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EFFECTS OF ALKALINE AND ACID TREATMENT TO THE YIELD AND QUALITY OF CHITOSAN EXTRACTED FROM *Absidia* sp. dr

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Abstract. Chitosan was extracted from 60-hour old biomass of *Absidia* sp. dr that was subjected to different alkaline and acid treatment in the extraction protocol. In both alkaline and acid treatment, the temperature, incubation period and concentration of acid or alkaline significantly affects the production and quality of the chitosan produced ($P<0.05$). The type of acid used was also important in the production of chitosan. Quality analysis was performed to determine the degree of deacetylation, molecular weight and colour of chitosan. Usage of hydrochloric acid in the extraction of chitosan gave a significantly higher degree of deacetylation (DD) compared to acetic acid and formic acid. The average molecular weight of selected chitosan samples obtained ranged from 6.765×10^4 Da to 2.757×10^5 Da. Employment of strong acid, high acid concentration and high temperature produced darker coloured chitosan whereas milder treatments gave lighter coloured chitosan.

Keywords: Chitosan, *Absidia* sp. dr, degree of deacetylation, molecular weight

Abstrak. Kitosan diekstrak daripada biojisim kulat *Absidia* sp. dr yang berusia 60 jam dengan menggunakan rawatan alkali dan rawatan asid yang berbeza. Dalam kedua-dua rawatan tersebut, suhu, masa pengeraman dan kepekatan asid dan alkali memberikan kesan yang signifikan terhadap penghasilan dan kualiti kitosan yang diperoleh ($P<0.05$). Dalam rawatan asid, jenis asid juga didapati penting dalam penghasilan kitosan. Analisis kualiti bagi menentukan darjah penyahasetilan, berat molekul dan warna kitosan juga turut dilakukan. Penggunaan asid hidroklorik dalam pengekstrakan kitosan memberikan darjah penyahasetilan (DD) yang lebih tinggi berbanding asid asetik dan asid formik. Kitosan kulat terpilih mempunyai berat molekul purata dalam julat 6.765×10^4 Da hingga 2.757×10^5 Da. Penggunaan asid kuat, kepekatan asid yang tinggi dan suhu eraman yang tinggi menghasilkan kitosan dengan warna yang gelap sementara perlakuan yang lebih lembut menghasilkan kitosan dengan warna yang lebih cerah.

Kata kunci: Kitosan, *Absidia* sp. dr, darjah penyahasetilan, berat molekul

1.0 INTRODUCTION

Chitosan, a polymer rarely found in nature, is a non-toxic, biodegradable, biocompatible and highly polycationic biopolymer comprising of (1,4)-linked amino-

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deoxy-β-D-glucan. It is found primarily as the main component of the fungal cell wall, especially in Zygomycetes [1]. On a commercial scale, chitosan is extracted from the exoskeleton of crustaceans employing harsh chemical treatments. However, this extraction process, together with the variability in source material leads to inconsistent physicochemical characteristics of the chitosan produced. These characteristics make fungi a promising chitosan source as the physical properties of the extractable chitosan from fungi can be manipulated through the regulation of factors such as growth media composition and processing parameters in the extraction protocol [1]. The degree of deacetylation and molecular weight determines the characteristics of chitosan [1,2]. 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 [2].

This study aims to increase fungal chitosan production through modifications of the extraction protocol developed by White *et al.* [3]. In addition, this study was conducted to ascertain the effects of the alkaline and acid treatment used in the extraction protocol towards the quality of chitosan in terms of degree of deacetylation, molecular weight and colour of the chitosan produced.

2.0 MATERIALS AND METHODS

2.1 Fungi and Culture Conditions

This study was conducted on a local fungal (Zygomycete) isolate designated as *Absidia* sp. dr which was obtained from the culture collection of the School of BioSciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. A complex growth media, YPG (yeast extract 3 g/L peptone 10 g/L, glucose 10 g/L) was prepared and 1 mL/L trace element solution (trace element solution per 500 mL: $FeSO_4.7H_2O$ [5 g], $ZnCl_2$ [1.66 g], $CoCl_2.6H_2O$ [2 g], $MnSO_4.7H_2O$ [1.96 g] and hydrochloric acid 12 M [10 mL]) was added to the medium prepared. YPG was autoclaved at 110°C, 15 psi for 10 minutes as 195 mL aliquots in 500 mL Erlenmeyer flasks [1]. Inoculum was prepared aseptically by adding sterilized distilled water onto the fungal mycelia grown on potato dextrose agar (PDA) plates and 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 1×10^7 spores/mL. 5 mL of spore suspension was inoculated into every flask. *Absidia* sp. dr was then grown as submerged batch cultures at 30°C and with agitation of 150 rpm for 60 hours and then harvested.

2.2 Chitosan Extraction

The fungal biomass obtained were dried in a freeze dryer (Labconco Model 216004) and weighed. Lyophilized fungal biomass was then subjected to chitosan extraction protocol employing the standard method of White *et al.* [3]. This extraction protocol includes an alkaline treatment which is followed by an acid treatment. In the optimization of the extraction protocol, freeze-dried fungal biomass were subjected to modified alkaline treatment followed by the standard acid treatment in the White *et al.* [3] method of extraction. Meanwhile in the acid treatment, fungal biomass was subjected to the standard alkaline treatment prior to the modified acid treatment [4].

2.2.1 Alkaline Treatment

For the study on the effects of alkaline concentration on chitosan extraction, IM, 2M, 3M, and 4M sodium hydroxide (NaOH) solutions were used to treat samples at 121°C for 15 minutes. For the study on the the effect of temperature and incubation period, three temperatures, 95°C, 110°C and 121°C, and five incubation period, 10, 15, 20, 25 and 30 minutes were used with NaOH 1M as the treatment solution.

2.2.2 Acid Treatment

Three different acids, acetic acid (AA), formic acid (FA) and hydrochloric acid (HCl) were used as the extracting solution. Acid treatment were performed at 2%, 6% and 10% acid concentrations; incubation period at 3, 6 and 12 hours; temperature at 60°C and 95°C. The acid treatment was performed using a general linear experimental design.

2.3 Determination of Degree of Deacetylation

The degree of deacetylation was determined by the first derivative UV spectrophotometry method (FDUVS) [5]. The degree of deacetylation for the chitosan samples was determined based on percentage calculations of the glucosamine content in the samples [5].

2.4 Determination of Molecular Weight

The average molecular weight was resolved using the intrinsic viscosity method [6]. Curves for η_{sp}/concentration versus concentration (whereby η_{sp} - specific viscosity) were plotted and extrapolated in order to obtain the intrinsic viscosity, [η] ([η]= $[\eta_{sp}/c]_{c\rightarrow 0}$). The average molecular weight was then calculated using the Mark-Houwink equation:

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

K and a are the coefficients related to the the Ubbelohde tube and the molecular weight of sample [6].

2.5 Assessment of Colour

The colour of chitosan was assessed using a chromameter (Minolta Model CR300, Japan) and the Hunter value for lightness (L) was recorded [7]. Data obtained was analyzed statistically using the Jandel Scientific SigmaStat statistical package.

3.0 RESULTS AND DISCUSSION

3.1 Chitosan Yield

3.1.1 Alkaline Treatment

In the alkaline treatment, it was observed that significantly higher amounts ($P<0.05$) of chitosan was extracted with the increase in incubation period and temperature. The highest yield of chitosan was obtained at incubation temperature 121°C and incubation period of 30 minutes (19.7% chitosan/biomass). The utilization of different NaOH concentrations (1M, 2M, 3M and 4M) did not give significant differences to the chitosan yield (Table 1).

Table 1 Effect of concentration of NaOH solution on the extraction yield of chitosan

Alkaline treatment Molarity of NaOH (a)	Chitosan (mg)
Standard treatment (b)	139.25
2M (b)	138.69
3M (b)	141.11
4M (b)	139.38
1M (c)	140.22
(Alkaline treatment performed 3X)	

(a) All treatments were done at 121°C for 15 minutes
(b) Treatments with 1M NaOH in the standard method
(c) This treatment was done at 121°C for 15 minutes and was repeated twice

It was observed that the yield of chitosan increased significantly with the increase of temperature and incubation period especially for 25 minutes and 30 minutes incubation period (Table 2). Chitosan production showed significant differences even with small increments of temperature and incubation period ($P<0.05$). At 121°C, a raise of the incubation period from 15 minutes to 30 minutes yielded a significant increase ($P<0.05$) of chitosan yield (41.29%). A decrease in the temperature to 95°C

Table 2 Effect of temperature and incubation period on the extraction yield of chitosan with 1M NaOH

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	$^{a}90.00^{d}$	$^{b}134.25^{d}$	$^{b}139.25^{de}$
20	$^{a}95.50^{d}$	$^{b}136.25^{de}$	$^{b}148.50^{de}$
25	$^{a}102.25^{d}$	$^{b}140.55^{de}$	$^{c}159.00^{e}$
30	$^{a}118.50^{e}$	$^{b}145.25^{e}$	$^{c}196.75^{f}$

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

at standard incubation period (15 minutes) showed a reduction of 35.37% in the chitosan produced. Meanwhile, alkaline treatment at 115°C and incubation period of 15 minutes did not render significant differences when compared to the standard alkaline treatment (121°C, 15 minutes). Higher temperatures and longer incubation periods were necessary to enable effective interactions between NaOH and the constituents of the fungal cell wall thus making it possible to extract higher levels of chitosan [8]. Longer incubation period also gave longer reaction time for NaOH to act on the chitin and chitosan structure in order to separate chitosan from other cell wall polysaccharides [7]. Thus it can be concluded that increasing the incubation period to 30 minutes and maintaining the temperature at 121°C yielded high amounts of chitosan when alkaline treatment was used (Table 2).

3.1.2 Acid Treatment

In 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. The highest chitosan yield was obtained with 6% formic acid at incubation period of 12 hours at 95°C (Figure 1). 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 ($P<0.05$) (data not shown). 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 ($P<0.01$) (data not shown).

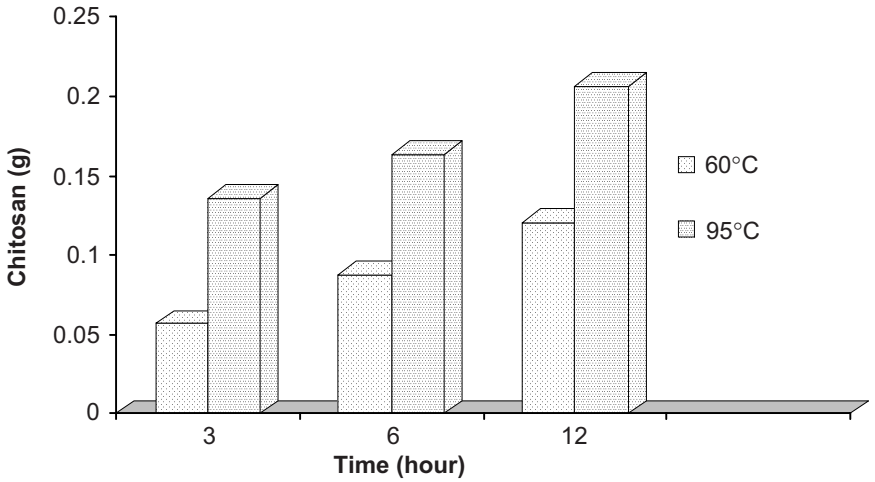

Figure 1 Extraction of chitosan using formic acid 6% at different incubation period and temperature

3.2 Degree of Deacetylation

3.2.1 Effects of Alkaline Treatment to the Degree of Deacetylation of Chitosan

The increase of the alkaline concentration in the alkaline treatment increased the degree of deacetylation (DD) of the chitosan extracted (Table 3). This is in accordance with the study conducted by Rigby and Nemours [7] which stated that the level or extent of deacetylation is influenced and controlled by alkaline concentration, temperature, incubation period, particle size and density. Higher alkaline concentrations may cause alkaline hydrolysis to occur at a higher rate in the AIM resulting in a higher degree of deacetylation [9]. Repetition of the standard alkaline treatments to the same AIM samples for up to 3 times also produced chitosan with

Table 3 Effect of concentration of NaOH solution on the degree of deacetylation of chitosan

Alkaline treatment Molarity of NaOH (M) (a)	Degree of deacetylation
1M (Standard treatment)	82.56%
2M	83.97%
3M	85.20%
4M	86.75%
1M (b) (Alkaline treatment performed 3X)	83.69%

(a) All treatments done at 121°C for 15 minutes. Treatment with 1M NaOH is considered the standard method
(b) Treatments done at 121°C for 15 minutes and repeated twice

a higher DD as compared to chitosan extracted using the standard alkaline treatment (Table 3) [9]. This treatment repetitively exposed the acetamido group at C2 to the hydrolytic action of NaOH thus increasing the probability of deacetylation of the remaining amide group.

3.2.2 Effects of Acid Treatment on Deacetylation of Chitosan

The degree of deacetylation (DD) for chitosan extracted by the various acid treatment were also determined (Table 4). The general trend observed was that DD increased with the increase in 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 10% hydrochloric acid and 12 hours incubation period at 95°C. Meanwhile, the lowest DD (76.16%) was obtained with 2% formic acid and 3 hours incubation period at 60°C. Hydrochloric acid, being a strong acid in comparison to acetic acid and formic acid, caused a greater extent of hydrolysis towards the acetyl moieties, in addition to the hydrolysis within the network of monomers in the chitosan polymer [6]. DD was found to be highest for all acids at 10% acid concentration. In the acid treatment, it was observed that varying individual

Table 4 Degree of deacetylation for fungal chitosan extracted using different acid treatments

Acid concentration	Incubation period	Temperature acid	Acetic acid	Formic acid	Hydrochloric acid
2%	3 hours	60°C	$^{a}76.89\%^{c}$	$76.16\%^{c}$	$^{b}86.26\%^{c}$
6%	3 hours	60°C	$^{a}81.72\%^{d}$	$^{a}82.41\%^{d}$	$^{b}86.78\%^{d}$
10%	3 hours	60°C	$^{a}83.98\%^{e}$	$^{a}82.82\%^{e}$	$^{b}88.23\%^{e}$
2%	3 hours	95°C	$^{a}81.61\%^{f}$	$^{a}81.19\%^{f}$	$^{b}86.88\%^{f}$
6%	3 hours	95°C	$^{a}83.85\%^{g}$	$^{a}85.26\%^{g}$	$^{b}88.22\%^{g}$
10%	3 hours	95°C	$^{a}85.02\%^{h}$	$^{a}86.28\%^{h}$	$^{b}89.93\%^{h}$
2%	6 hours	60°C	$^{a}77.73\%^{i}$	$^{a}77.62\%^{i}$	$^{b}86.41\%^{i}$
6%	6 hours	60°C	$^{a}82.78\%^{j}$	$^{a}83.01\%^{j}$	$^{b}87.10\%^{j}$
10%	6 hours	60°C	$^{a}84.16\%^{k}$	$^{a}83.17\%^{k}$	$^{b}88.67\%^{k}$
2%	6 hours	95°C	$^{a}82.05\%^{l}$	$^{a}81.86\%^{l}$	$^{b}87.26\%^{l}$
6%	6 hours	95°C	$^{a}84.55\%^{m}$	$^{a}86.66\%^{m}$	$^{b}89.21\%^{m}$
10%	6 hours	95°C	$^{a}85.89\%^{n}$	$^{a}86.97\%^{n}$	$^{b}90.21\%^{n}$
2%	12 hours	60°C	$^{a}79.68\%^{o}$	$^{a}78.65\%^{o}$	$^{b}86.63\%^{o}$
6%	12 hours	60°C	$^{a}83.55\%^{p}$	$^{a}83.71\%^{p}$	$^{b}87.73\%^{p}$
10%	12 hours	60°C	$^{a}84.57\%^{q}$	$^{a}84.41\%^{q}$	$^{b}89.03\%^{q}$
2%	12 hours	95°C	$^{a}82.56\%^{r}$	$^{a}82.02\%^{r}$	$^{b}87.31\%^{r}$
6%	12 hours	95°C	$^{a}84.83\%^{s}$	$^{a}86.86\%^{s}$	$^{b}89.88\%^{s}$
10%	12 hours	95°C	$^{a}86.15\%^{t}$	$^{a}87.12\%^{t}$	$^{b}90.45\%^{t}$

$^{a\text{-}b}$ Mean values in the same row bearing different superscripts have significant statistical difference

$^{c\text{-}t}$ Mean values in the same column bearing different superscripts have significant statistical difference

factors (acid concentration, temperature and incubation period) did not seem to give consistent results whereas the varying/manipulation of particular parameter factor gave mixed results. However, the 3 way ANOVA performed showed that the trend of the DD values was dependent on the interaction between factors and also the type of acid utilized (Table 4). Multiple paired comparison (Student-Newman-Keuls) shows that the DD of chitosans extracted using hydrochloric acid were significantly different compared to chitosan extracted using acetic acid and formic acid ($P<0.05$). There was no significant difference between the DD values of chitosan extracted using acetic acid and formic acid ($P>0.05$) (Table 4).

3.2.3 Molecular Weight of Chitosan

The average molecular weight of selected fungal chitosan (6.765×10^4 Da to 2.757×10^5 Da) was found to be very much lower compared to crustacean chitosan (2.216×10^6 Da to 8.099×10^6 Da) (Table 5). These values were similar to the results obtained by Pochanavanich and Suntornsuk [10] where the molecular weight ranged from 2.7×10^4 Da to 1.9×10^5 Da. This study found that the different acid treatment affected the molecular weight of the chitosan extracted [7]. In general, it was found that the use of an organic acid (acetic acid and formic acid) yielded chitosan with higher molecular weights as compared to mineral acid (hydrochloric acid). This was due to the fact that organic acids were weak agents for hydrolysis. Therefore, the utilization of organic acids as the extracting solution would not cause extensive degradation in the polymer chain of chitosan [7]. Chitosan sample extracted using hydrochloric acid, a good hydrolyzying agent, had the lowest molecular weight

Table 5 Molecular weight of chitosan

Chitosan	Intrinsic viscosity [η]	Degree of deacetylation (%)	Values of K and a	Molecular weight, M (Da)
AA 2% 12 hr 95°C	205.7	82.56	1.424×10^{-3}, 0.96	2.370×10^5
AA 10% 12 hr 95°C	237.88	86.15	1.424×10^{-3}, 0.96	2.757×10^5
AA 2% 3 hr 60°C	76.828	76.89	0.104×10^{-3}, 1.12	1.737×10^5
FA 2% 12 hr 95°C	101.61	82.02	1.424×10^{-3}, 0.96	1.137×10^5
HCl 2% 12 hr 95°C	61.741	87.31	1.424×10^{-3}, 0.96	7.366×10^4
LMW SIGMA	1759.2	85.56	1.424×10^{-3}, 0.96	2.216×10^6
HMW SIGMA	6103.8	85.79	1.424×10^{-3}, 0.96	8.099×10^6

Description of chitosan sample:
AA – chitosan extracted using acetic acid
FA – chitosan extracted using formic acid
HCl – chitosan extracted using hydrochloric acid
LMW SIGMA – low molecular weight commercial chitosan
HMW SIGMA – high molecular weight commercial chitosan

(6.765×10^4 Da). Therefore, the differences in the molecular weight might be attributed to the difference in the degree of hydrolysis and degradation of the chitosan polymer chain by the acids. Factors such as incubation period, temperature and acid concentration also influenced the molecular weight of the fungal chitosan. Long incubation periods and high temperatures would further promote the hydrolysis and degradation of the polymer chain [7].

3.3 Colour Assessment

This study found that the utilization of acid as the extracting solution at low concentrations, short incubation period and low temperature produced lighter-coloured chitosan (Table 6). The strength of acid also significantly affected the colour of chitosan, where weaker acids produced lighter coloured chitosan as compared to strong acids ($P<0.05$). Chitosan was found to have the highest lightness value (L = 81.08) when treated using 2% formic acid, 3 hours incubation period and temperature of 60°C. The lowest lightness value (L = 62.57) was obtained with 10% hydrochloric acid, 12 hours incubation period and temperature of 95°C (Table 6). Being a carbohydrate based polymer, the drying process at high temperature over a long period may darken the colour of the resulting chitosan due to caramelization [11].

Table 6 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
6%	3 hours 60°C	76.37	78.36	71.58
	6 hours 60°C	75.18	78.09	71.02
	12 hours 60°C	75.91	76.88	68.53
	3 hours 95°C	71.51	77.13	69.79
	6 hours 95°C	70.32	75.89	68.74
	12 hours 95°C	71.29	76.70	70.26
10%	3 hours 60°C	74.08	74.15	69.57
	6 hours 60°C	73.40	72.55	67.36
	12 hours 60°C	73.07	72.96	64.61
	3 hours 95°C	72.15	68.34	67.16
	6 hours 95°C	70.23	70.22	65.23
	12 hours 95°C	71.58	68.97	62.57

4.0 CONCLUSION

In the alkaline treatment, the highest chitosan yield (19.67% chitosan/biomass) was obtained with 30 minutes incubation period and temperature of 121°C. Meanwhile, the highest yield for the acid treatment (20.6% chitosan/biomass) was obtained with 6% formic acid, 12 hours incubation period and temperature of 95°C. The highest DD (90.45%) was obtained using hydrochloric acid 10%, incubation period of 12 hours and temperature at 95°C. Chitosan with the highest lightness value (L = 81.08) was obtained when treated using formic acid 2%, incubation period of 3 hours and temperature at 60°C. The results showed that the extraction protocol can be modified to give higher chitosan yields and variability in the quality of the chitosan produced. However, the desired properties of chitosan will determine the combination of treatments to be used.

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