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Advances in extraction and biological activities of crawfish chitosan and its application in decolorization of synthetic dyes

[*Avanços na extração e atividades biológicas da quitosana de lagostim e sua aplicação na descoloração de corantes sintéticos*]

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ABSTRACT

An eco-friendly method of extraction for chitin and chitosan extracted from crawfish was our goal. Chitin is always present with proteins, minerals, and other components. This study used an eco-accommodating, novel technique for chitin and chitosan extraction. *Lactobacillus lactis* was used for the deproteinization and demineralization of chitin in a single stage by *Saccharomyces cerevisiae* (BB: biological–biological extraction) to convert chitin into chitosan. BB is a more environmentally friendly method of producing chitosan than deacetylation with NaOH (BC: biological–chemical extraction). Chitosan was characterized by FTIR. A high degree of deacetylation (%) was observed. The UV spectrum for chitosan was similar at 0.788, 0.415, and 1.150 for CC, BC, and BB, respectively. The results show that chitosan (BB) has potential applications in the biomedical fields such as antioxidant activity, anticancer activity against human liver cancer (HepG2), breast cancer (MCF-7) and human hepatocellular carcinoma (HCT) cell lines. The results in terms of water treatment and removal of dyes using chitosan (BB) are valuable in terms of its application in industrial wastewater treatment and demonstrate that it can be used as a biosorbent.

Keywords: chitin, chitosan, crustacean, anticancer, antioxidant, water treatment*; Lactobacillus lactis, Saccharomyces cerevisiae*

RESUMO

O objetive deste trabalho era criar um método de extração ecologicamente correto para a quitina e a quitosana extraídas do lagostim. A quitina sempre está presente com proteínas, minerais e outros componentes. Este estudo usou uma técnica nova e ecologicamente correta para a extração de quitina e quitosana. Lactobacillus lactis foi usado para a desproteinização e desmineralização da quitina em um único estágio por Saccharomyces cerevisiae (BB: extração biológico-biológica) para converter a quitina em quitosana. O BB é um método mais ecológico de produção de quitosana do que a desacetilação com NaOH (BC: extração biológico-química). A quitosana foi caracterizada por FTIR. Foi observado um alto grau de desacetilação (%). O espectro de UV para quitosana foi semelhante a 0,788, 0,415 e 1,150 para CC, BC e BB, respectivamente. Os resultados mostram que a quitosana (BB) tem aplicações potenciais nos campos biomédicos, tais como atividade antioxidante, atividade anticancerígena contra o câncer de fígado humano (HepG2), câncer de mama (MCF-7) e linhas celulares de carcinoma hepatocelular humano (HCT). Os resultados em termos de tratamento de água e remoção de corantes usando quitosana (BB) são valiosos em termos de sua aplicação no tratamento de águas residuais industriais e demonstram que ela pode ser usada como biossorvente.

Palavras-chave: quitina, quitosana, crustáceo, anticâncer, antioxidante, tratamento de água; Lactobacillus lactis, Saccharomyces cerevisiae

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INTRODUCTION

Crawfish and crustacean waste, and even the entire organic- rich shellfish no longer need to be considered as disposable "waste" products with low economic value. On the contrary, they should be thought of as profitable alternatives that can lead to valuable commercial products (Fernandez-Kim, 2004). Nowadays wastes from the world's fisheries go beyond twenty million tons, which almost equal twenty-five percentage of the total fisheries production (Hayes *et al*., 2008).

Crawfish waste represents a significant and renewable major resource in the form of the biopolymer's chitin and its deacetylated form, chitosan (Hamdi *et al*., 2024; Hamed *et al*., 2016) Permitted limits, high costs, and environmental complications regarding the disposal of marine processing waste have led to improved importance in biotechnology research regarding the sorting as well as extraction of further high-quality, low-volume biomolecules produced from shellfish waste treatments. Waste containing exoskeletons o crustacean is now considered as the chief source of biomass for many important industries such as chitin production (Abd El-Ghany *et al*., 2023).

Chitosan is recognized as partially deacetylated chitin form, but it may be with higher water soluble than chitin, and much more easily to be processed. Therefore, the comparatively small size chitosan are the molecules that are planned for multiple applications in many fields such as: agriculture; water and wastewater treatment; nutrition and drinks; feed; chemicals; and personal care (Zuber *et al*., 2013; Rinaudo, 2006). Additionally, chitosan has been considered as bioactive compounds [8], offering potential for application in, for instance, wound dressing and cosmetics. Chitosan is considered as important biomaterials. However, the process including extraction as well as purification of chitin and its subsequent conversion to chitosan (oligomers) need numerous processing steps (Zargar *et al*., 2015; Bastiaens *et al*., 2019).

A chemical extraction process is usually common for chitin extraction which comprises three main steps including demineralization, deproteination, and finally deacetylation, by using concentrated acids and alkalis under raised temperatures. Such processing conditions require high amounts of energy and are associated with several negative implications such as an increase in the chitin purification cost and impaired physiochemical properties of the extracted product (Dhillon *et al*., 2013).

Biological extraction is defined as green extraction processes that are centered on the concept of 'green chemistry' which is gaining more consideration, by using microbes and/or microbial enzymes for chitin and chitosan extraction.

Chitosan and chitosan products have many applied applications in a diverse range of fields, comprising the food and nutrition industries, aquaculture, agriculture, medicine, pharmacy, biomedicine, cosmetology, bio-imaging, veterinary medicine, the paper industry, the textile and fiber industries, chromatography, the beverage industry, photography, wastewater treatment, sludge dewatering, biotechnology, and nanotechnology (Morin-Crini *et al*., 2019; Zhao *et al*., 2010; Hamdi *et al*., 2022a).

Nowadays, much more research studies are needed on both extraction of chitin and its subsequent bioconversion to chitosan from crustacean wastes, principally on the conditions necessary to reach high-grade chitosan. The objectives of this study are to determine the ideal conditions for chitosan synthesis from crawfish waste, as well as to investigate the qualities, properties, and uses of chitosan in the treatment of tap water, contamination of which is the primary cause of communicable illnesses. Germs and chemicals can enter drinking water after it has been treated, either at the source or in the distribution system. Harmful microorganisms and chemicals can contaminate tap water from a diversity of sources, including chemical fertilizers, insecticides, and other chemicals applied to land near the water. Therefore, tap water treatment is required to eradicate these dangerous bacteria and biologically extracted chitosan is considered good candidate for water treatment (Sarbon *et al*., 2014).

MATERIALS AND METHODS

Local fishermen gathered the crawfish *Procambarus clarkia* (Hamdi *et al*., 2022b) from the Nile River, and stored and delivered them on

ice. The muscles and viscera of crawfish were removed from the exoskeletons, followed by a tap-water wash. Exoskeletons were heated to a boil for 15 minutes, dried, and then incubated at 50°C for ten hours. The samples were then dried at room temperature before being crushed in a grinder. Samples were stored out of the light in clear nylon bags with silica preservation sacks (Girard, 1852).

Bacteria (*Lactobacillus lactis*—ACADC 178—accession number: LS991409), and fungus (*Saccharomyces cerevisiae*—accession number: 006-001) were purchased from the Centre of Microbiological Resources (Cairo Mircen), Faculty of Agriculture, Ain Shams University, Egypt (Hamed *et al*., 2016).

The subculture of *L. lactis* was carried out on MRS Agar medium containing: Meat extract 8.0 g, $MnSO_4$ 0.04 g, yeast extract 4.0 g (MERCK), $MgSO_4$ 0.2g, casein peptone 10.0 g, $C_6H_{14}N_2O_7$ 2.0 g, CH_3COONa 5.0 g, D(+)glucose 20.0g and tween 80 1.0g. Then, the liquid medium is usually solidified by adding 15g/L and incubating it for 48–72 h in the presence of 5% CO_2 (Khanafari *et al*., 2007).

Czapek Dox agar medium was used for the maintenance and cultivation of *S. cerevisiae*; the medium composition is [K_2HPO_4 1g, sucrose 20g, KCl 0.5 g, $FeSO_4.7H_2O$ 0.01g, $NaNO_3$ 2g, $MgSO_4.7H_2O$ 0.5g, agar 15g] (Ali *et al*., 2012). The media was incubated for 72 h at 37 °C, and the solidification step was excluded. The two microbial isolates are approved for human consumption by the FDA (Wigner *et al*., 2022). In addition, *S. cerevisiae* is considered as safe and belonging to biosafety level one (Fialho *et al*., 2009).

Chemical extraction was accomplished in three steps. The first step was deproteinization, by adding 1 g of crustacean powder to 9 ml of 5% NaOH wt/vol.; the second step was demineralization, using 25mL of 1N HCl to deproteinize the powder; and the third step was deacetylation, where 20mL of NaOH 50%wt/vol. was added to the previous flask (Arbia *et al*., 2013).

Lactobacillus lactis was subcultured and maintained using sterilized M.S. media. *Lactobacillus lactis* was used to deproteinize and demineralize the sample with crustacean shells. It was then filtered, and *Saccharomyces cerevisiae* performed an eco-friendly deacetylation process, instead of NaOH 50%wt/vol. (Zhao *et al*., 2010).

Lactobacillus lactis was subcultured and maintained using sterilized M.S. media. *Lactobacillus lactis* was used to deproteinize and demineralize the sample with crustacean shells. It was then filtered, and after a period of time, the deacetylation step was carried out using 50% NaOH (Pal *et al*., 2014).

The spectra of FTIR of the commercial chitosan and extracted chitosan (biological, chemical) were obtained to confirm the structure of chitosan by FTIR graph analysis, according to the method described by Fernandes Queiroz *et al*. (2014).

The extracted chitosan elemental analysis by different methods was performed using an elemental analyzer, and approximately three mg of chitosan was used (Ming-Tsung *et al*., 2009). The elemental analysis procedure was usually used for determination of the percentage of carbon, hydrogen, and nitrogen in samples using the three extraction methods.

After the samples were dried, their solubility was evaluated to make sure that chitin had been converted to chitosan Bauer *et al*., 1966).

$$\text{Solubility} = \frac{500 - m1}{0.5\text{m1} = \text{ mass of undissolved solids (mg)}}$$

After adding 0.1g of each sample, they were dissolved in 1% and 5% acetic acid. The remaining undissolved chitosan was then collected using gravity filtration, rewashed with acetone, and then dried at 40°C under vacuum overnight before being weighed.

Using double-distilled water at 25°C and stirring for 5 hours to create a saturated solution, the sample was then suspended. Undissolved solids were at that point recovered by gravity filtration, washed with acetone, and dried at 40°C under vacuum overnight before being weighed.

The degree of deacetylation of chitosan was determined using infrared spectroscopy as per

the method described by Ibrahim *et al*. (2019). After weighing 0.1g chitosan powder, then dissolving it in 25mL of 0.06 M for 1 h at room temperature using a magnetic stirrer, the solution was diluted to 50 ml, titrated against 0.1 NaOH to pH 3.75 with continuous stirring, and the volume of NaOH at pH 3.75 was recorded. Titration to pH 8 was then carried out and the NaOH volume was recorded (Ibrahim *et al*., 2019).

$$DD = \frac{161.16(v2 - v1)N}{w1}$$

DD: degree of deacetylation 161.16: mass of chitosan monomer
V_1,V_2: NaOH volumes N: strength of NaOH (0.1M)
W1: mass of sample after correction for moisture (300g)

The thermal stability of the biologically extracted chitosan sample (BB extracted) was measured in the National Research Center for Housing and Building, Egypt, using a Shimadzu TGA-50H instrument under a nitrogen atmosphere with a warming rate of 10°C/min (Voitovich *et al*., 1994).

The viscosity was measured for the biologically extracted chitosan (BB extracted) using an Ostwald capillary viscometer (Shehata, *et al.*, 2023). The relative viscosity was calculated as follows: Chitosan polymer was dissolved in 5% acetic acid and 1 M KCl to obtain the intrinsic viscosity used in molecular weight calculations. Chitosan viscosity was measured for different concentrations (0.1%, 0.2%, 0.3%, 0.4 %, 0.5 %) to establish a standard curve; then, the intrinsic viscosity was calculated.

The molecular weight was calculated using the following equation:

$$(\eta) = KMa.$$

(K) and (a) are empirical viscometric constants, at which, K= 1.81 X 10-3 ml/g and a= 0.93.

The antioxidant potentiality of the extracted chitosan from crawfish exoskeletons was estimated against a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described by Navarro-Hoyos *et al*. (2018). To evaluate the potential antioxidant activity of the extracted chitosan, the following steps were performed:

Sample preparation: The sample was prepared at a final concentration of 7.8125, 15.625, 31.25, 62.5, and 125µg/mL in distilled water. Trolox standard preparation: A solution with concentration 20µg/mL of Trolox was prepared in methanol as stock solution, from which, five concentrations were set with concentration 12.5, 9.375, 6.26, 3.125, and 1.5625µg/mL.

Procedure: DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical assay was performed according to the following procedure: Briefly, 100µL of freshly prepared DPPH reagent (0.1% in methanol) was added to 100 µL of the sample in 96-well plate (n=6); the reaction was incubated at room temperature for 30 min in the dark (Al-Qaysi *et al*., 2021). At the end of the incubation time the resulting reduction in DPPH color intensity was measured at 540 nm. Data are represented as means according to the following equation: percentage inhibition = ((Average absorbance of blank-average absorbance of the test)/(Average absorbance of blank))*100

Cell lines were purchased from the National Cancer Institute and were maintained as a "monolayer culture" using RPMI medium augmented with 10% FBS and 2% Pen/Strep. The incubation temperature was set at 37 °C in 5% CO2 in a high-humidity atmosphere in an incubator (Thermo Fisher Scientific USA) as mentioned by Zhao *et al*. (2010).

The lines were repetitively sub-cultured to retain them in the log phase. Sterile conditions were achieved by working in an equipped laminar flow cabinet (Microflow Laminar flow cabinet, MDH limited, Hampshire SP10 5AA, U.K.). Cells were grouped into the control group and treatment groups with different concentrations of the drug (12.5, 25, 50, and 100µg/mL)

After twenty-four hours, we added ten µl of the MTT reagent (0.5 mg/ml) to each well. The microplate was then incubated for four hours. 100 µl of the prepared solution was added into each well. Finally, we measure the absorbance of the samples using a microplate (ELISA) reader after whole solubilization of the purple formazan crystals at 570nm. The percentage of cell

viability was calculated using the following equation:

The viability of cells (%) = [ODS/ ODC] × 100.

ODS stands for the sample's mean optical density, while ODC is the control's mean optical density (Zhao *et al*., 2010).

The parameters for water quality were assayed in the Micro-analytical Center, Cairo University.

The pH (potential hydrogen) was measured by a pH meter (George *et al*., 2013). EC (electrical conductivity) was measured using an EC instrument electrode. TDS (total dissolved solids), CO_3, HCO_3, Cl, and SO_4 were measured using titration processes Ca, Mg, Na, and K concentrations were measured by atomic adsorption, following the method described by the authors. Residual chlorine and COD (chemical oxygen demand) were obtained by the titration processes (Fouad *et al*., 2022). BOD (biological oxygen demand) was measured using a BOD instrument, following the method described by the authors in (Fouad *et al*., 2022). TSS (total suspended solid) measurements were performed using gravimetry, as described by the authors in (Fouad *et al*., 2022).

The biologically extracted chitosan was tested to remove the color of five synthetic dyes. The test was carried out at 47.5^{o}C, pH=4.5. The reaction solution concentration was 1 g/L of biologically extracted chitosan along with the dye: 25, 50 or 100mg/L (Malachite green, Bromothymol blue, Eosin, Congo red, and Crystal violet), all dyes were purchased from Sigma Aldrich. The percentage of dye decolorization, calculated as decolorization percentage, using the following formula: decolorization (%) = [(Ai− At)/Ai] × 100, where Ai: initial absorbance of the dye and At: absorbance of the dye over time (Zhuo *et al*., 2015).

All the presented data were the mean of three replicates. The SPSS 22 software (SPSS Inc., Chicago, IL) was used to determine the standard deviation (SD); p>99%.

Crawfish is one of the commonly used sources of food for humans. Following the common procedure, about eighty percent of crayfish shells become discarded, and approximately hundreds of thousands of tons of crawfish exoskeletons are generated every year (Arvanitoyannis and Kassaveti, 2008). The random discarding of crayfish shells naturally or through landfill may cause environmental pollution (Marei *et al*., 2016). In contrast, suitable discarding of this waste can be expensive, for example, in Australia it reaches up to 150 dollar per ton (Abd El-Ghany *et al*., 2023). Markedly, the crayfish shell has a unique composition and contains three basic compounds, namely protein (20%–30%), calcium carbonate (from 30% to 40%), and chitin from 20% to 30% (Zuber *et al.*, 2013). Some minor elements have been identified, comprising lipids, astaxanthin, and other ingredients. Reasonable utilization of the rich crayfish shell resources is now attractive for scientists (Arvanitoyannis and Kassaveti, 2008).

In this study, BB extraction was considered as another approach, employing proteolytic bacteria such *as Lactobacillus lactis* and *Saccharomyces cerevisiae*. *Lactobacillus lactis* deproteinizes and demineralizes chitin in a single process, and *Saccharomyces cerevisiae* deacetylates chitin to produce chitosan. *Saccharomyces cerevisiae* produces chitin deacetylase enzyme to deacetylate chitin. Chitosan is consequently formed as a green alternative that has more homogenous properties. Chitin's N-acetyl-D-glucosamine residues are deacetylated by chitin deacetylase enzyme. *S. cerevisiae* has been shown to include the genes CDA1 and CDA2 for chitin deacetylase (Zhao *et al*., 2010).

The extracted chitosan was characterized in terms of "Fourier transform infrared" or FTIR spectroscopy, elemental analysis, solubility, degrees of deacetylation (DA), and mass spectroscopy (Marei *et al*., 2016).

FTIR spectroscopy was used to explore the vibrational properties of amino acids and cofactors that are subject to minute structural changes. The infrared spectra of the commercial and extracted chitosan from crawfish are presented in Figure 1 and Table 1. The IR spectrum of crawfish chitosan showed the presence of characteristic bands such as those at 3423 cm^{-1}, 3400 cm^{-1}, 3400 cm^{-1}, and 1628 cm^{-1} for the chitosan standard, BB-, BC-, and CC-extracted chitosan, respectively. These bands were in the same range of bands characteristic of chitosan (Hamdi *et al*., 2022a) These

corresponded to (NH_2) in primary amines in the samples tested. Bands at 3423 cm^{-1}, 2900 cm^{-1}, 2900 cm^{-1}, and 2651 cm^{-1} for the chitosan standard, BB-, BC-, and CC-extracted chitosan, respectively, corresponded to (OH) in the pyranose ring. CH_2 bands in CH_2OH (C-H) in the pyranose ring of the crawfish chitosan standard, BB-, BC-, and CC-extracted chitosan were at 2880-2923cm^{-1}, 1450cm^{-1}, 1600cm^{-1}, and 1400cm^{-1},, respectively. On the other hand, bands resembling (C=O) in NHCOCH3 (amide I band) were as follows: 1667-1629cm^{-1}, 1550cm^{-1}, 1450cm^{-1}, and 1156cm^{-1} for the chitosan standard, BB-, BC-, and CC-extracted chitosan, respectively; furthermore, bands resembling (CH_2) in the CG_2OH group were at 1422cm^{-1}, 1650cm^{-1}, 1300cm^{-1}, and 1025cm^{-1}, respectively, while the bands (CH_3) in $NHCOCH_3$ (amide functional group) were at 1380cm^{-1}, 1300cm^{-1}, 1150cm^{-1}, and 531cm^{-1}, respectively. Similarly (C-H) bands in the pyranose ring complex of the NHCO group (amide III band) were at 1322cm^{-1}, 1100cm^{-1}, 1050cm^{-1}, and 578cm^{-1}; (C-O-C) glycosidic linkage bands were at 1155cm^{-1}, 900cm^{-1}, 700cm^{-1}, and 1628cm^{-1}; and aromatic compounds bands were at 1155cm^{-1}, 900cm^{-1}, 700cm^{-1}, and 1628cm^{-1} for the crawfish chitosan standard, BB-, BC-, and CC-extracted chitosan, respectively.

The structural integrity of the chitosan extracted using the CC, BB, and BC methods was verified by FTIR analysis, as shown in Figure 1.

The resulting differences in reaction-induced FTIR spectra were used to determine the IR fingerprints of each relevant residue using a variety of methods. Therefore, we compared the three different extraction methods with commercial standard chitosan and realized the following: in CC-extracted chitosan, we cannot see (CH3) in NHCOCH3 (amide functional group); in BC-extracted chitosan, we cannot see (CH3) in NHCOCH3 and (C-O-C) glycosidic linkages; and finally, for BB-extracted chitosan, we cannot see (CH2) CG2OH group and (C-O-C) glycosidic linkages. We can see from the FTIR test results that almost all the three methods are similar with slightly more acceptance for the CC extraction method. By comparing the results from previous studies and the standard (Abd El-Ghany *et al*., 2024), the CC extraction method resulted in a carbon, hydrogen, and nitrogen percentage of 17.21, 3.53, and 4.28 %, respectively. BC-extracted chitosan had carbon, hydrogen, and nitrogen percentages of 29.78, 5.9. and 4.83%, respectively, and BB-extracted chitosan had carbon, hydrogen, and nitrogen percentages of 32.74, 6.37, and 5.68 %, respectively.

The biological extraction process was better than the chemical method because it not only preserves the chitin structure but is also safe to the environment. Deproteination and demineralization of crawfish shells can also be achieved via a biological method by employing proteolytic bacteria (*Lactobacillus lactis*). Next, the deacetylation process was used to convert chitin into chitosan using *Saccharomyces cerevisiae*, which is a green alternative for chitosan production in comparison with deacetylation with NaOH. The biological extraction of chitin involves simpler manipulation, a lower energy input, and greater reproducibility in comparatively less time and with a lower solvent consumption (Khanafari *et al*., 2008; Yadav *et al*., 2019).

It is quite clear that the spectrum of the commercial chitosan standard and that obtained utilizing biological methods tended to be similar in that the bands that were seen virtually exactly identified the chitosan structure.

After comparing the percentages of nitrogen, carbon, and hydrogen for the three extraction techniques and a chitosan standard, the carbon percentages for the CC and BC extraction methods were very good, while the hydrogen and nitrogen percentages for BB extraction were comparable to the standard percentage. Therefore, the procedure of biological procedure for chitin extraction is gaining immense attention as it is cleaner, eco-friendly, and economic, along with it being able to produce chitin and chitosan with the desired properties (Khanafari *et al*., 2008). The chitosan nitrogen content rises with elongated deacetylation reaction and a more efficient deacetylation of chitin with a high chitosan deacetylation (Kumari *et al*., 2015). When comparing the results with the crawfish chitosan standard, the carbon, hydrogen, and nitrogen percentages were 35.04, 6.57, and 6.73, respectively. We can conclude that CC produces non-acceptable results. Furthermore, the BB extraction method produced the best result, and it

was similar to the standard, so we can admit that the BB extraction method is the best method for the extraction of chitosan from crawfish.

The percentage solubility of chitosan extracted by three different methods in water were as follows: 97%, 92%, and 75% for the BB extraction of chitosan, BC extraction of chitosan, and CC extraction of chitosan, respectively.

The percentage solubility of chitosan extracted by three different methods in acetic acid were as follows: 98%, 90%, and 77%, respectively, for the BB extraction of chitosan, BC extraction of chitosan, and CC extraction of chitosan, respectively.

When comparing the degree of acetylation for the three separate extractions, each with the volume specified above, the findings showed a high degree of deacetylation (%), with percentages of 89.6, 90.8, and 93.4% for the CC, BC, and BB extractions, respectively.

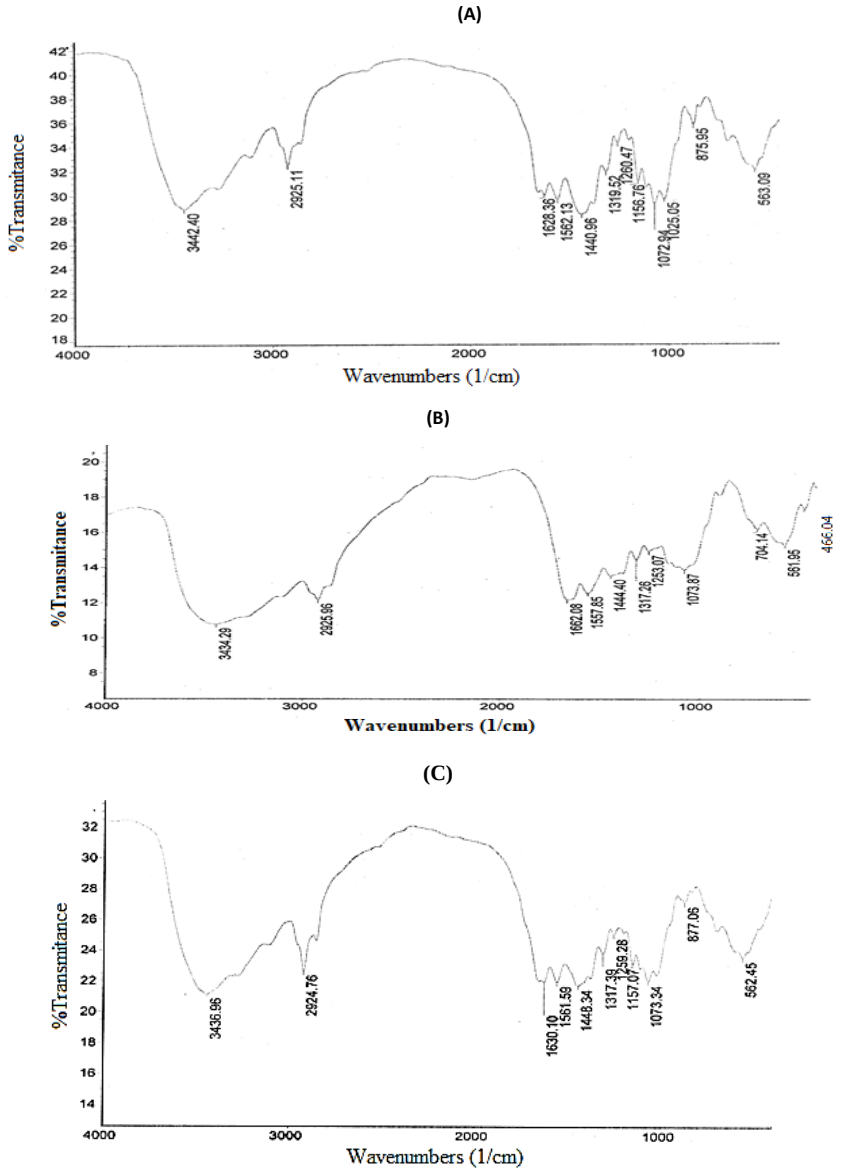

Figure 1. FTIR chart of extracted chitosan: (A) chemical–chemical extraction, (B) biological–chemical extraction, and (C) biological–biological extraction.

Table 1. FTIR spectra characteristics and functional groups of chitosan extracted by three methods of extraction: (A) chemical–chemical extraction, (B) biological–chemical extraction, and (C) biological–biological extraction

CC extraction (1/cm)	BC extraction (1/cm)	BB extraction (1/cm)	Crawfish chitosan Standard (1/cm)	Vibration mode
3442	3400	3400	3423	(NH2) in primary amines
2925	2900	2900	3423	(OH) in pyranose ring
1628	1600	1450	2923-2880	(CH2) in CH2OH (C-H) in pyranose ring
2651	1450	1550	1667-1629	(C=O) in NHCOCH3 (amide I band)
1440	1300	1650	1422	(CH2) CG2OH group
1156	1150	1300	1380	(CH3) in NHCOCH3 (amide functional group)
1025	1050	1100	1322	(C-H) in pyranose ring complex of NHCO group (amide III band)
531	700	900	1155	(C-O-C) glycosidic linkage
578	550	550	800-400	Aromatic compounds

Table 2. Carbon, nitrogen, and hydrogen percentages in the three different methods of extraction: (A) chemical–chemical extraction, (B) biological–chemical extraction, (C) biological–biological extraction

Sample Compounds	Carbon %	Hydrogen %	Nitrogen %
CC extraction	17.21	3.53	**4.28**
BC extraction	29.78	5.9	**4.83**
BB extraction	32.74	6.37	**5.68**
Standard	35.04	6.57	**6.73**

Table 3. Degree of deacetylation in the three extraction methods and UV peaks of: (A) chemical–chemical extraction, (B) biological–chemical extraction, and (C) biological–biological extraction. All data are ± standard deviation

Type of extraction	Degree of deacetylation (%)	UV peaks
CC extraction	89.6 ± 1.1	0.210 at 280 nm 0.788 at 295 nm
BC extraction	90.89 ± 1.3	0.415 at 285 nm
BB extraction	93.47 ± 1.5	1.150 at 299 nm

The CC extraction of chitosan produced two peaks of 0.210 and 0.788 at 280 and 295, respectively. The BC extraction of chitosan produced many peaks from 200 to 300 nm and one peak of 0.415 at 285 nm as shown in table (3). The BB extraction of chitosan had one peak of 1.150 at 299nm. Chitosan can also be determined based on its ultraviolet–visible (UV–Vis) spectroscopy. This characterizes molecules by using absorption spectroscopy in the ultraviolet and visible wavelength ranges of 180-380 nm and 380-750 nm, respectively. This is one of the most fundamental strategies that must be used when characterizing an analyte. Chromophores are light-absorbing functional groups found in all major families of biomolecules. When these chromophores absorb UV–Vis light, they are stimulated from their ground state to a higher energy level, producing unique spectra that aid in the identification of particular molecules (Boly *et al*., 2016).

The UV spectrum was measured for chitosan extracted from crawfish using BB, BC, and CC extraction methods at 295nm.

The maximum absorbance was 1.150 for BB-extracted chitosan, 0.415 for BC-extracted chitosan, and 0.788 for CC-extracted chitosan; the highest absorbance (1.150) was observed for biologically extracted chitosan, which indicates

that it was the highest sample in terms of yield concentration.

Another way to characterize the extracted chitosan is by X-ray diffraction (XRD), which is a versatile, non-destructive analytical technique used to analyze physical properties such as phase composition, crystal structure, and the orientation of amorphous polymer when it has a disordered structure or crystalline high-order structure of chitosan. The crystalline structure has a higher degree of deacetylation and a lower number of impurities than the amorphous structure (Lalitha, 2004).

The XRD of chitosan is characteristic of an amorphous polymer when it has a disordered structure or crystalline high-order structure [42]. The XRD for CC extraction showed no peak 2theta at 10 and had a broad peak at 20, with other peaks of 22, 26, 30, 40, 49, 55, and 66. The XRD for BC extraction showed no peaks at 10 and a broad peak at 20, rather than other peaks at 29, 32, 34, 36, 40, 43, 57, 59, and 56 as shown in Fig. 2. The XRD for the BB extraction method had a peak at 10, and a broad band at 20, 26, 33, 34, and 38. The peaks around 9.63 and 20.53 were related to crystal-I and crystal-II in the chitosan structure, and both peaks were attributed to a high degree of crystallinity of the prepared chitosan (Günister *et al*., 2007). BB extraction had very good XRD result, with 2theta peaks at 10 and 20, and only a few other peaks.

TGA curves of biologically extracted chitosan were used as measurement of the thermal stability of chitosan, as shown in Fig. 3. Two endothermic peaks were observed. The first peak appeared at 77.9 °C and corresponded to the loss of water. The second emerged at 298.68 °C. This result is in accordance with the results of Kumar and Koh (2012), who stated that the TGA curve of pure chitosan shows that the two stages of weight loss are in the range from 47 to 450 °C and the difference may be due to some impurities. The molecular weight of biologically extracted chitosan was calculated to be 78.18 Da, using the intrinsic viscosity. Some of the factors that affect chitosan viscosity are: ionic strength, temperature, molecular weight, degree of deacetylation, pH, and bleaching. Most commercial chitosans have molecular weights ranging from 50 to 2000 kDa, with an average DDA of 50–100% (commonly 80–90%). Based on molecular weight, chitosan can be grouped into low molecular weight (<100 kDa), medium molecular weight (100–1000 kDa), and high molecular weight >1000 kDa (Santoso *et al*., 2020).

Chitosan is used in drug delivery systems, as its molecular weight is sufficiently low to allow it to be excreted by the kidney. The amino and carboxyl groups of the chitosan molecule (Zargar *et al*., 2015) can create a hydrogen bond with glycoproteins in mucus, resulting in an adhesive action. As mucoproteins in mucus are positively charged, chitosan and mucus are attracted to each other, which increases drug retention and continuous drug release in vivo, while also improving drug bioavailability (Le Page *et al*., 2015).

Oxidation, which is among the chief factors in chemical spoilage, can also have an impact on food, where it can lead to rancidity and/or a decline in the nutritional value, color, flavor, texture, and safety of the food (Bauer *et al*., 1999; Bae *et al*., 1999). The crawfish exoskeleton, containing chitosan that can be extracted by BB extraction using *S. cerevisiae*, shows that when the concentration of the sample is increased, the DPPH scavenging rises, as shown in Figure 4. The IC_{50} value of the sample was 56.2 µg/mL, compared to strong antioxidant control like Trolox, for which the IC_{50} was 79.4 µg/ml, as shown in Fig. 5. The significance of oxidation in the human body and in food has long been understood. Cells must be able to carry out oxidative metabolism to survive. The generation of free radicals and other reactive oxygen species, which leads to oxidative alterations, is a side effect of this reliance. There is mounting evidence that these species participate in numerous typical in vivo regulation systems (Santoso *et al*., 2020). Upon oxidation of some cell components such as DNA, cellular proteins, membrane lipids, and enzymes, free radicals can overcome protective enzymes like superoxide dismutase, catalase, and peroxidase and produce destructive and lethal cellular effects (such as apoptosis), which blackout cellular respiration. However, it appears that cell signaling pathways are affected by reactive oxygen species in ways that are not fully understood (Le Page *et al*., 2015). Based on estimations, deteriorative postharvest responses cause the failure of more than 50% of the world's

fruit and vegetable crops (Colbert and Decker, 1991). The interactions of different antioxidants offer protection mechanisms against the destructive effects of oxidation, and the need to determine antioxidant activity is a must. Chitosan is known to stimulate mitosis, late-stage apoptosis, and S-phase cell cycle arrest in HCT116 cells. It has also inhibited the development of HCT116 cells in vitro and in vivo through promoting BAK mRNA expression and decreasing BCL-2 and BCL-xL (Shahidi and Wanasundara, 1992). When comparing our results, BB-extracted chitosan had a high antioxidant activity compared with other extraction methods and compared with the Trolox standard, with an IC_{50} of 7.353 µg/mL. The IC_{50} value is the concentration of an antioxidant required to scavenge 50% of the DPPH radicals in a solution. The lower the IC_{50} value, the more potent the antioxidant (Ngo and Kim, 2014; Tian *et al*., 2007). The antioxidant effect of chitosan has been reported to decrease the oxidation of lipids by chelating ferrous ions in crawfish. This eliminates the prooxidant activity of ferrous ions by preventing their conversion to ferric ion (Muthu *et al*., 2021). In the same way, chitosan obtained from Tunisian marine shrimp (*Penaeus kerathurus*) waste, crab (*Carcinus mediterraneus*) shells, and cuttlefish (*Sepia officinalis*) bones exhibited antioxidant properties (Chlif *et al*., 2021).

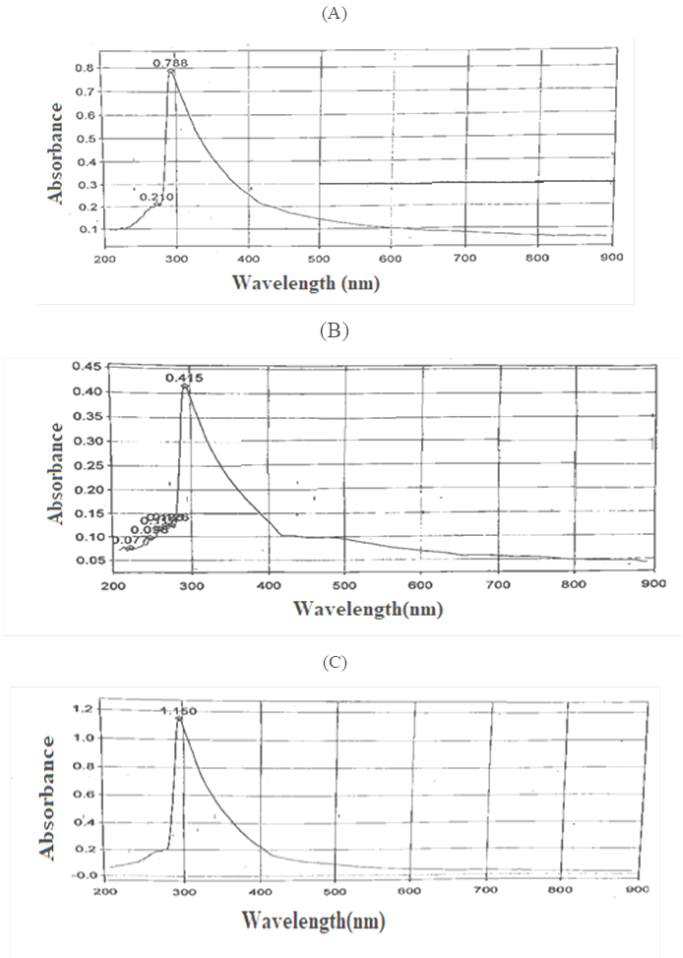

Figure 2. XRD analysis chart of extracted chitosan: (A) chemical–chemical extraction, (B) biological–chemical extraction, and (C) biological–biological extraction.

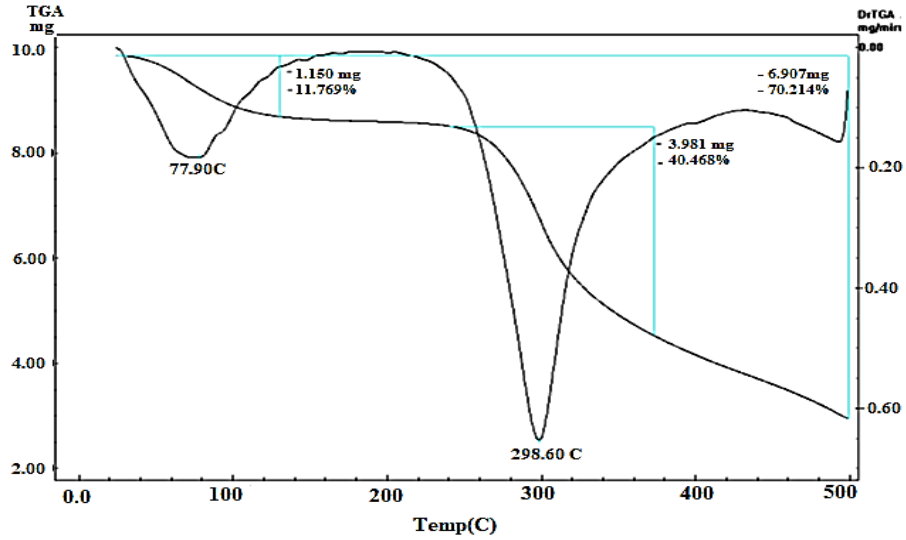

Figure 3. Thermal gravimetric analysis (TGA) of biologically extracted chitosan from crawfish

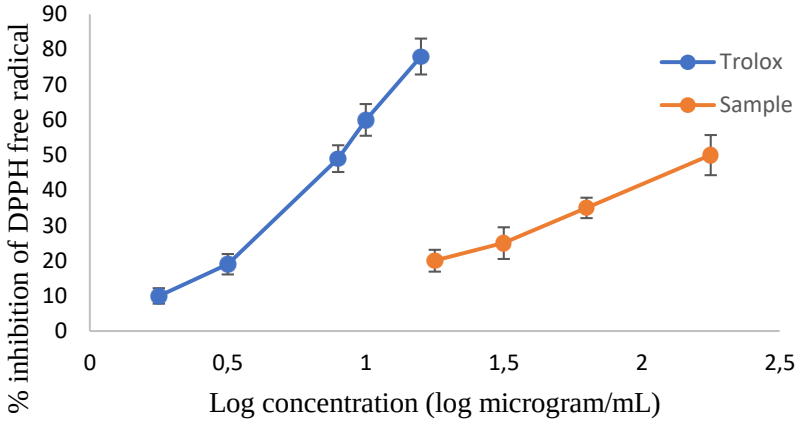

Figure 4. Antioxidant activity of BB-extracted chitosan when compared to Trolox.

As is shown in Figures 5, 6, and 7, cell viability decreased with the gradual increase in concentration of extracted chitosan for the three cancer cell lines HepG-2, HCT, and MCF-7. The IC_{50} values for the CC, BC, and BB extraction of chitosan on HepG2 (49.9 ug/mL, 51.9 ug/mL, and 26.9 ug/mL, respectively), HCT (54 ug/mL, 59 ug/mL, 59 ug/mL, respectively), and MCF-7 (31 ug/mL, 33 ug/mL, and 35 ug/mL, respectively) decreased as the concentration of extracted chitosan increased. Chitosan has the capacity to affect tumor cells directly by interfering with cell metabolism, limiting cell growth, or triggering cell death. It also has an anticancer effect by boosting immunological function. Chitosan's anticancer properties in vitro and in vivo point to its promising use as a supplemental anti-cancer medication and drug carrier (No *et al.*, 2007). On the tumor cell surface, it displays selective adsorption and neutralizing actions. It can target the liver, spleen, lungs, and colon as a drug carrier. The potential applications of chitosan include use in the biomedical field to utilize its antioxidant activity and anti-cancer activity against HepG-2, HCT, and MCF-7 cell lines, using it to improve food safety, and many other applications (Morin-Crini *et al.*, 2019).

According to our findings, the BB extraction technique had the lowest IC_{50} values for the three cell lines tested: HepG2, HCT, and MCF-7.

Water is a vital natural resource on earth that is critical for agricultural, domestic, industrial, and several recreational activities (Dudhani and Kosaraju, 2010). Chitosan, a biosorbent, plays an important role in water treatment (Hajji *et al*., 2015). The water treated with chitosan had a reduced chlorine content from 35.4 to 30.08mg/L (4 mg/L); effective continuous disinfection is provided by free chlorine residuals in water distribution systems of 0.5 to 2.0mg/L.

The pH of tap water treated with extracted chitosan was lowered from 6.98 to 6.88 to be close to the normal level; the normal pH of drinking water lies within the range 6.5–8.5. The HCO_3 was also reduced from 207.45 to 61.017mg/L (Cao *et al*., 2005). Also, the concentrations of Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+} were reduced from 55.7, 30.624, 38.62, and 13.293 to 40.08, 15.65, 18.16, and 2.13mg/L, respectively. These results are in line with those of other studies in which treatment with chitosan led to a lowered pH and reduced mineral concentrations to a safe level (Bhatt *et al*., 2023).

These tests were performed for tap water samples, including different elemental concentrations and parameters for water in the sample, before and after treatment with chitosan. Finally, water treated with active carbon and modified with a chitosan filter went through the same tests (different elemental concentrations and parameters for water quality) to provide an obvious comparison between the three water samples.

In terms of drinking water characteristics such as taste and odor, the secondary maximum contaminant level (SMCL) for sulphate in drinking water is now 25 milligrams per liter (mg/L). According to the EPA estimates, 3% of the nation's public drinking water systems may have sulphate levels of 25mg/L or more (Dudhani and Kosaraju, 2010). In this study, sulphate concentration decreased from 25.9 to 14.2mg/L, which is acceptable in accordance with the standard.

In the USA and Canada, the calcium content of water ranges from 1 to 135mg/L, and in this study the calcium concentration was decreased from 55.7 to 40.08mg/L. With an average calcium content of 21.8mg/L, spring water is generally found to have a comparatively low calcium content (Bhatt *et al*., 2023). Magnesium makes up around 25% of the human body, with 60% of that amount found in bones, and 40% in the muscles and tissues. The WHO guidelines state that the maximum amount of magnesium that should be present in water is 50mg/L (Bhatt *et al*., 2023).

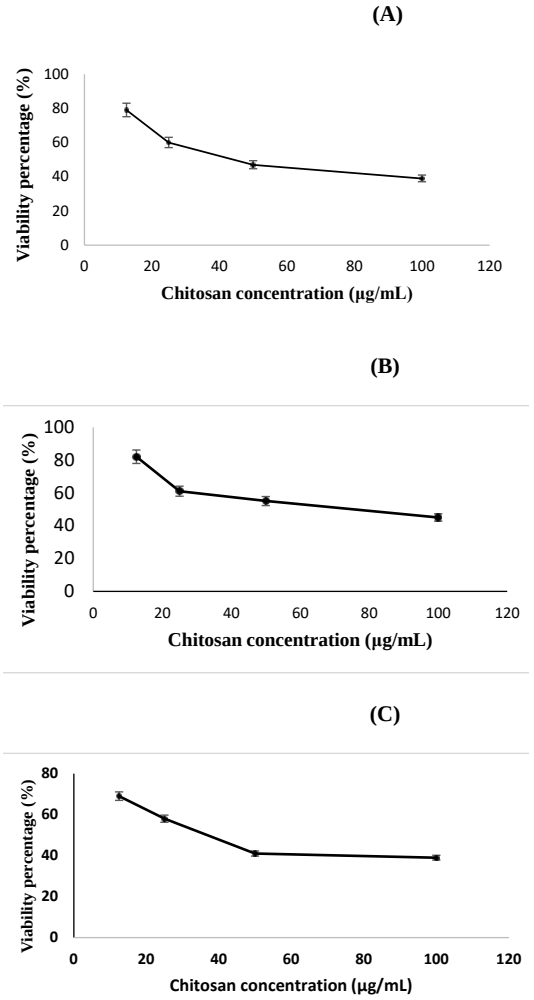

Figure 5. Effect of chitosan extracted by different methods on HepG-2 cell line: (A) chemical–chemical extraction, (B) biological–chemical extraction, and (C) biological–biological extraction.

Most water supplies contain less than 20 mg of sodium per liter (Meride and Ayenew, 2016), and after chitosan treatment, it was found that the concentration of sodium decreased from 38.62 to 18.16mg/L. Furthermore, the maximum allowable concentration of added potassium is 20mg/L according to drinking water advisory (2003).

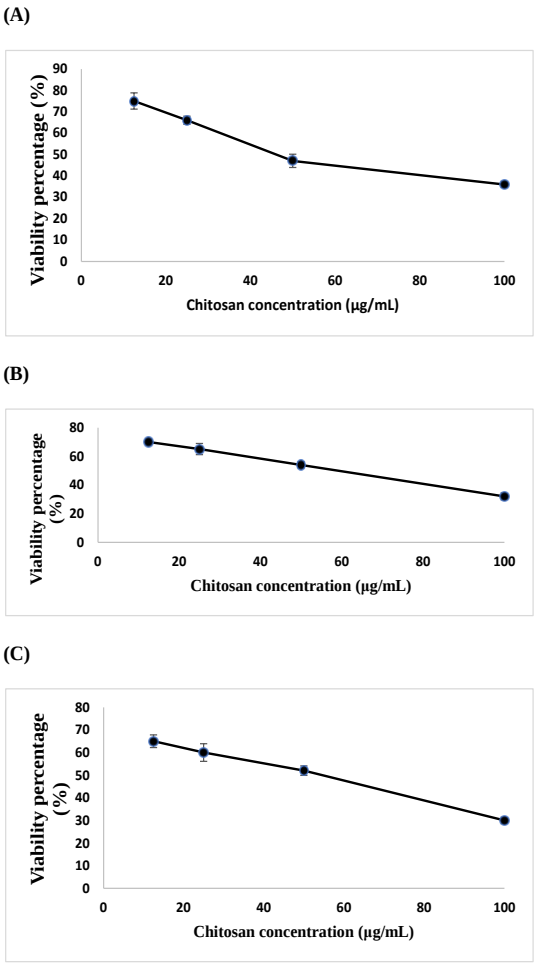

Figure 6. Effect of chitosan extracted by different methods on HCT cell line: (A) chemical–chemical extraction, (B) biological–chemical extraction, and (C) biological–biological extraction.

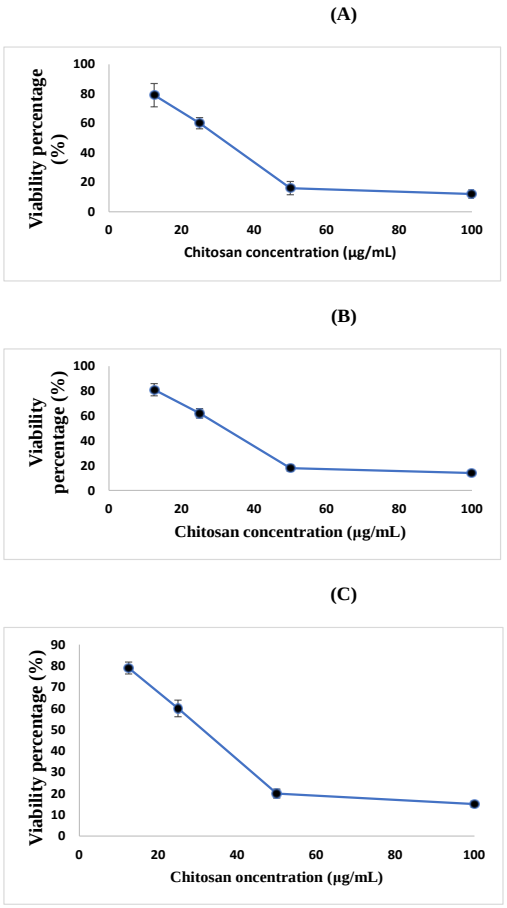

Figure 7. Effect of chitosan extracted by different methods on MC7 cell line: (A) chemical–chemical extraction, (B) biological–chemical extraction, and (C) biological–biological extraction.

Table 4. Chemical comparison between tap water and after treatment with chitosan filter; all data are ± standard deviation

Parameter	Untreated Tap water (mg/L)	After treatment with chitosan (mg/L)
Co_3^{2-}	ND	ND
HCO_3^-	207.5±5.1	61.0±2.7
Cl^-	35.5±3.8	30.1±2.9
So_4^{2-}	25.9±1.9	14.1±1.3
Ca^{2+}	55.7±4.2	40.1±4.1
Mg^{2+}	30.6±3.7	15.7±2.4
Na^+	38.6±3.4	18.2±2.6
K^+	13.3±1.6	2.1±0.3
Residual chlorine	7.44±0.2	ND

The ability of a solution to convey current through its ionic process is measured by electrical conductivity (EC). WHO guidelines state that the EC value should not be greater than 0.4 ds/ml according to WHO (Potassium…, 2009). The EPA recommends a TDS concentration in water of 500mg/L (500 mg/L) according to Department of Health Annual

Report 2017-2018. The annual report provides detailed information about the Department of Health's financial and non-financial performance for 2017–18. It has been prepared in accordance with the Financial Accountability Act 2009, the Financial and Performance Management Standard 2009, and the annual report requirements for Queensland Government agencies.

Our results of BOD decreased from 10mg/L to zero when a chitosan filter was used; the same trend was observed with COD, which decreased from 115.9mg/L to 32.65mg/L. The greater the organic matter contamination in water, the greater the COD value according to (Drinking Water Advisory, 2003). The most frequent use of COD is to quantify the number of oxidizable pollutants present in surface water according to (Potassium…, 2009). Biochemical oxygen demand (BOD) is a measure of how much oxygen is consumed by bacteria and other microorganisms during the aerobic (oxygen-containing) decomposition of organic matter at a given temperature. One thing you cannot see when looking at water in a lake is oxygen. Although the WHO does not set particular limits for BOD or COD, lower values are preferred (Xu *et al*., 2020). Our results agree with the standards, where COD and BOD levels up to 30mg/L may be appropriate. The BOD decreased from 10mg/L to zero when the chitosan filter was used, and the same trend was observed with COD, which decreased from 115.9mg/L to 32.65mg/L. On the other hand, TSS levels under 10mg/L are regarded good for drinking water according to (World…, 2011), which was in line with our results after treatment with chitosan, where the TSS level decreased from 0.062 to 0.05mg/L. This is also approved by Meride who used chitosan only and found, when using the selected dose of 8mg/L, that the BOD value reached 8.0, and the COD value reached approximately 10.00mg/L (45% removal rate). The TOC reached 3.3 mg/L (removal rate of 45%) according to (Bhatt *et al*., 2023). TSS level decreased from 0.062 to 0.05mg/L. after treatment with chitosan.

The biologically extracted chitosan was used to decolorize five synthetic dyes (bromothymol blue, malachite green, eosin, Congo red, or crystal violet.). Decolorization was monitored by measuring the absorbance of the reaction mixture at a concentration of 25, 50, or 100mg/L.

As is shown in Figure 8, biologically extracted chitosan could efficiently decolorize different synthetic dyes. Decolorization efficiency showed the same trend at the three tested concentrations. However, increasing the dye concentration was somehow associated with less decolorization. Bromothymol blue, malachite green, eosin, Congo red, or crystal violet (100mg/L) could be decolorized up to 80%, 84.1%, 65%, 60.9%, and 78%, respectively, within 15 hours. The biologically extracted chitosan displayed the maximum decolorization percentage against the malachite green dye.

Table 5. Water characteristics before and after treatment with chitosan filter. All data are mean ± standard deviation

Parameter	Untreated Tap water	After treatment with chitosan
pH	6.98±0.13	6.88±0.18
EC	0.36±0.04ds/m	0.23±0.03ds/m
TDS	230.4±1.7mg/L	120.8±1.9mg/L
COD	115.9±0.81mg/L	32.65±0.83mg/L
BOD	10±0.78mg/L	ND
TSS	0.062±0.01mg/L	0.05±0.02mg/L

Commonly, physical or chemical methods for dye removal are expensive, have low efficiency, and sometimes generate other pollutants. Modified chitosan is a good candidate to be used as a sorbent for dye decolorization that has been successfully applied to remove anionic amaranth red and cationic methylene blue dyes from colored effluents (Couture *et al*., 2018).

Kyzas *et al*., 2017, used modified chitosan in the same way and found it has a very high sorption potential for some dyes such as reactive red and amaranth red dyes. Eichlerova *et al*., 2006, found that, compared to other dye groups, triphenylmethane dyes are resistant to enzymatic treatment and need a longer time for decolorization (Muzzarelli, 1973). The selected pH was 4.5 because the decolorization efficiency drastically decreased above pH 5.0 due to the

deprotonation of the amide groups of the grafted chitosan (Labidi *et al*., 2019). On the other hand, at a pH above 8.0, the decolorization efficiency decreased due to the presence of OH− ions in the basic solution acting as competitive ions for dye removal (Bellaj *et al*., 2024). Also, the increase in reaction temperature could greatly accelerate the dye removal (Kaczorowska and Bożejewicz, 2024), so the selected temperature was set to 45°C; most industrial effluents with dyes have a slightly elevated temperature around 40-45.

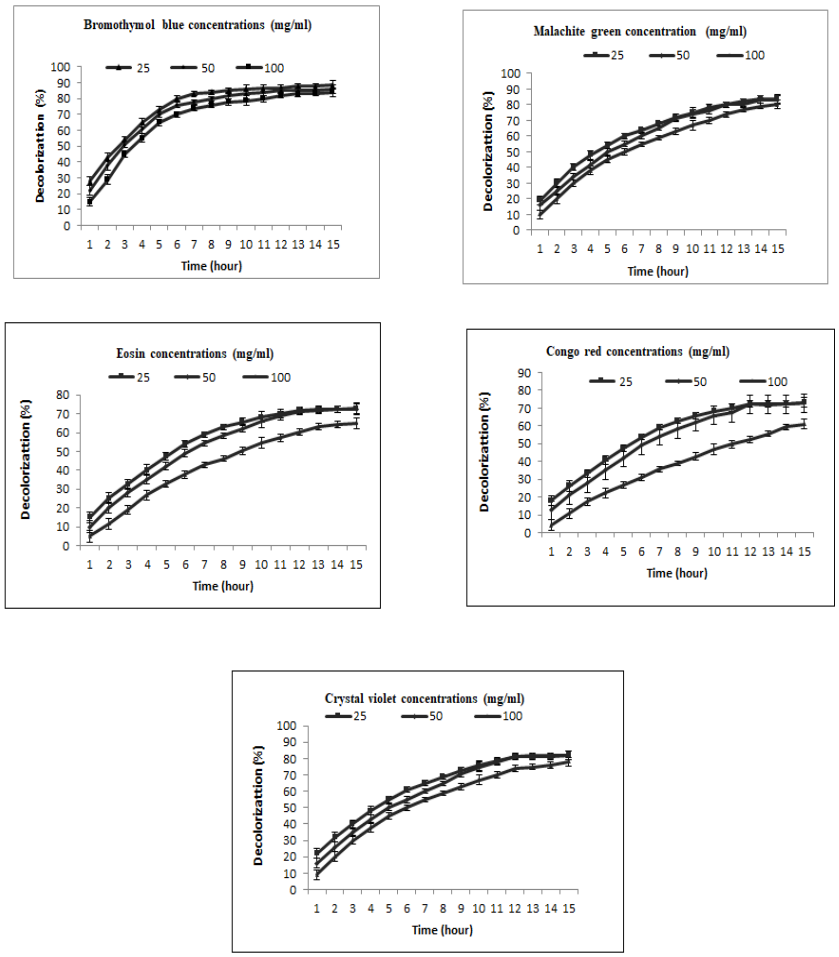

Figure 8. Decolorization of different dyes by biologically extracted chitosan: bromothymol blue, malachite green, eosin, Congo red, or crystal violet. All data are mean ± standard deviation.

CONCLUSIONS

Our research led us to the conclusion that the novel eco-friendly method of extraction of chitosan from the exoskeleton waste of crawfish is efficient. Extracted chitosan is considered a non-toxic, biodegradable polymer that is utilized in a variety of industries, including food, agriculture, wastewater treatment, medicines, cosmetics, and textiles. In comparison to chemical extraction, the biological extraction of chitin and chitosan by utilizing probiotics like *Lactobacillus lactis* and *Saccharomyces cerevisiae* is more economical and environmentally benign. Chemical extraction techniques may cause chitin to become denaturized and involve the use of hazardous organic solvents that harm the environment. The present study opens a new avenue for the eco-friendly extraction of chitosan from chitin that is proficient and environmentally sustainable, which can be used for different applications, especially in dye decolorization.

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