.90	Pupal exuviae
	1552.59	Adult

The spectra of all four BSF chitin samples showed no absorption bands at 1798 cm^{-1}, 1420 cm^{-1}, and 876 cm^{-1}, in which, according to Mohammed, Williams and Tverezovskaya (2013), were the evidences for the removal of mineral substances ($CaCO_3$) from the chitin compounds after the demineralization process. The lack of bands at 1540 cm^{-1} was associated with the absence of protein residues in the analyzed chitin compounds (Waśko et al. 2016). The FTIR spectra of chitin extracted from BSF in this study were similar to the absorption bands of chitins isolated from honeybees and marine crustaceans as reported in previous studies (Al Sagheer et al. 2009; Kaya et al. 2015b; Mohammed, Williams & Tverezovskaya 2013). In view of these literatures, the identity of the chitin compounds isolated from BSF samples can be confirmed.

FTIR SPECTROSCOPIC ANALYSIS OF CHITOSAN

The FTIR analysis of chitosan derived from various life cycle stages of BSF was performed in the frequency region of 4000 - 650 cm^{-1} as well (Figure 2). Figure 2 also displays the FTIR analysis for commercial chitosan. In general, although there were some minor differences found in the absorption spectra, the positions of the characteristic peaks of BSF chitosan were nearly the same as those of the BSF chitins, with only the intensities of most of the absorption peaks becoming weaker.

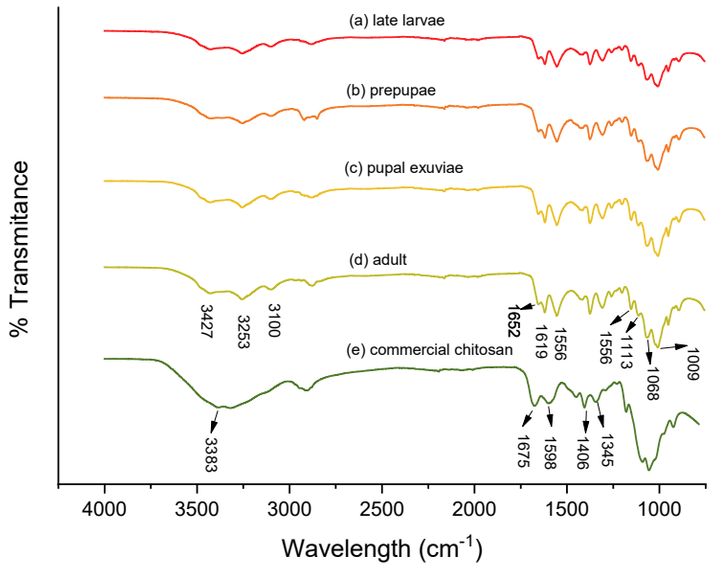


FIGURE 2. FTIR absorption spectra of the chitosan prepared from (a) late larvae, (b) prepupae, (c) pupal exuviae, (d) adult BSF and (e) commercial chitosan (Sigma Aldrich)

The chitosan samples exhibited weak bands around 3430 cm^{-1}, which corresponds to the -OH stretching vibration, N-H stretching vibrations of free amino groups, and intermolecular hydrogen bonds (Zhang et al. 2011). The NH-bending (amide II) and CO-stretching (amide I) bands are seen around 1598 (amide II) and 1675 cm^{-1} (NH_2 bending) in commercial chitosan, whereas the amide II and amide I on the BSF samples are seen at 1619 cm^{-1} and 1556 cm^{-1}, respectively. The N-H and O-H stretching bands in the 3000–3600 cm^{-1} region are seen to be more complex for chitin than for chitosan, as similarly seen in Triunfo et al. (2022). The wavenumber at 3383 cm^{-1} was attributed to the -OH bending, while C-O stretching vibrations in alcohol were examined at 1153 cm^{-1} and 1068 cm^{-1} (Song et al. 2013). A noticeable weakening of the band emerging at 1619 cm^{-1} (C=O stretching vibrations) was observed, demonstrating the effect of the N-deacetylation process (Song et al. 2013). The intensities of both peaks at 1620 cm^{-1} and 1556 cm^{-1} were almost the same in the chitin FTIR spectra (Figure 1), but for chitosan, the bands at 1556 cm^{-1} were stronger than the bands at 1620 cm^{-1} (Figure 2), which indicated a certain degree of deacetylation in the chitosan compounds (Qi et al. 2004).

According to Mohammed, Williams, and Tverezovskaya (2013), the FTIR spectrum of chitosan would show additional bands in the region between 1606 cm^{-1} and 1566 cm^{-1} due to the presence of primary amine groups. However, such changes were not observed in the FTIR spectra of both BSF chitosan and commercial chitosan in this study. This may be due to differences in the chemical extraction method, as well as the distinct species involved. Apart from that, research suggested that there should be no absorption peaks at 3260 cm^{-1} and 3110 cm^{-1}, as the amide groups were lost from the chitin during the deacetylation process. In the current study, there were still peaks at 3256 cm^{-1} and 3100 cm^{-1} found in the spectra of BSF chitosan, but with a much weaker intensity. It could be postulated that BSF chitin compounds were not fully deacetylated after the alkaline treatment. Besides, the nearly identical spectra between chitin and chitosan samples might be due to the fact that certain chitin compounds underwent partial deacetylation during the deproteinization and demineralization processes with the use of strong acids and bases, resulting in less obvious changes.

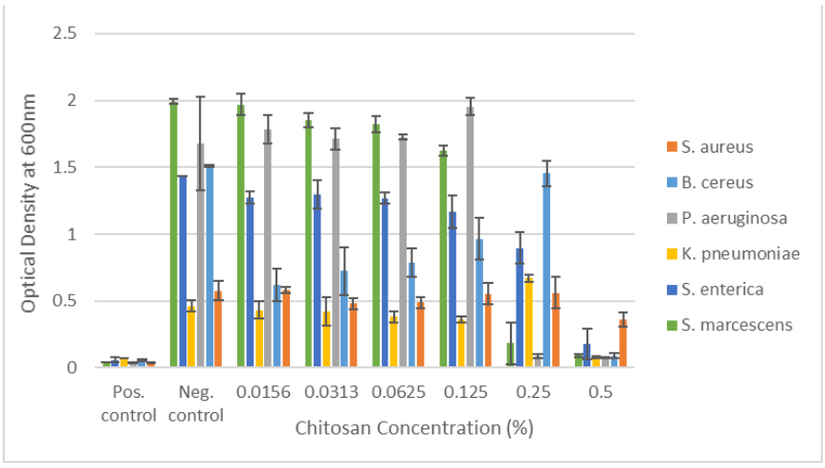
ANTIBACTERIAL PROPERTIES OF BSF CHITOSAN AND THEIR MIC VALUES

The efficient antibacterial concentration of BSF chitosan was investigated in detail, with the activity data evaluated in terms of OD values of corresponding bacterial suspension in different chitosan concentrations and the minimum inhibitory concentration (MIC) of each type of chitosan being obtained. Table 3 presents the MIC values of different chitosan against the six pathogenic bacterial strains, including *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella enterica*, and *Serratia marcescens*. There were no significant differences between the different types of BSF samples and their MIC values ($p > 0.05$).

TABLE 3. MICs of different chitosan samples against various bacteria

Bacteria tested	MIC of different chitosan samples (%, w/v)				
	Late larvae	Prepupae	Pupal exuviae	Imagoes	Commercial
Staphylococcus aureus	-	-	-	-	0.0625
Bacillus cereus	0.5000	0.5000	0.5000	0.5000	0.0625
Pseudomonas aeruginosa	0.2500	0.2500	0.2500	0.2500	0.0625
Klebsiella pneumoniae	0.5000	0.5000	0.5000	0.5000	0.0313
Salmonella enterica	0.5000	0.5000	0.5000	0.5000	0.5000
Serratia marcescens	0.2500	0.2500	0.2500	0.2500	0.1250

a


Optical Density at 600nm
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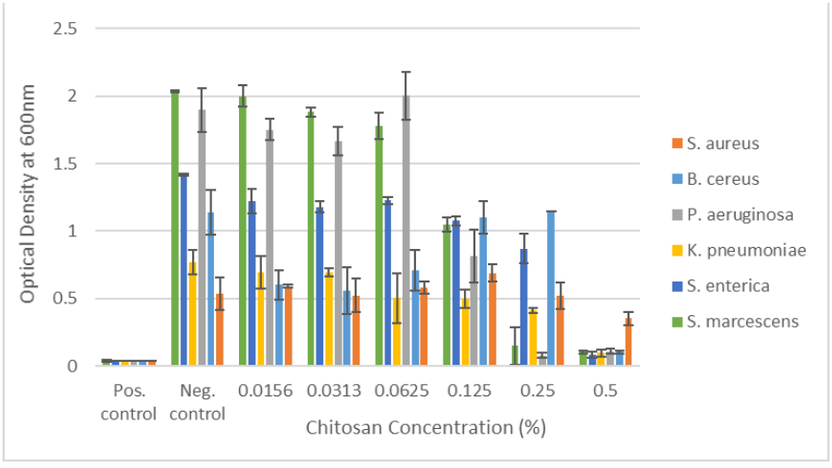
2
1.5
1
0.5
0
Pos. control
Neg. control
0.0156
0.0313
0.0625
0.125
0.25
0.5
Chitosan Concentration (%)
S. aureus
B. cereus
P. aeruginosa
K. pneumoniae
S. enterica
S. marcescens

b


Optical Density at 600nm
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Pos. control
Neg. control
0.0156
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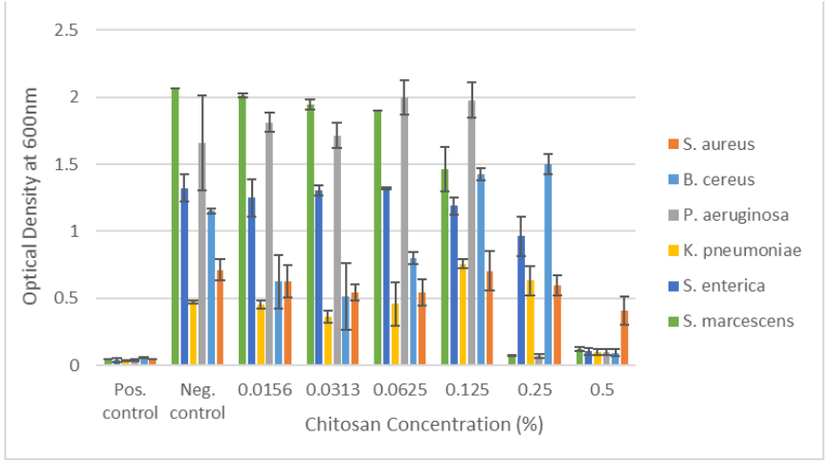
Chitosan Concentration (%)
S. aureus
B. cereus
P. aeruginosa
K. pneumoniae
S. enterica
S. marcescens

c


Optical Density at 600nm
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2
1.5
1
0.5
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Pos. control
Neg. control
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0.125
0.25
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Chitosan Concentration (%)
S. aureus
B. cereus
P. aeruginosa
K. pneumoniae
S. enterica
S. marcescens

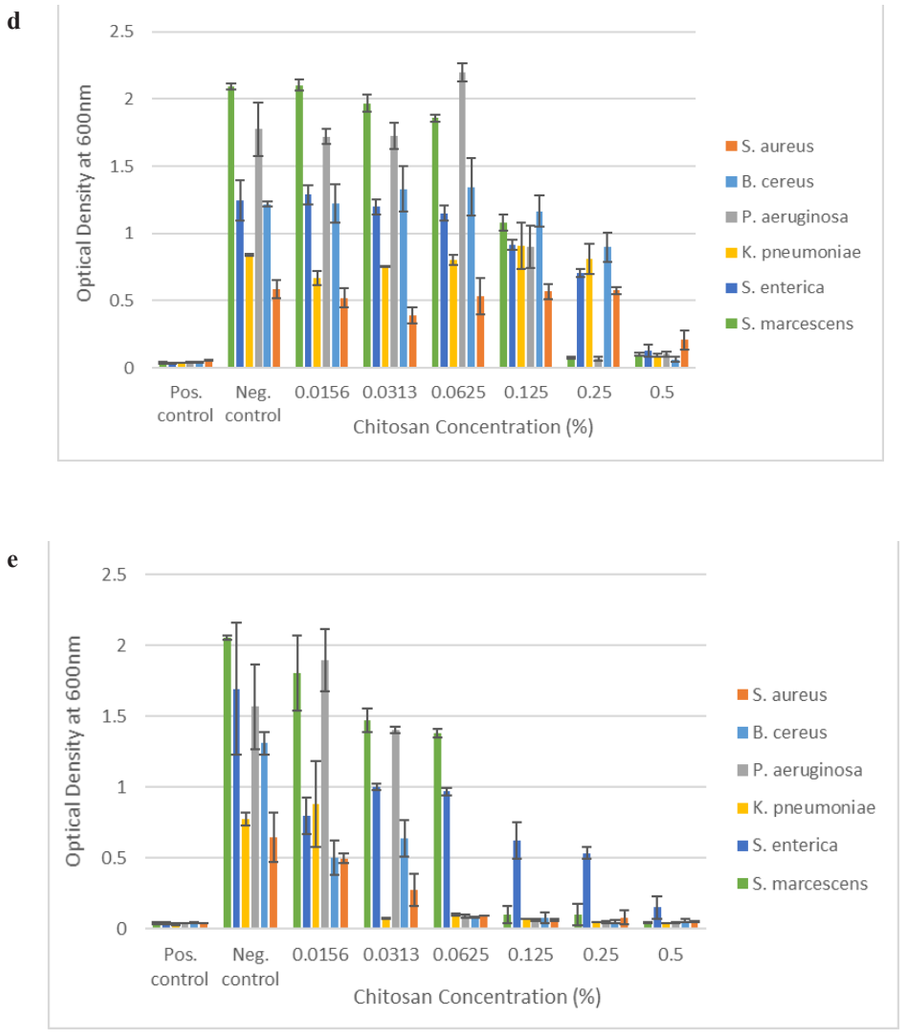

FIGURE 3. Effects of different concentrations of chitosan from the late larvae (a), prepupae (b), pupal exuviae (c), imagoes (d) of *Hermetia illucens* and commercial chitosan (e) against six different bacterial strains as measured by the optical density at 600 nm. All data were expressed as mean with SD (n = 3)

Figure 3 summarizes the data by chitosan groups, in which the antibacterial effects of each chitosan were compared between the different bacterial strains tested, to assess their relative antibacterial efficiency. The optical densities (ODs) for *S. aureus* growth fluctuated between 0.4 and 0.7 for the four BSF chitosan concentrations, ranging from 0.016% to 0.25%. Although there was a reduction in the growth of *S. aureus* in 0.5% BSF chitosan solutions, the OD readings in the range of 0.3 to 0.4 were still considered much higher as compared to that of the positive control group containing antibiotics. The results showed that BSF chitosan had no effective antibacterial activity against *S. aureus*, at least at the concentrations employed in this current study, and the possible MIC values could be much higher than 0.5%. On the other hand, commercial chitosan was shown to have inhibited the proliferation of *S. aureus* at concentrations of 0.063%, 0.125%, 0.25%, and 0.5%, where the ODs were constantly maintained at values below 0.1 (0.051 - 0.094). For *B. cereus*, the growth rate at 0.016% and 0.031% of chitosan prepared from late larvae, prepupae, and pupal exuviae had dropped to a lower value compared to the

concentration of 0.125% and 0.25%. Such variations may be caused by the formation of non-homogenous suspensions during bacterial inoculum preparation due to the nature of *B. cereus* that tends to form precipitates in solution. It was observed that all BSF chitosans were able to prevent the growth of *B. cereus* at 0.5% concentration, which was demonstrated by the decrease in OD readings to below 0.1. Commercial chitosan had a similar effect on *B. cereus* as on *S. aureus*, in which the compound started to inhibit the growth of the bacteria at the concentration of 0.063% and above.

Both *P. aeruginosa* and *S. marcescens* exhibited similar survival patterns when cultured in different concentrations of BSF chitosan. The ODs varied between 1.4 and 2.1 in the concentration range of 0.016% - 0.063%. At 0.125% chitosan, the OD readings showed a slight decrease, and when the concentrations were at 0.25% and 0.5%, BSF chitosan showed antibacterial activity against both *P. aeruginosa* and *S. marcescens*. For commercial chitosan, both the bacteria had slight differences in their reactions to the increasing chitosan concentration. Referring to their OD readings, the growth of *P. aeruginosa* was hindered at 0.063% and above, while the growth of *S. marcescens* was only restricted when the concentration was equal to or more than 0.125%. The OD values measured for *K. pneumoniae* fluctuated between 0.3 and 0.9 at 0.016% - 0.25% of the four BSF chitosan, suggesting that chitosan with a concentration of 0.25% or lower exerted no antibacterial effect on *K. pneumoniae*. However, there was an immediate reduction in ODs to a value below 0.1 at 0.5% BSF chitosan, indicating the growth of *K. pneumoniae* could only be inhibited by 0.5%. Besides, *K. pneumoniae* is shown to be the most susceptible to the antibacterial effect of commercial chitosan, in which the ODs were constantly maintained at a value of under 0.1 at a concentration of 0.031% and above (0.044 - 0.101). In the case of *S. enterica*, the OD readings were markedly reduced at 0.5% chitosan solutions, suggesting it to be the effective concentration for inhibiting bacterial growth. Unlike other bacterial species, *S. enterica* showed the lowest susceptibility to the antibacterial effect of commercial chitosan, as the OD reading for its growth only decreased to the value of 0.154 when the chitosan concentration was raised to 0.5%.

In general, all the four different BSF chitosans (prepared from late larvae, prepupae, pupal exuviae, and imagoes) had no discernible differences in their effects against the six bacteria. When the MIC values were evaluated at the individual strain level, the majority of the differences between the bacterial species were only two-fold. Neither of the BSF chitosans was active against any bacterial species at a concentration lower than 0.25%. It could be said that among the bacteria tested, *S. aureus* was the most resistant towards the antibacterial activities of BSF chitosan, followed by *B. cereus*, *K. pneumoniae*, *S. enterica*, and the most susceptible species were *P. aeruginosa* and *S. marcescens*. In addition, BSF chitosan also showed inferior antibacterial activity in comparison to commercial chitosan. Different concentrations of commercial chitosan had successfully inhibited the proliferation of a variety of bacterial species, with the minimum concentration of 0.031% was found. The six bacterial species tested could be classified in the following order of sensitivity towards commercial chitosan: *K. pneumoniae* > *S. aureus* = *B. cereus* = *P. aeruginosa* > *S. marcescens* > *S. enterica*. In this study, the results showed that both BSF chitosan and commercial chitosan could reduce bacterial growth in which the chitosan concentration was confirmed to play an essential role in their antibacterial activities, though their MICs varied accordingly with the bacterial species, suggesting variable sensitivities of different microbes to the antibacterial agents.

Previous studies presented MIC values of 0.75 mg/mL, 0.01 mg/mL, and 0.25 mg/mL for *S. aureus*, *K. pneumoniae*, and *Salmonella typhi*, respectively (Younes et al. 2014). Benhabiles et al. (2012) reported MIC values of 0.1 mg/mL and 0.5 mg/mL for *B. cereus* and *P. aeruginosa*. On the other hand, Wang (1992) obtained the result with a chitosan concentration of 1 - 1.5% for complete inhibition of *S. aureus*. Others had documented the antibacterial activity of chitosan against a wide range of microorganisms, with MICs ranging from 0.1% to 1.5% (w/v) (Kanatt, Chander & Sharma 2008; No et al. 2002; Sagoo et al. 2002).

The MIC values for BSF chitosan obtained in this study were relatively higher than the commercial chitosan, as well as those reported in previous literature. This could be due to the use of BSF chitosan compounds consisting of poorly characterized mixtures in testing of their bioactivities, which resulted in poor reproducibility of the biological response. The presence of impurities in the compounds could have attributed to a higher concentration needed to inhibit the bacterial growth. Apart from that, the high MICs might be due to the fact that BSF chitosans could not attain 100% solubility in the 0.1% acetic acid solution due to their relatively high molecular size and weight. Smaller amounts of BSF chitosan are expected to exhibit antibacterial activity if the purified forms of fine chitosan compounds could be produced.

It was worth noting that the antibacterial activity of chitosan was a complicated process, and both intrinsic and extrinsic factors, such as the distinct experimental conditions, the bacteria and chitosan sources, molecular weight (MW) and degree of deacetylation (DD) of chitosan, the interacting pH, and the solvents employed were among the other variables that probably accounted for the aforementioned differences in the MIC values. The MW and DD of chitosan often vary accordingly with the factors governing the extraction process, including the time of reaction, temperature, chemical concentrations, and shear stress (Terbojevidh & Cosani 1997), thereby affecting the solubility, chemical and biological reactivity of the final chitosan biopolymers, particularly their antibacterial activities in the present study. As a result, it is very difficult to ascertain the definitive MICs required to exhibit effective inhibitory action due to the variability in the DD and polymerization of the chitosan batches.

Conclusion

The objectives of the study were achieved with the successful isolation of chitin and chitosan from BSF as well as the determination of the antibacterial activities of the chitosan products. Chitin polymers were successfully extracted from different growth phases of the BSF. Chitin content was found to gradually increase when transitioning from larvae to adult flies. The pupae and imagoes of BSF were considered more suitable than larvae as the alternative chitin source due to their higher chitin contents. The antibacterial tests carried out by turbidity measurements demonstrated that the four BSF chitosans exhibited identical antibacterial activities against all the pathogenic bacterial strains tested, except for *Staphylococcus aureus*. Compared to that of the commercial chitosan, BSF chitosan showed relatively weaker antibacterial activity in the conditions here tested, in which their MIC values were much higher than that of the commercial chitosan. The apparent antibacterial activity of BSF chitosan against pathogenic bacteria provided a promising input for the purification of effective antibacterial compounds of natural origin from black soldier flies. Further work of characterization is needed to produce a more detailed chemical profile for the chitosan compounds and as an informative guide for subsequent utilization of BSF chitosans in various applications. It is recommended to further explore other possible biological activities of the BSF chitosan (e.g., antioxidant and antitumor activities) for enhanced exploitation of their commercial value.

Acknowledgements

This research was supported by funding from Fundamental Research Grant Scheme (FRGS) by the Ministry of Higher Education (FRGS/1/2019/STG05/UCSI/03/1) and an internal university grant (PSIF grant No. PROJ-2019-In-FAS-063) of UCSI University KL. The authors are grateful to Mr Giva Kuppusamy from Crops for the Future (CFF) for supplying us with BSF and to Mr. Dewainjeran for his assistance in the laboratory during this research.

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