British Biotechnology Journal
4(11): 1212-1222, 2014
ISSN: 2231–2927

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Production of Some Polysaccharides from Kojic Acid Producing *Aspergillus oryzae* var. *effusus* NRC14 Biomass

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Authors' contributions

This work was carried out in collaboration between all authors. This work was a part of M.Sc. thesis by assistant researcher author EII. Author AMS designed the study. Authors AMS, HMH and MMH supervised the study and managed the literature searches, wrote the first draft. All authors read and approved the manuscript.

Article Information

DOI: 10.9734/BBJ/2014/13740
Editor(s):
(1) Chung-Jen Chiang, Department of medical laboratory Science and Biotechnology, China Medical University, Taiwan.
Reviewers:
(1) Anonymous, Cukurova University, 01330 Adana, Turkey.
(2) Anonymous, Federal Rural University, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=688&id=11&aid=6576

Original Research Article

Received 1st September 2014
Accepted 27th September 2014
Published 22nd October 2014

ABSTRACT

Aims: The present investigation describes the utilization of *Aspergillus oryzae* var. *effusus* NRC14 biomass producing kojic acid in the production of fine chemicals that have valuable applications such as chitin, chitosan, β-1, 3 glucan and /or their derivatives.
Methodology: Experiments of cell growth and substrate utilization were conducted in static cultures with identified medium composition. The technology involved in the extraction of their biopolymers is quite simple and the size scale is compatible with those

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currently used. This work was performed in Department of Microbial Chemistry, NRC.
Results and Conclusion: The utilization of *A. oryzae* var. *effusus* NRC14 biomass waste actually will cover a part of the cost production of kojic acid; therefore, clean cell walls preparations were isolated from the fungus biomass at different stages during kojic acid fermentation in a glucose salts medium. The major chitin constituent was extracted from the prepared cell walls from six days cultured *A. oryzae var. effusus* NRC14 biomass by cold conc. HCl or by 5% of LiCl/ DMAA solvent in yields 4.78% and 2.44%, respectively. Among the chitin family, chitosan has received special attention since it has several applications. Chitosan was extracted from alkali insoluble material (cell wall) obtained from 12 days cultured biomass by using different concentrations of acetic acid (0.5-2.5%) the results showed that the highest yield of chitosan was 4.65% at a concentration of 2% acetic acid. The isolated product was characterized by infrared spectrum. Chitosan-glucan complex was isolated in 17.94% yield by treating the *A. oryzae var. effusus* NRC14 biomass produced from kojic acid production for 12 days with 40% aqueous NaOH for 6h. at 95℃. Chitosan-glucan complex was fractionated into its components. The amount of extracted chitosan was 3.5%; the residue obtained after solubilization of chitosan by acid treatment was characterized as being mostly insoluble glucan (94%). Chitin-glucan complex was isolated from 6 days cultured *A. oryzae* var. *effusus* NRC14 biomass in yield 17.034%. The lyophilized product was fractionated into its components (chitin and glucan) in yields 16.36% chitin, 82.72% β, 1-3 glucan. The infrared spectra of the isolated products as well as of standard samples proved the identity of the isolated materials as chitosan, chitin and glucan.

Keywords: Asprgillus oryzae var. effusus; kojic acid; polysaccharides; chitin; chitosan and glucan.

ABBREVIATIONS

DMMA- N,N-dimethyl acetamide ; NRC- National Research Center ; PDA- Potato dextrose agar ; d.w- distilled water ; CGC- chitin(chitosan)-glucan complex ; T- Transmittance % of (chitin- chitosan or glucan); conc.- concentrated; SDS-sodium dodecyl sulphate.

1. INTRODUCTION

The composition and architecture of fungal cell wall is now well known in a variety of fungi. Chemically the fungal cell wall is 80 to 90 percent polysaccharides with most of the remainder consisting of proteins, lipids and other compounds such as glucan, chitin, chitosan and mannan [1-3]. The composition of fungal cell walls are variable, depending on their age and environmental factors [4].

Initial analysis of cell wall composition by alkali solubilization showed a mediley of polysaccharides that were either alkali soluble or alkali insoluble. The alkali- soluble polymers from a variety of fungi included mannoproteins and some α-and β-(1, 3)–D-glucan. The insoluble polymers include β-(1,3)-D-glucan and β-(1,6)-D-glucan, chitin, chitosan, polyglucuronic acid and cellulose. In general, the insoluble polysaccharides convery mechanical strength to the cell. The soluble polysaccharides link interstitial components that connect and restrict the skeletal polymers. Both components play a key role in the function of the cell wall [5].

Traditionally waste mycelium of *Aspergillus niger* from citric acid production is considered as an industrial chitin (chitosan) - glucan complex source. There are several reports on the other Micoromycetes belonging to genus Ascomyceta and Zygomycta utilization for chitin (chitosan) - glucan complex production. Fungal CGC is considered as an alternative source of chitin/ chitosan [6,7] as well as a potent agent for application in medicine for wound – healing management [7,8], for improvement of desquamation process and xerosis reduction in diabetic patients [9]. For reduction of aortic fatty streak accumulation [10], etc.

2. MATERIALS AND METHODS

2.1 Microorganism

In a previous work Hazzaa et al. [11] *Aspergillus oryzae* var. *effusus* NRC14 was isolated from Egyptian soil and identified by the Regional Center for Mycology and Biotechnology, Al-Azhar Univ., Cairo, Egypt based on hyphal morphology and colony characters using an image analysis system [12].

2.2 Culture Conditions

The strain was maintained on PDA slants at 4℃ and sub-cultured at intervals from 15-30 days.

2.3 Culture Media

Glucose salts liquid medium [13] was initially used for the production of kojic acid and fungal biomass. It has the following composition (g/l); glucose, 100; NH_4NO_3, 1.125; $MgSO_4.7H_2O$, 0.5; KCl, 0.1; H_3PO_4, 0.063ml.

The fungal strain was inoculated (one disk of mycelial growth, 6 days old) into 250 ml flasks, each containing 50 ml medium. The cultures were incubated static at 30℃±2 for different time intervals. All cultures were run in duplicates. The medium was decanted; the mycelium washed several times with d.w and dried in the oven at 80℃ for 24h.

2.4 Cell Wall Preparation

Cell wall was prepared by using two methods.

The method of White et al. [14] was used. *A. oryzae* var. *effusus* biomass was collected by filtration using two layers of cheesecloth, and washed twice with d.w. The biomass was blotted between filter paper, and weighed, the wet biomass was homogenized by blender in d.w, and then autoclaved in 1.0 N NaOH for 15min. at 121℃. The ratio of wet biomass to NaOH solution was 1:20 (w: v). After cooling the suspension was filtered through a Buchner funnel using Whatman No.1 filter paper. The extracted material was washed several times with d.w until neutral pH was obtained (pH, 7.0) and three times with 95% ethanol, once with d.w and blotted between filter paper. The extracted material was then dried at 60℃ for 48h, weighed and stored at room temperature until use.

The method described by Kisser et al. [15] for the preparation of cell walls was used. The mycelium was collected by filtration using two layers of cheesecloth, and washed twice with distilled water, then blotted between filter paper, and weighed. The wet biomass was

homogenized by blender in d.w, and then blotted between filter paper. The wet biomass (10 g) was stirred with 100 ml 1% (w/v) SDS at room temperature for 24h. The suspension was then frozen thawed and homogenized. After filtration, the procedure was repeated with the residue. The final debris suspended in 25ml of tap water, stirred for 10min. and filtered. Washing repeated until the filtrate showed no absorption at 260nm (as a criterion for cell wall purity). The residue was then washed with absolute methanol and dried at room temperature, weighed and stored at room temperature until use.

2.5 Extraction of Chitosan

Chitosan extraction was carried out by a modified method of Rane and Hoover [16] and Crestini et al. [17]. The dried cell wall was powdered in kitchen mill for 3min. and refluxed with 0.5-2% acetic acid (1:40) (w: v) at 95℃ for 8h. To determine the time of extraction of chitosan, fresh cell wall treated with fresh 2% acetic acid (six times) for 12h. The extracted slurry was centrifuged at 6000g for 15 min. and the insoluble fraction was discarded. The pH of the supernatant fluids was adjusted to pH 10.0 with 2N NaOH, the solution was centrifuged under cooling at 6000g for 15min., and the precipitated chitosan was then washed with d.w, 95% ethanol (1:20) and acetone (1:20) (w:v), respectively, lyophilized and characterized.

2.6 Extraction of Chitin

Chitin was extracted by using two methods.

Chitin extraction was carried out by the method of Winkler [18]. The dried cell wall was treated with cold conc. HCl and left in the refrigerator overnight. The mixture was then filtered by using a fritted glass filter. Chitin was precipitated from the cold solution by adding 50% cold ethanol. The precipitate was washed several times with cold d.w until neutral pH was obtained, then lyophilized.

Also, chitin was extracted by the method of Teng et al. [19]. The dried cell wall was placed in 5% LiCl/DMAA solvent at a ratio of 1.0g/150ml with constant stirring at room temperature for 48 h., the viscous suspension was centrifuged at 6000g for 5 min., the supernatant containing dissolved chitin was collected, and d.w (1:1) was added to the supernatant to precipitate the chitin over 24h. The precipitate was recovered as chitin by centrifugation. The recovered chitin was washed with d.w to remove remaining LiCl/DMAA, freeze- dried, weighed and characterized.

2.7 Extraction of Chitin-Glucan Complex from Fungal Biomass

A modification of the procedure of Stagg and Feather [20] was used. The mycelium mat obtained from kojic acid production was used for the extraction of chitin-glucan complex. The wet biomass (35.5g wet weight = 5.33g dry weight) was suspended in 1.0 liter of 10% of NaOH to dissolve soluble glucan and proteins, and stirred at 25℃ for 36h., the suspension was centrifuged at 6000g for 15min., the residue was washed several times with d.w until neutral pH was obtained. The neutralized extract was washed once by 5% acetic acid to remove chitosan (which could possibly result from the deacylation of chitin from the NaOH treatment), then washed twice more with d.w to eliminate the acid then once with ethanol and lyophilized. The lyophilized product was fractionated into its components according to the method of Winkler [18].

2.8 Extraction of Chitosan-glucan Complex from Fungal Biomass

The method described by Muzzarelli et al. [21] was used. In a typical experiment, wet mycelium (50.0g wet weight = 7.5g dry weight) was refluxed with 40% NaOH (w: v) (1:30) solution for 6h., at 95℃ (internal temperature). Such a treatment simultaneously deacylates the chitinous fraction, dissolved proteins, removes the soluble glucan, and hydrolyzed the lipids. The mixture was then filtered, and the residue was washed several times with d.w until neutral pH was obtained and once with ethanol (95%) then twice with d.w. The product was dried by lyophilized for 72h. The lyophilized product was fractionated into its components and characterized according to the method of Muzzarelli et al. [21].

2.9 Physical Characterization for the Extracted Products

Infrared spectra for extracted biopolymers were performed in KBr discs at Central Services Laboratory, NRC by using infrared spectrometer (JAS. Co., FT/IR- 6100).

3. RESULTS AND DISCUSSION

In a previous work [11] *A. oryzae* var. *effusus* NRC14 was found to be the best organism for high kojic acid crystals production when grown statically on a glucose salts medium, therefor the fungal biomass waste was used for the production of some polysaccharides. The fungal cell wall of filamentous fungi including *A. oryzae* consists of polysaccharides (over 90% of the cell wall) and small amount of proteins. Chitin is the major structural polysaccharide found in cell walls of filamentous fungi accounting for 10-20% of the cell wall, and contributes significantly to the integrity of the cell wall [1,22-25].

Because chitin (poly N- acetyl- glucosamine) is one of the major components of cell walls in *A. oryzae*, its present during the early stage of culture in the cell wall preparations from the mycelium, and assumed to be of sufficient quantity in the cell wall regardless of the culture period [25]. Therefore, clean cell walls preparations were isolated from *A. oryzae var. effuses* biomass NRC14 at different stages during kojic acid fermentation on a glucose salts medium. The cell walls accounted for a major portion (51.63-56.11%) of the biomass dry weight. The major chitin constituent was extracted by using cold conc. HCl or by LiCl/DMAA solvent from the cell walls isolated at 3-12 days of growth. Data presented in Table 1 indicate that a high chitin content (4.78%) obtained from cold conc. HCl treatment was observed in isolated cell wall from six days cultured *A. oryzae var. effusus* biomass, while the extracted chitin by 5% LiCl/DMAA solvent was lower than that obtained from cold conc. HCl treatment (2.44%). The amount of extracted chitin from *A. oryzae var. effusus* cell wall was low compared with values (10-20%) reported by some researchers [1,22-24]. This lower in the amount of extracted chitin retained to some of N- acetyl- glucosamine units are deacetylated and depolymerization of chitin during the extraction process of the cell wall by NaOH and chitin by HCl, the deacetylated glucosamine content generally varies between 8% and 15% in chitin [25,19]. The insoluble residues obtained from extraction of chitin by two methods mentioned above were treated with 2% acetic acid to extract of chitosan, the extracted chitosan yields increased as the cell walls age increased until reached to the maximum yields (5.9, 5.87%) at 12^{th} day of cultivation. This fact emphasizes the importance of culture age for the extraction of chitin and chitosan. Some researchers [3,2] reported that the composition of fungal cell walls is variable, depending on their age and environmental factors.

Table 1. Chitin extraction from cell wall of *A. oryzae var. effusus* by cold conc. HCl

Cell wall source	Cell wall yield%	Chitin yield%	Cell wall residue%
3 days cultured biomass	51.63	3.940	96.0
6 days cultured biomass	53.36	4.780	95.0
9 days cultured biomass	53.41	3.355	96.5
12 days cultured biomass	56.11	0.170	99.8

The isolated chitin was identified by the infrared spectrum, the result in Fig. 1, showed that the isolated material as well as of standard chitin proves the identity of the isolated material as chitin. The most characteristic bands of chitin, 1650cm^{-1} (Amide I); 1560cm^{-1}(Amide II);3400-3480cm^{-1} that responded to OH-3 and intra - intermolecular hydrogen bonds are evident in the spectra of the isolated material [26].

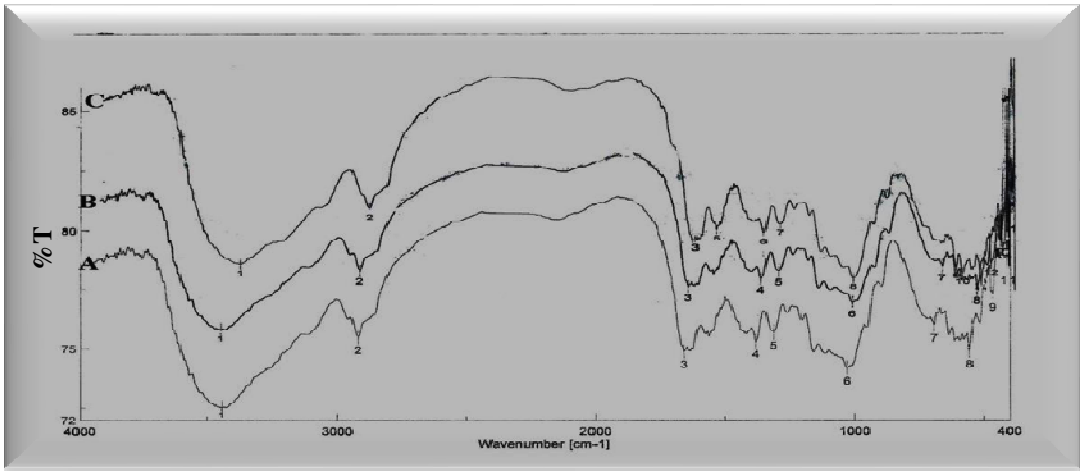

Fig. 1. I.R. spectra of chitin standard (curve, A), extracted chitin by cold conc. HCl, (curve, B) and extracted chitin by LiCl/DMAA (curve, C). Where %T meaning: Transmittance % of chitin

Among the chitin family, chitosan has received special attention since it has several applications [27,28]. Chitosan was extracted from alkali insoluble material (cell wall) obtained from 12 days (late stage) cultured biomass by using different concentrations of acetic acid (0.5-2.5%). Data presented in Table 2, showed that the highest yield of chitosan was 4.65%, at a concentration of 2.0% acetic acid after 8h (Table 3).

Table 2. Chitosan extraction from *A. oryzae var. effusus* cell wall using different concentrations of acetic acid for 8h at 95°C

Acetic acid concentration%	Chitosan yield%	Cell wall residue%
0.5	0.43	99.5
1.0	0.49	99.0
1.5	2.50	97.0
2.0	4.65	96.2
2.5	2.76	97.0

Table 3. Chitosan extraction from *A. oryzae var. effusus* cell wall using 2% acetic acid for different times at 95℃

Time(h)	Chitosan yield%	Cell wall residue%
2	1.50	98.2
4	2.00	97.8
6	2.30	97.4
8	5.00	94.6
10	1.30	98.0
12	0.30	99.6

The isolated product was characterized by infrared spectrum (Fig. 2). The infrared spectrum of the isolated product as well as of standard chitosan proves the identity of the isolated material as chitosan (Fig. 2). In both compounds, the most characteristic band of chitosan, 1640cm^{-1}, this absorption band is due to associated with water or amide band and a broad band near 1590cm^{-1}was present [26,29]. Several methods have been developed for the extraction of chitosan from the fungal mycelia. Most methods used 1N NaOH to remove protein and other cell wall materials and then the chitosan was extracted with 2% acetic acid. The yield of chitosan produced from the fungal mycelia treated in this way is very low. The extraction procedure for high yield production of pure chitosan from the fungal cell wall material has not yet been accomplished up to 2001 [30,31].

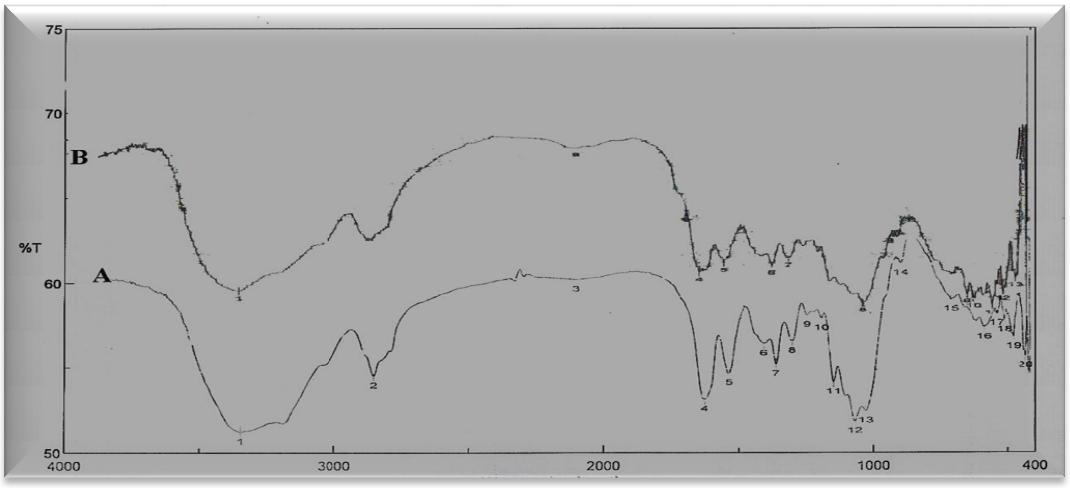

Fig. 2. I.R. spectra of chitosan standard (curve, A), extracted chitosan by acetic acid (curve, B)

Some researchers [32-34] have investigated the bond between chitosan and glucan in fungal cell wall and developed a powerful enzymatic method for the production of excellent quality chitosan from the fungal mycelia in a high yield.

Chitosan- glucan complex was isolated in 17.94% yield by treating the *A. oryzae var. effusus* biomass produced from kojic acid fermentation for 12 days with 40% aqueous NaOH for 6h.at 95℃. Chitosan- glucan complex was fractionated into its components. The amount of extracted chitosan was 3.5%. The residue obtained after the solubilization of chitosan by acid treatment was characterized as being mostly insoluble glucan (94%). The

infrared spectrum of the isolated products supports the identity of the isolated complex as chitosan- glucan complex (Fig. 3). The most characteristic bands of chitosan-glucan complex are 1650cm^{-1} C=N, 1550, 1375, 894 and 2920cm^{-1}. Absorption at 1550cm^{-1} was observed in the alkaline-insoluble glucan and chitosan. From a previous study reported by Muzzarelli, et al. [21], they found that the significant characteristics of animal chitosan are preserved in the chitosan-glucan complex isolated from *A. niger*. The formation of the chitin/ chitosan- glucan complex chains results in a rigid cross- linked network in the cell wall [35-37] and causes a considerable problem for the extraction of intact chitosan and glucan. It does not break down easily under mild extraction condition [32,38,39]. Therefore most of the researchers are trying to find the link-digestion with different enzymes [40,41,32].

β- 1, 3-glucan is another major structural component of the cell wall and is highly branched with β-1,6 linkage. Chitin, galactomannan, and β-1, 3/1, 4- glucan are covalently bound to β-1, 3 glucan. The alkali insolubility of β-1,3 glucan is due to its covalent linkages with chitin [42] chitin-glucan complex was isolated from 6 days cultured *A. oryzae var. effusus* biomass by using the methods of Stagg and Feather [19] or Feofilova et al. [43]. The alkali resistant residue was obtained in 17.034% and 16.36%, respectively. The lyophilized products were fractionated into its components (chitin and glucan) in yields 16.36% chitin, 82.72% glucan. Vysotskaya et al. [44] reported that the ratio between chitin and glucan varies depending not only on the source but also, on the conditions of cultivating the same fungus species.

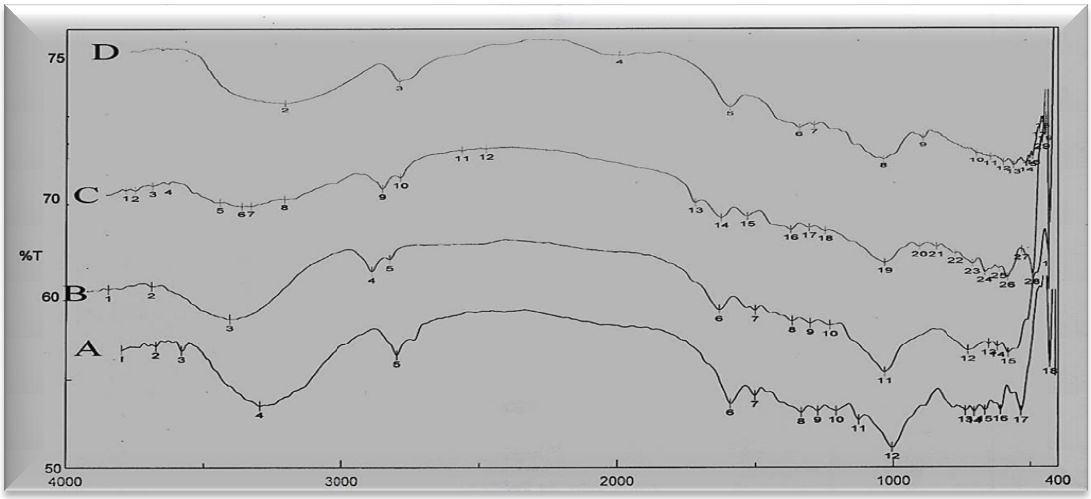

Fig. 3. I.R. spectra of laminarin standard (curve, A), β-1,3 glucan from chitin- glucan complex extracted from biomass (curve, B), β-1,3 glucan from chitin-glucan complex extracted by 10% NaOH for 36h (curve, C) and β-1,3 glucan from chitin-glucan complex extracted from chitosan- glucan (curve,D)

The infrared spectrum of isolated product supports the identity of the isolated complex as chitin- glucan complex (Fig. 3).Bands characteristic of chitin 1660cm^{-1}(Amide I); 1555cm^{-1} and 1315 are evident in the spectra of chitin. Absorption maxima : 888 indicating a β-glycosidic linkage,1035,1070,1150,1197,1228,1360,1530,1625,3845 and 2915cm^{-1} of β-glucan was almost identical to that of β,1-3 glucan *A. oryzae* var. *effusus* and laminarin (Sigma) (Fig. 3). These were no absorption bands at 810,820 and 845cm^{-1}, indicating the absence of α-1, 3 glucan.

4. CONCLUSION

The utilization of the fungal biomass waste actually will cover a part of the cost production of kojic acid. *A. oryzae var. effusus* NRC14 biomass contain about 18% chitosan-glucan-complex. The extracted chitosan and glucan yield from chitosan-glucan complex was 5% and 92.0%, respectively, and this in turn will allow the isolation of higher yield of polysaccharides.The technology involved in the extraction of their biopolymers is quite simple and the size scale is compatible with those currently used.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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