[BIO22] Production and quality of chitosan extracted from local fungal isolates

Dawn Carmel Paul, Kalaivani Nadarajah, Abdul Jalil Abdul Kader

School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor Darul Ehsan

Introduction

Chitosan is a non-toxic, biodegradable, biocompatible and highly polycationic biopolymer comprising of (1,4)-linked amino-deoxy-β-D-glucan. It is rarely found in nature and is found primarily as the main component of the fungal cell wall, especially in Zygomycetes (Arcidiacono & Kaplan 1992). At present, chitosan is commercially extracted from the exoskeleton of crustaceans using strong alkali. However, this harsh extraction process, together with the variability in source material leads to inconsistent physicochemical characteristics. Physical properties of chitosan extracted directly from fungi can be manipulated by control of factors such as growth media composition in fermentation and processing parameters in the extraction protocol (Arcidiacono & Kaplan, 1992; Jaworska & Konieczna, 2001). The degree of deacetylation and molecular weight dictates the behaviour of chitosan (Arcidiacono & Kaplan, 1992). Chitosan has a massive range of applications in industries such as cosmetics, pharmaceutical, textile, food, biotechnology and agriculture. The absence of allergenic proteins which are associated with crustacean chitosan makes fungal chitosan a suitable candidate as a food preservative.

This study aims to compare the chitosan production of local fungal isolates grown on different growth media and to increase fungal chitosan production through modifications of the extraction protocol developed by White *et al*., (1979). Quality analyses were performed to determine the degree of deacetylation, molecular weight and colour of the chitosan produced. Comparison between the antibacterial properties of fungal chitosan and crustacean chitosan was also conducted.

Materials and Methods

Fungi and culture medium

This study was conducted on three local fungal (Zygomycete) isolates designated as *Absidia* sp. DR, *Absidia* sp. 2a1 and *Rhizopus* sp. which were obtained from the culture collection of the school of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. Three growth media, YPG (yeast extract 3gm/l,peptone 10gm/l, glucose 10gm/l and 1 ml/l trace elements), BG (nutrient broth 8gm/l; yeast extract 0.1gm/l; glucose 5gm/l; KCl 0.1gm/l; $MgSO_4.7H_2O$ 0.25gm/l; $MnCl_2.4H_2O$ 0.002gm/l and $FeSO_4.7H_2O$ 0.00029 gm/l), and TVB (glucose 20gm/l; $(NH)_4SO_4$ 1.4gm/l; KH_2PO_4 2 gm/l; $CaCl_2$ 0.3gm/l; $MgSO_4.7H_2O$ 0.3gm/l; molybdic acid 85% 0.01gm/l and 1ml/l trace elements (per 500ml: $FeSO_4.7H_2O$ [5gm], $ZnCl_2$ [1.66gm], $CoCl_2.6H_2O$ [2gm], $MnSO_4.7H_2O$ [1.96gm] and hydrochloric acid 12 M [10ml]) were prepared. All three growth media were autoclaved at 110°C, 15 psi for 10 minutes. Growth media were prepared as 195ml aliquots in 500ml Erlenmeyer flasks (Arcidiacono & Kaplan 1992). Inocula was prepared aseptically by adding sterilized distilled water onto the fungal mycelia grown on potato dextrose agar plates (PDA) and then carefully scraping the spores from the mycelia using a stab wire. Spore suspension was filtered into a sterilized flask and spore count was performed using a haemacytometer (Improved Neubauer, 0.100 mm deep, brightline Hemacytometer, USA). The spore suspension was prepared as $1x10^7$ spores/ml. 5 ml of spore suspension was inoculated into every flask. The fungi were grown as submerged batch cultures at 30°C and with agitation of 150 rpm for 96 hours. Cultures were harvested at 12-hour intervals.

Extraction

The fungal biomass obtained from each 12-hour sampling point were dried in a freeze dryer (Labconco Model 216004) and weighed. Lyophilized fungal biomass were then subjected to chitosan extraction protocol employing the standard method of White *et al*. (1979). For the optimization of the extraction protocol, 60–hour old *Absidia* sp. DR biomass grown on YPG medium were freeze-dried and subjected to modified alkaline and acid

treatments in the White *et al*. method of extraction (1979). Three different acids, acetic acid (AA) formic acid (FA) and hydrochloric acid (HCl) were used as the extracting solution. Acid treatment were performed at acid concentration 2%, 6% and 10%; incubation period 3, 6 and 12 hours; temperature 60°C and 95°C.

Quality and statistical analysis

The degree of deacetylation was determined by the first derivative UV spectrophotometry method (FDUVS) (Muzarelli & Rochetti, 1985). The degree of deacetylation for the chitosan samples was determined based on calculations for the percentage of the glucosamine content in the samples (Muzarelli & Rochetti, 1985). The average molecular weight was resolved using the intrinsic viscosity method (Rege & Block, 1999). Curves for η_{sp}/concentration versus concentration (whereby η_{sp} - specific viscosity) were plotted and extrapolated in order to obtain the intrinsic viscosity, [η] ($[\eta]= [\eta_{sp}/c]_{c\rightarrow 0}$). The average molecular weight was then calculated using the Mark-Houwink equation:

$$[\eta] = KM^a$$

whereby K and a are coefficients related to the the Ubbelohde tube and the molecular weight of sample. The colour of chitosan was assessed using a chromameter (Minolta Model CR300, Japan) and the Hunter values for lightness (L) was recorded (Roberts, 1992). Data obtained was analyzed statistically using the Jandel Scientific SigmaStat statistical package.

Antibacterial test

Five fungal chitosan samples produced from the different treatments in the extraction protocol designated as CAA1(TVB), CAA1, CAA2, CFA1 and CHCl1 were selected. With the exception of CAA1(TVB), all fungal chitosan were extracted from mycelia harvested from the YPG. AA, FA and HCl correspond to acetic acid, formic acid and hydrochloric acid respectively. The numbers 1 and 2 refers to chitosan extraction using acid concentration 2%, incubation period 12 hours, temperature 95°C and acid concentration 10% incubation period 12 hours, temperature 95°C respectively. Crustacean chitosan (crab) with low (CLMW) and high (CHMW) average molecular weight were obtained from SIGMA whereas shrimp (CMMW) chitosan was obtained from Chitochem (Perak, Malaysia). The test bacteria comprising of three Gram positive bacteria (*Bacillus cereus, Staphylococcus aureus* and *Streptococcus faecalis*) and four Gram negative bacteria (*Enterobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa* and *Serratia marcescens*) were obtained from the culture collection of the Malaysian Institute of Medical Research (IMR), Kuala Lumpur.

Antibacterial properties of fungal and crustacean chitosan were determined through the Kirby-Bauer disc diffusion method and the determination of MIC values (Sudarshan *et al.* 1992). Measurement of optical density for the growth of bacteria were done at 450nm for *S. aureus* and 600 nm for other bacteria.

Results and Discussion

Fungal Growth and chitosan production

In general, all three isolates exhibited the best growth on YPG (Figure 1), followed by BG and TVB. There was no significant difference between the biomass and chitosan production for *Absidia* sp. DR and *Absidia* sp. 2a1. However the average production of chitosan for both isolates grown on YPG were 56.85% higher than the chitosan production of *Rhizopus* sp. grown on the same medium ($P<0.05$) (Figure 1).

Chitosan production was also significantly higher for fungi grown on YPG as compared to BG and TVB ($P<0.05$) (Figure 2). This study is in agreement with the fact that high biomass yields result in high chitosan production (Jaworska & Konieczna 2001). Among the three media utilized, YPG was found to contain the highest amount of nitrogen, 1.69gm/l of peptone, and yeast extract. Followed by TVB with 1.4gm/l of NH_4SO_4 and 1.24gm/l of nutrient broth and yeast extract in BG. According to Andrade *et al*. (2000), high nitrogen content increases the synthesis of enzymes involved in chitin biosynthesis. Therefore the highest yield from YPG was expected due to its high nitrogen content as compared to TVB and BG. Based on the absolute values obtained in this study, *Absidia* sp. DR produced 18% chitosan/biomass (60 hours), which was the highest amount of chitosan followed by *Absidia* sp. 2a1 and *Rhizopus* sp.

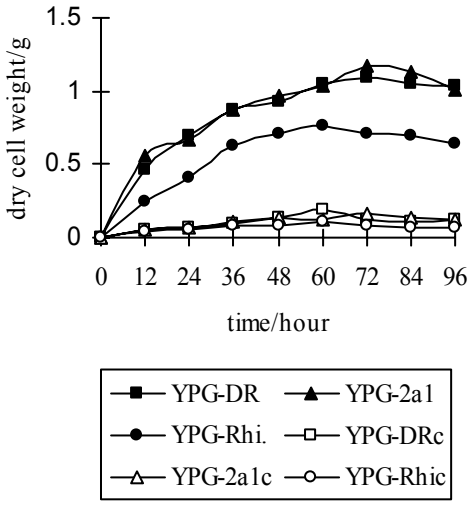

FIGURE 1 Profile of biomass and chitosan production from fungi grown on YPG.

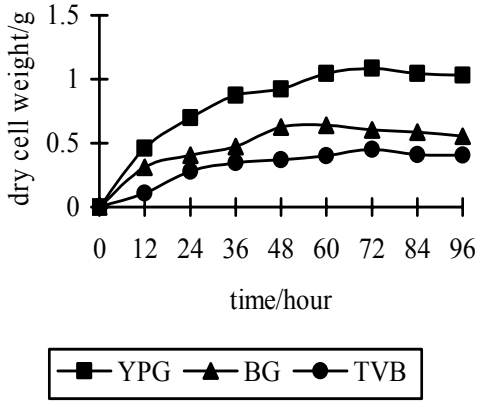

FIGURE 2 Profile of *Absidia* sp. DR biomass grown on different growth media

In the duration of the 96 hours, the mycelial biomass increased with the incubation period. The extractability of chitosan increased with the increment of biomass until the 60th or the 72nd hour of culturing whereby the fungi is in the late exponential phase of growth and the highest amount of chitosan is extracted. Beyond this time point, chitosan extraction exhibited a gradual decrease although fungal biomass continued to increase (Tan *et al*. 1996). This is attributed to the strong binding of chitin and chitosan to other cell wall components (Tan *et al*. 1996).

Extraction

In the alkaline treatment, five incubation period and three incubation temperature were used (Table 1). It was observed that significantly higher amount of chitosan was extracted with the increase of incubation period and temperature ($P<0.05$). The highest yield of chitosan was obtained at incubation temperature 121°C and incubation period 30 minutes (19.7%. chitosan/biomass).

TABLE 1 Extraction of chitosan from 1 gram biomass using alkali at different incubation period and temperature

Inc. period (min)	Chitosan (mg)		
	95°C	115°C	121°C
10	$^{a}87.75^{d}$	$^{b}117.50^{d}$	$^{b}129.00^{d}$
15	$^{a}90.00^{d}$	$^{b}134.25^{d}$	$^{b}139.25^{d\,e}$
20	$^{a}95.50^{d}$	$^{b}136.25^{d\,e}$	$^{b}148.50^{d\,e}$
25	$^{a}102.25^{d}$	$^{b}140.55^{d\,e}$	$^{c}159.00^{e}$
30	$^{a}118.50^{e}$	$^{b}145.25^{e}$	$^{c}196.75^{f}$

$^{a-c}$ Mean values in the same row bearing different superscripts have significant statistical difference.
$^{d-f}$ Mean values in the same column bearing different superscripts have significant statistical difference.

In the acid treatment, it was observed that the utilization of acetic acid and formic acid as the extracting solution yielded higher amounts of chitosan as compared to hydrochloric acid.

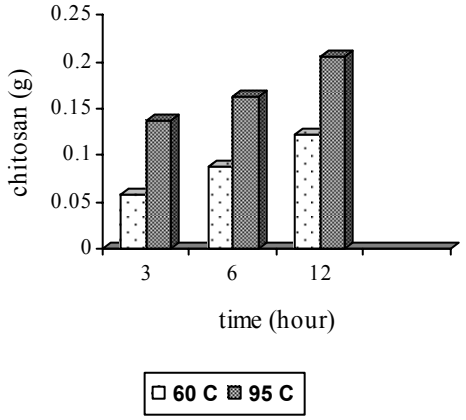

FIGURE 3 Extraction of chitosan using formic acid 6% at different incubation period and temperature

The highest chitosan yield was obtained with formic acid 6% at incubation period 12 hours and temperature 95˚C (Figure 3). This study also observed that the same incubation period, temperature and acid concentration rendered different effects and interactions when different acids were used as the extracting solution. For example, in the utilization of acetic acid, it was found that only the incubation period played a significant role in

affecting the amount of extractable chitosan ($P<0.05$). Whereas for formic acid, temperature was the main determining factor for chitosan extraction ($P= 0.034$) and for hydrochloric acid, acid concentration was found to significantly affect chitosan extraction ($P<0.01$).

Quality analyses

The degree of deacetylation for chitosan extracted from *Absidia* sp. DR grown on the different media were determined. This study found that the growth medium affects the degree of deacetylation of chitosan. Fungi grown on TVB and BG produce chitosan with a higher degree of deacetylation as compared to YPG (Table 2).

TABLE 2 Degree of deacetylation for chitosan extracted from *Absidia* sp. DR grown on different media

Growth medium	DD
YPG	82.56%
BG	83.79%
TVB	84.55%

This suggests that the differences in medium composition and the presence of certain elements may affect the activities of chitin deacetylase (Jaworska & Konieczna 2001). TVB contains 2000 ppm potassium ions and 300 ppm magnesium ions whereas BG contains 100 ppm potassium ions and 250 ppm magnesium ions. In addition, TVB also contains calcium ions. Microelements such as ferum, manganese and cobalt ions are able to influence the activities of chitin synthase and chitin deacetylase (Jaworska & Konieczna 2001). Therefore the presence of potassium and calcium ions in BG and TVB were able to catalyze the activities of chitin deacetylase whereby more acetyl moieties of chitin and chitosan chain were hydrolyzed, thus increasing the degree of deacetylation of the chitosan produced. DD increased with the temperature, incubation period and acid concentration.

The degree of deacetylation (DD) for chitosan extracted by the various acid treatment were also determined (Table 3). DD increased with the increase of incubation temperature, incubation period and acid concentration. DD was also found to be significantly higher ($P<0.01$) when hydrochloric acid was used as the extracting solution compared to acetic acid and formic acid. The highest DD (90.45%) was obtained with hydrochloric acid 10%, incubation period 12 hours and temperature 95°C meanwhile the lowest DD (76.16%) was obtained with formic acid 2%, incubation period 3 hours and temperature 60°C. Hydrochloric acid being a strong acid in comparison to acetic acid and formic acid, caused a higher degree of hydrolysis towards the acetyl moieties, in addition to hydrolysis between the monomers in the chitosan polymer.

Studies by Arcidiacono and Kaplan (1992) also showed that the type of growth medium utilized affects the average molecular weight of the chitosan extracted from fungi. According to Arcidiacono and Kaplan (1992), fungi grown on defined medium produces chitosan with significantly higher molecular weight as compared to chitosan extracted from fungi grown on complex medium. This study however found that the average molecular weight for chitosan extracted from fungi grown on complex medium was higher (2.370×10^5 Da in YPG) than chitosan extracted from fungi grown on defined (7.66×10^4 Da in TVB) and semi defined medium (6.765×10^4 in BG). The lower molecular weight conferred by fungi grown on defined medium might be due to the presence of elements such as ferum and manganese ions or certain substrates that may cause the activities of chitin synthase to slow down (Jaworska & Konieczna 2001) or maybe even to activate the activities of chitinase enzymes. The slow down in the chitin synthase activities affects the building of chitin polymer chain, accounting for the derivation of relatively smaller chitosan polymers. The activation of chitinase on the other hand promotes the cleaving of chitin polymer chain and therefore shortening the length of the chitin polymer chain and reducing the molecular weight of the chitin and chitosan produced.

This study found that the utilization of acid as the extracting solution at low concentrations, short incubation period and low temperature gave rise to lighter-coloured chitosan (Table 4). The strength of acid also significantly affected the colour of chitosan whereby weaker acids produced lighter coloured chitosan as compared to strong acids

TABLE 3 Degree of deacetylation for fungal chitosan extracted using different acid treatments

Acid concentration	Incubation period	Temperature	Acetic acid	Formic acid	Hydrochloric acid
2 %	3 hours	60°C	[a]76.16%[c]	[a]76.89%[c]	[b]86.26%[c]
6 %	3 hours	60°C	[a] 82.41%[d]	[a] 81.72% [d]	[b] 86.78% [d]
10 %	3 hours	60°C	[a] 82.82%[e]	[a] 83.98% [e]	[b] 88.23% [e]
2 %	12 hours	95°C	[a] 82.02%[f]	[a] 82.56% [f]	[b] 87.31% [f]
6 %	12 hours	95°C	[a] 86.86%[g]	[a] 84.83% [g]	[b] 89.88% [g]
10 %	12 hours	95°C	[a] 87.12%[h]	[a] 86.15% [h]	[b] 90.45%[h]

[a-b] Mean values in the same row bearing different superscripts have significant statistical difference.
[c-h]Mean values in the same column bearing different superscripts have significant statistical difference.

TABLE 4 Hunter Lightness (L) values of chitosan extracted using different acid treatments

Acid concentration	Treatment	Acetic acid (L)	Formic acid (L)	Hydrochloric acid (L)
2%	3 hours 60°C	75.60	81.08	80.72
	6 hours 60°C	74.82	80.56	76.34
	12 hours 60°C	74.45	78.43	72.97
	3 hours 95°C	73.05	78.92	70.26
	6 hours 95°C	72.18	79.04	69.23
	12 hours 95°C	76.03	78.86	68.60

($P<0.05$). Chitosan was found to have the highest lightness value ($L>80$) when treated using formic acid 2%, incubation period 3 hours and temperature 60ºC. Chitosan extracted from fungi grown on defined media was also found to have higher lightness values compared to samples extracted from fungi grown on complex medium.

Antibacterial properties

Fungal and crustacean chitosan had similar bacterial inhibition trends whereby Gram positive bacteria were more susceptible to the action of chitosan as compared to Gram negative bacteria. In the disc diffusion test, the diameters for the zones of inhibition ranged from 7.0 to 9.5 mm for all fungal and crustacean chitosans. Fungal chitosan CAA1 (82.56%, 2.370×10^5 Da) gave the best overall results. In past studies(Sudarshan *et al.,*1992), chitosan with higher degree of deacetylations were said to yield better inhibitory effects as compared to chitosan with lower degree of deacetylation. However this study found that CAA1 (82.56%) rendered higher inhibition as compared to CAA2 (87.12%) and CHCl1 (87.31%), both with higher degree of deacetylation (Table 5). This finding is supported by Park et al. (2004). In the MIC test CAA1 gave the lowest MIC value for Gram positive bacteria whereas CAA2 yielded the best inhibitory effect towards Gram negative bacteria. Higher degree of deacetylation and higher molecular weight may enable chitosan to inhibit Gram negative bacteria as compared to the lower values for Gram positive bacteria. The lack of uniformity in the MIC results indicated that the effectiveness of the antimicrobial activity of chitosan is dependent on its physicochemical attributes and also the morphology of the test bacteria. The MIC values given by CAA1 were comparable to that of the crustacean chitosans except for *E. coli* and *P. aeruginosa.*. This study also found that

TABLE 5 Zones of inhibition (mm) for fungal and crustacean chitosan towards *S. aureus*

Concentration/Chitosan	CAA1	CAA2	CHCl1	CMMW
10 mg/ml	8.50	8.00	8.00	8.00
5 mg/ml	8.75±0.25	8.25±0.25	7.75±0.25	8.25±0.25
2.5 mg/ml	9.00	7.50	7.50	8.00
1.25 mg/ml	7.25±0.25	7.00	7.00	8.00
0.625 mg/ml	7.25±0.25	-	-	-

fungal chitosan produced from various treatments have antibactericidal effects towards *B. cereus* at different concentrations (Table 6). The crustacean chitosan used in this study did not exhibit antibactericidal effect towards *B. cereus.* It is possible that the crustacean chitosan were unable to cause further bacterial damage due to their high average molecular weight ($2.216x10^6$ Da to 8.099×10^6)

TABLE 6 Minimum bactericidal concentration towards *Bacillus cereus*

Chitosan	MBC values (mg/ml)
CTVB	5.00
CAA1	1.25
CAA2	2.50
CFA1	5.00
CHCl1	0.625

Acknowledgement

The authors would like to thank the Ministry of Science, Technology and Innovation for the National Science Fellowship awarded to Dawn Carmel Paul and for IRPA Grant awarded to Kalaivani Nadarajah and Abdul Jalil Abdul Kader.

References

Andrade, V. S., Neto, B.B., Souza, W and Campos-Takaki, G.M. (2000). A factorial design analysis of chitin production by *Cunnighamella elegans*. *Canadian Journal of Microbiology* 46: 1042-1045.

Arcidiacono, S. and Kaplan, D.L. (1992). Molecular Weight Distribution of Chitosan Isolated from *Mucor rouxii* under Different Culture and Processing Conditions. *Biotechnology and Bioengineering* 39: 281-286

Jaworska, M.M. and Konieczna, Z. (2001) The Influence of Supplemental Components In Nutrient Medium on Chitosan Formation by the Fungus *Absidia orchidis*. *Applied Microbiology and Biotechnology* 56: 220-224

Muzzarelli, R. A.A. and Rochetti, R. (1985). Determination of the degree of acetylation of chitosans by first derivative ultraviolet spectrophotometry. *Carbohydrate Polymers* 5: 461-472.

Park, P.J., J.Y. Je, H.G. Byun, S.H. Moon and Kim, S.K. (2004). Antimicrobial activity of hetero-chitosans and their oligosaccharides with different molecular weights. *Journal of Microbiology and Biotechnology* 14: 317-323

Rege, P.R. and Block, L. H. (1999). Chitosan processing: Influence of process parameters during acidic and alkaline hydrolysis and effect of the processing sequence on the resultant chitosan's properties. *Carbohydrate Research* 321: 35-245.

Roberts, G.A.F. (1992). Chitin Chemistry. London: The Macmillan Press Ltd.

Sudarshan, N.R., Hoover, D.G. and Knorr,D. (1992). Antibacterial action of chitosan. *Food Biotechnology* 6:257-272.

Tan, S.C, Teck, K.T., Sek, M.W. and Khor, E. (1996). The Chitosan Yield of Zygomycetes at their Optimum Harvesting Time. C*arbohydrate Polymers* 30: 239-242.

White, S.A., Farina, P.R. and Fulton, I. (1979). Production and Isolation of Chitosan from *Mucor rouxii. Applied and Environmental Microbiology* 38: 323-328