SINAI Journal of Applied Sciences 12 (3) 2023 389-398



Available online at <u>www.sinjas.journals.ekb.eg</u>
SCREENED BY SINAI Journal of Applied Sciences
IThenticate
Print ISSN 2314-6079

Print ISSN 2314-6079 Online ISSN 2682-3527



NANOFIBERS LOADED WITH NANOCHITOSAN OF FUNGAL ORIGIN: SYNTHESIS, CHARACTERIZATION, AND ANTIMICROBIAL ACTIVITY OF NANOCHITOSAN FROM *Aspergillus niger*

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ARTICLE INFO Article history: Received: 06/05/2023 Revised: 19/05/2023 Accepted: 01/06/2023

Keywords: Fungal chitosan, Nanoparticles, Nanofibers, Physical properties, Antimicrobial activity. ABSTRACT Natural biopolymers are attracting the researcher's attention because of their magnificent properties and various biological activities. Among them, chitosan is the deacetylated form of chitin which is present in the fungal cell wall. In the present study, chitosan was extracted from Aspergillus niger on malt yeast broth (MYB) with yield 8%, degree of acetylation 69.5 % and molecular weight 2223.11 Da. Nano-chitosan was prepared through ionic gelation method then characterized through dynamic light scattering which revealed that the zeta potential was 37 mV and polydispersity index (PDI) reached 0.48 while transmission electron microscope (TEM) showed the spherically shaped prepared nanoparticles had particle size ranged from 1.77 to 8.17 nm. Thermoplastic Polyurethane (TPU) fibers loaded with chitosan and nano-chitosan were synthesized through electrospinning then morphologically characterized by scanning electron microscope (SEM) showing that chitosan loaded TPU fibers were comparatively rougher than that of nano-chitosan loaded TPU fibers which were quite smooth. The Limiting oxygen index (LOI) value of chitosan loaded TPU fibers was only 20.8% and the UL-94 resulted in V-1. However, the LOI value of nanochitosan loaded TPU fibers increased up to 26.6% and the UL-94 result achieved V-0 rating. Moreover, the tensile strength of the chitosan loaded TPU fibers was 12.3 ± 0.2 MPa, while the nano-chitosan loaded TPU fibers was 17.9 ± 0.5 MPa. Finally, nano-chitosan loaded TPU fibers showed the maximum antibacterial activity with inhibition zone diameter reached 40 mm and MIC value 15.6 µg/mL.



INTRODUCTION

Chitin is the main precursor of chitosan which is a natural biodegradable biopolymer. Various types of applications had been monitored *e.g.* water engineering, food and nutrition, medical applications biotechnology and in gene therapy recently (**Thambiliyagodage** *et al.*, **2023**). Due to seasonal and limited supply, processing challenges, particularly with the large amount of waste of concentrated alkaline solution causing environmental pollution, and inconsistent physicochemical properties, chitosan production commercially by crustacean chitin deacetylation with strong alkali seems to have a low acceptance for industrial potential. However, chitosan from fungi has been focused on in new studies. A larger possibility for more reliable products exists with the manufacture and purification of chitosan from the cell walls

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of fungus cultivated under controlled conditions (Tayel *et al.*, 2011).

Nowadays, citric acid industrial scale production is almost exclusively accomplished by *Aspergillus niger*. Annual global production exceeds 600,000 metric ton (**de Oliveira** *et al.*, **2022**). Isolation of chitin and/or chitosan could be done by fermentation industries of fungi with their mycelia produced as a promising source (**Tayel** *et al.*, **2011**).

In our daily lives, synthetic polymeric materials are employed extensively. Despite this, chitosan has a poor microbial resistance and flammable upon exposing to a heat source (Andrew and Dhakal, 2022), several additives have been employed to enhance the antimicrobial activity and eliminate flammability effect (Qi et al., 2022). Chitosan possesses the capacity of promoting the char formation of the matrix during combustion due to its carbon and composition. nitrogen Chitosan nanoparticles, being biocompatible, nontoxic, versatile, and biodegradable, attracted researchers the attention of in the biomedical field (Ahmed et al., 2021; Anderson et al., 2022). Mohamed (2022) tested chitosan nanoparticles' different concentrations efficacy in enhancing the cold shelf life of kareish cheese against microbial contamination. It was reported that chitosan nanoparticles showed high antimicrobial effect in a dose dependent manner.

The present study aimed to synthesize Thermoplastic Polyurethane (TPU) nanofibers loaded with nano chitosan of fungal origin for biomedical applications.

MATERIALS AND METHODS

Fungal Isolation and Identification

The fungal strain (*Aspergillus niger*) used in this study was isolated from soil and identified with GenBank Accession

number MT597434.1 (Abdelalatif *et al.*, 2023).

Cultivation Media and Culture Conditions

Malt yeast broth (MYB) was used for the cultivation of *Aspergillus niger*. The incubation period was 7 days at 28°C to allow the formation of fungal mats (**George** *et al.*, **2011**).

Chitosan Extraction and Purification

The fungal mycelia were harvested and 50 ml of 1N sodium hydroxide (NaOH) solution was added per g (dry weight) of mycelia and homogenized. The content was sterilized at 121°C for 20 minutes (alkali treatment). Centrifugation at 6000 rpm for 20 min was done to collect the alkali insoluble materials (AIM), AIM was then washed with distilled water until the pH was neutralized (pH 7) followed by dryness at 40°C. Dried AIMs were treated (1:30 W/V) with 2% acetic acid (chitosan solvent), under reflux conditions for 8 hours at 95°C. The insoluble part was separated by centrifugation at 6000 rpm for 15- 20 min while the supernatant (containing the chitosan) was collected and treated with 2N NaOH solution until the pH reached 10 to precipitate the fungal chitosan. The flocculated chitosan was then centrifuged at 6000 rpm, for 15 min. The isolated chitosan was washed four to five times with distilled water to neutralize it. After that, ethanol (96%) and acetone were employed to rinse the chitosan and then it was dried in a vacuum oven dryer at 60°C (Chatterjee et al., 2005). The crude chitosan yield was calculated from the following equation:

Chitosan yield (%) = [dry wt. of obtained chitosan/dry wt. of sample] x 100

Chitosan Characterization

Chitosan produced samples were characterized in KBr pellets by using FTIR (Model FTIR-6100) in the range of 400 to 4000 cm^{-1} (George *et al.*, 2011). As well as the estimation of degree of deacetylation

and chitosan molecular weight (**Pochanavanich and Suntornsuk, 2002**).

Chitosan Nanoparticles Preparation

TPP solution in deionized water at a concentration of 1.0 mg/mL, pH 5.0 was prepared. In addition to chitosan (3.0 mg/mL) was mixed in diluted acetic acid (0.5%) and stirred at room temperature for 20 minutes. After bringing the pH down to 5.0, the solution was filtered using Gooch crucible (AG 1 x 3) vacuum filtration to remove any remaining insoluble particles. Under magnetic stirring at 600 rpm for 60 minutes, chitosan solutions were added dropwise to TPP solutions in a 3:1 ratio, creating chitosan nanoparticles. After adjusting the cycle and amplitude (using Hielscher Ultrasonics GmbH, Teltow, Germany), the mixture was sonicated for 5 minutes before being analyzed (Hejjaji et al., 2018).

Characterization of Nano-chitosan Particles

Nano chitosan particles were characterized Using Transmission electron microscopy (JEM-100 CX Joel) (Lee *et al.*, 2014), FTIR (George *et al.*, 2011), zeta potential and poly-dispersity index (Hejjaji *et al.*, 2018).

Preparation of Chitosan and Nanochitosan Loaded TPU fibers

Melt mixing and hot compression molding were used to create samples of TPU composites. A micro twin-screw extruder (Wuhan Rayzong Ming Plastics Machinery Co., Ltd., China) at 40 rpm was used to melt and mix TPU and chitosan or nano-chitosan. The extrusion temperatures ranged from 170°C to 175°C to 180°C. For 20 minutes at 10 MPa and 175°C, the pelletized extrude was hot pressed in a molding machine. The sample was then subjected to 20 minutes of cold pressing at 20 MPa at room temperature (**Zhang et al., 2018**).

Characterization of Chitosan and Nano-chitosan Loaded TPU Fibers

Morphological characterization

Fiber morphology was observed with a scanning electronic microscope (SEM) (JSM-5600) at an accelerated voltage of 10 kV (**Huang** *et al.*, **2011**).

Flammability test

The limiting oxygen index (LOI) values were determined by using a JF-3 instrument according to ASTM D2863-97. While the UL-94 vertical burning tests were conducted by using a CZF-3 instrument according to ASTM D3801 with a sample thickness of 3.2 mm (Liu *et al.*, 2019).

Tensile strength

TPU fibers loaded with chitosan or nano-chitosan were tested for tensile strength in three different orientations: random, parallel, and perpendicular. All samples were the same size (30 mm x 10 mm), and the tests were run at room temperature (20°C) and humidity (65%) using a universal materials tester (H5 K-S, Hounsfield, UK) equipped with a 50 N load cell. For each sample, the cross-head speed was set at 10 mm/min (**Liu** *et al.*, **2019**).

Antibacterial Activity of Chitosan, Nano-chitosan, Chitosan Loaded TPU Fibers and Nano-chitosan Loaded TPU Fibers

Antibacterial activity was carried out using disc-diffusion method according to CLSI guidelines (**Abbey and Deak, 2019**). By measuring the lowest inhibitory concentration (MIC), minimum bactericidal concentration (MBC) (**Elshaer** *et al.*, **2022**).

RESULTS

Chitosan Production and Characterization

The growth of *A. niger* on MYB was observed for 7 days. The chitosan yield was 18% while the molecular weight was 2223.11 Da and the degree of acetylation was 69.5%. Extracted chitosan structure was confirmed by FTIR analysis. In general, chitosan shows bands at 3000-3500 cm⁻¹ attributed to O–H stretching and a significant band of amide I and amide II are located at 1653 cm⁻¹ and 1436 cm⁻¹ respectively. Finally, the C–N fingerprint band appears at the range of 800 - 810 cm⁻¹ (Fig. 1).

Nano-chitosan Preparation and Characterization

The prepared nano-chitosan was characterized by TEM revealing that the particle size ranged from 1.77 - 8.17 nm (Fig. 2). Moreover, the zeta potential was 37 mV and the PDI was 0.48.

Preparation and Characterization of Chitosan and Nano-chitosan Loaded TPU Fibers

SEM of chitosan and nano-chitosan loaded TPU fibers

According to the results shown in Fig. 3, the surface of chitosan loaded TPU fibers was noticeably rougher than that of nanochitosan loaded TPU fibers. We can show that nano-chitosan has the potential to increase the spinnability of polymer solutions by analyzing their surface morphologies.

Flammability test

Chitosan and nano-chitosan loaded TPU fibers' LOI and UL-94 findings are shown in Table 1. Chitosan-loaded TPU fibers only achieved a LOI of 21% and a UL-94 rating of V-1. The UL-94 result for nano-

chitosan loaded TPU fibers, on the other hand, allows for a V-0 grade, which indicates a much higher LOI value (up to 26.6%). In general, nano-chitosan is more effective than chitosan for lowering TPU's flammability.

Tensile strength

The tensile strength of the chitosan loaded TPU fibers was 12.3 ± 0.2 MPa, while the nano-chitosan loaded TPU fibers was 17.9 ± 0.5 MPa (Table 2). Therefore, nano-chitosan enhances the characteristics of TPU by increasing the average tensile strength and showing better elasticity than the chitosan loaded TPU fibers.

Antibacterial Activity of Chitosan, Nano-chitosan, Chitosan Loaded TPU Fibers and Nano-chitosan Loaded TPU Fibers

Results in Table 3 reveal that the inhibition zone diameters (IZD), MIC and MBC of all tested samples ranged from 12.0 - 40.0 mm, 15.6 - 500 µg/ml and 31.2 - 1000 µg/ml, respectively. Nano-chitosan loaded TPU fibers has showed the highest IZ diameter and the lowest MIC value 40 mm and 15.6 µg/ml, respectively against *Escherichia coli* 25922 and *Klebsiella pneumonia* 13883. Therefore, nano-chitosan loaded TPU fibers has a potent antibacterial activity followed by chitosan loaded TPU fibers, nanochitosan and chitosan.





Fig. 1. FTIR of chitosan extracted from Aspergillus niger on MYB



Fig. 2. Nano-chitosan particles under transmission electron microscope







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Fig. 3. SEM of chitosan (a & b) and nano-chitosan (c & d) loaded TPU nanofibers

Sample	LOI (%)	UL-94		
Chitosan loaded TPU fibers	21.0 ± 0.1	V-1		
Nano-chitosan loaded TPU fibers	29.9 ± 0.3	V-0		

Table 1. Flammability test of chitosan and nano-chitosan loaded TPU fibers

Table 2. Tensile strength of chitosan and nano-chitosan loaded TPU fibers

Sample	Average tensile strength (MPa)				
Chitosan loaded TPU fibers	12.3 ± 0.2				
Nano-chitosan loaded TPU fibers	17.9 ± 0.5				

Table 3. Antibacterial activity of chitosan, nano-chitosan, chitosan loaded TPU fibers and nano-chitosan loaded TPU fibers

Sample / Pathogen	Chitosan		Nano-Chitosan		Chitosan loaded TPU fibers			Nano-chitosan loaded TPU fibers				
	IZD (mm)	MIC (µg/ml)	MBC (µg/ml)	IZD (mm)	MIC (µg/ml)	MBC (µg/ml)	IZD (mm)	MIC (µg/ml)	MBC (µg/ml)	IZD (mm)	MIC (µg/ml)	MBC (µg/ml)
Escherichia coli	18 ±	± 125 3	250	25 ± 0.1	75	150	27 ± 0.4	50	100	40 ± 0.1	15.6	31.2
25922	0.3											
Proteus mirabilis	14 ±	250	750	22 ±	100	300	26 ±	75	150	35 ±	62.4	125
35659	0.2	2		0.4			0.5			0.3		
Staphylococcus aureus	17 ± 0.6	125	375	21 ± 0.2	150	450	27 ± 0.3	75	225	$\begin{array}{c} 35 \pm \\ 0.2 \end{array}$	31.2	93.6
25923												
Klebsiella pneumonia	12 ± 0.2	500	1000	20 ± 0.3	150	300	26 ± 0.2	100	200	$\begin{array}{c} 40 \pm \\ 0.1 \end{array}$	22	44
13883												

DISCUSSION

levels Because of its high of biocompatibility, biodegradability, and antibacterial characteristics (Yang et al., chitosan-based materials 2021). have garnered a lot of interest for usage in biomedical settings. Since chitin and chitosan are found in the cell walls of many fungi, including Aspergillus niger, their mycelium has been investigated as potential sources (Ke et al., 2022). Similar results were found by Ghormade et al. (2017) who reported that the yield of chitosan isolated from A. niger was 10%, the degree of acetylation was 64-90%, and the molecular weight was 2.7×10^3 Da. Also, compared to solid state fermentation, biomass production doubles during submerged fermentation (Crognale et al., 2022). Another research found that the A. niger chitosan production was 11.64 percent, with an acetylation level of 86.4 percent.

Peaks in the FTIR spectrum of chitosan were observed at 3406 cm⁻¹ (-OH stretch), 2920 cm⁻¹ (C-H stretch), 1651 cm⁻¹ and 1635 cm⁻¹ (N-H bend), and 1068 cm⁻¹ (C-O stretch) (Tavel et al., 2011). Nano-chitosan particles display a wide peak at 3419-3467 cm⁻¹ due to -OH and water stretching vibrations, which is consistent with the findings of Calderón et al. (2013), who reported that the size of nano-chitosan particles varied from 30-300 nm with PDI 0.5 and zeta potential +35 mV. C-H stretching vibrations caused peaks between 2870 and 2937 cm⁻¹. Furthermore, research has shown that the size of nano-chitosan particles is between 200 and 2500 nm (Sreekumar et al., 2018).

Zhang *et al.* (2018) reported that Nanochitosan loaded TPU achieved LOI of 28.6% and UL-94 V-0, whereas results for neat TPU were 20.8% and no rating in the UL-94 test. Additionally, TPU and nanochitosan loaded TPU demonstrated tensile strengths of 15.5 and 19.2 MPa, respectively. In contrast to the control, the morphological characteristics of the TPU surface loaded with fungal chitosan showed no evidence of agglomerated particles, suggesting uniform distribution of the fungal chitosan in the coating layer and the absence of undesirable agglomeration during coating formation (Tayel et al., 2011). Chitosan and nanochitosan were shown to be more effective against Gram-negative (E. coli) than Grampositive (S. aureus) bacteria in terms of their antibacterial properties. Gram-positive bacteria, in contrast, have a peptidoglycan cell wall that is both thicker and stiffer. Therefore, Gram-positive bacteria are less susceptible to antibacterial effects (Shirvan et al., 2014). Both fungal chitosan loaded TPU and nano-chitosan loaded TPU were shown to have MICs of 2.25 mg/ml and 1.75 mg/ml against E. coli, respectively, in a different investigation (Tayel et al., 2011).

Conclusions

A. niger produces high yield of chitosan with high molecular weight. Therefore, the synthesized nano-chitosan had stable and small particle size. Nano-chitosan has improved the properties of the TPU fibers by making it smother, improve spinnability of polymer solution, reduce the flammability, increase the average tensile strength, and increase the antibacterial activity.

Author Contributions: B.H.E., R.A.E., M.H.E. and M.El-K. Conceptualization, methodology, validation, resources, data curation, writing-original draft preparation. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

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

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الملخص العربى

ألياف نانوية محملة بنانو شيتوزان من أصل فطري: التخليق والتوصيف والنشاط المضاد للبكتيريا للشيتوزان النانومتري من فطر Aspergillus niger

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لقد جذبت البوليمر ات الحيوية الطبيعية انتباه الباحثين بسبب خصائصها الرائعة ونشاطها البيولوجي المتنوع. من بينها، الشيتوزان وهو الشيتين المنزوع الأسيتيل الموجود في جدر الخلايا الفطرية. في هذه الدراسة تم استخلاص الشيتوزان من دالتون. تم تحضير الشيتوزان النانومتري بطريقة الهلام الأيوني ثم تم تمييزه من خلال تشتت الضوء الديناميكي الذي أظهر أن جهد زيتا كان ٣٧ مللي فولت ووصل PDI إلى ٤٨، بينما أظهر مجهر الإلكترون النافذ (TEM) أن الجسيمات الناتومترية المحضرة كانت كروية بحجم جسيم يتراوح من ١٩،٧ إلى مهمر الإلكترون النافذ (TEM) أن الجسيمات الناتومترية المحضرة كانت كروية بحجم جسيم يتراوح من ١٩،٧ إلى ١٩،٧ مناومتر. تم تصنيع ألياف البولي يوريثين بالحرارة (TEU) المحملة بالشيتوزان والنانو شيتوزان من خلال الغزل الكهربائي ثم تم تمييزها شكيًا عن طريق المجهر الإلكتروني الماسح (SEM) مما وضح أن ألياف TPU المحملة بالشيتوزان كانت أكثر خشونة نسبيًا من ألياف المحملة بالحرارة (TEU) المحملة بالشيتوزان والنانو شيتوزان من خلال الغزل الكهربائي ثم تم تمييزها شكيًا عن طريق المجهر بعد والي الماسح (SEM) مما وضح أن ألياف TPU المحملة بالشيتوزان كانت أكثر خشونة نسبيًا من ألياف العولي بوزان المحملة بالنانو الشيتوزان والنانو شيتوزان من خلال الغزل الكهربائي ثم تم تمييز من ألياف البولي الاك بالشيتوزان (٢٠,٠٦ فقط وأدى الت عاصة تمامًا. كانت قيمة مؤشر الأكسجين المحدد (LOI) لألياف TPU المحملة بالشيتوزان المحملة بالنانو والتي كانت ناعمة تمامًا. كانت قيمة مؤشر الأكسجين المحدد (LOI) لألياف LOI المحملة بالشيتوزان بنسبة تصل إلى ٢,٢٦ ± ٢، ميجا باسكال، بينما كانت ألياف LOI المحملة بالنانو الشيتوزان بالشيتوزان المرابي المحملة بالنانو الشيتوزان ألما محملة بالنانو الشيتوزان المحملة بالنانو الشيتوزان بنسبة تصل إلى ٢,٦ ٢ لي حرام المان الياف تاصات المحملة بالنانو الشيتوزان المحملة بالنانو الشيتوزان بنسبة تصل إلى ٦,٦ ٢ ميجا باسكال، بينما كانت ألياف لكاح المحملة بالنانو الشيتوزان المحملة بالنانو الشيتوزان بنسبة مرح المرم الي المحملة بالنانو الشيتوزان ألم من ما محملة بالنانو الشيتوزان المحملة الشر لألياف للعال المحملة بالشيتوزان المحملة بالنانو الشيوزان ألم محملة بالنانو الشيتوزان المرضة مع قطر منطقة التثبيط بلغ عررا محمر المحمر مرمرم إلى العام العام المحما المحماد البكار مح

الكلمات الإسترشادية: الشيتوزان الفطري، الجسيمات النانوية، ألياف نانوية، الخصائص الفيزيائية، نشاط مضادات الميكروبات.