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Concurrent production of chitin from shrimp shells and fungi

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Abstract

Crustacean shells constitute the traditional and current commercial source of chitin. Conversely, the control of fungal fermentation processes to produce quality chitin makes fungal mycelia an attractive alternative source. Therefore, the exploitation of both of these sources to produce chitin in a concurrent process should be advantageous and is reported here. Three proteolytic *Aspergillus niger* (strains 0576, 0307 and 0474) were selected from a screening for protease activity from among 34 zygomycete and deuteromycete strains. When fungi and shrimp shell powder were combined in a single reactor, the release of protease by the fungi facilitated the deproteinization of shrimp-shell powder and the release of hydrolyzed proteins. The hydrolyzed proteins in turn were utilized as a nitrogen source for fungal growth, leading to a lowering of the pH of the fermentation medium, thereby further enhancing the demineralization of the shrimp-shell powder. The shrimp-shell powders and fungal mycelia were separated after fermentation and extracted for chitin with 5% LiCl/DMAc solvent. Chitin isolates from the shells were found to have a protein content of less than 5%, while chitin isolates from the three fungal mycelia strains had protein content in the range of 10–15%. The relative molecular weights as estimated by GPC for all chitin samples were in the 10^5 dalton range. All samples displayed characteristic profiles for chitin in their FTIR and solid-state NMR spectra. All chitin samples evaluated with MTT and Neutral Red assays with three commercial cell lines did not display cytotoxic effects.

Keywords: Fungi; Shrimp shells; Chitin; Fermentation; Cytotoxicity

1. Introduction

Chitin and its derivatives have been reported to be useful for biomedical applications such as wound healing and dressings, drug delivery agents, anti-cholesterolemic agents, blood anticoagulants, anti-tumor agents and immunoadjuvants.[1–3] In biotechnology, applications include wastewater treatment where chitin (as its derivative, chitosan) acts as an absorbent for heavy and radioactive metals, has use as food and feed additives, and in the manufacture of textile, paper, film and sponge sheet materials.[4]

The traditional and commercial source of chitin is from shells of crab, shrimp and krill

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that are wastes from the processing of marine food products. The annual worldwide crustacean shells production has been estimated to be 1.2×10^6 tons, and the recovery of chitin and protein from this waste is an additional source of revenue.[5] However, crustacean shell wastes can be limited and subject to seasonal supply. In recent years, chitin obtained by extraction from fungal mycelia is gaining importance. Fungal mycelia can be cultivated throughout the year by fermentation that is rapid, synchronized and can be organized in a closed or semi-closed technological circuit to comply with modern ecological requirements. In addition, fungal mycelia are relatively consistent in composition and are not associated with inorganic materials; therefore, no demineralization treatment is required to recover fungal chitin.[6]

In the recovery of chitin from shellfish sources, the removal of associated minerals and proteins normally requires the use of HCl and NaOH. These reagents can cause deacetylation and depolymerization of chitin. An alternative to these harsh chemical treatments is the use of proteolytic microorganisms, in particular extracellular proteases secreted from fungi. Hall and Silva reported that the fermentation of crustacean shells with lactic acid bacteria lowered the pH of the medium to approximately pH 4, facilitating the hydrolysis of proteins while leaving the associated chitin intact.[7] Bustos and Healy demonstrated that chitin obtained by the deproteinization of shrimp shell waste with various proteolytic microorganisms including *Pseudomonas maltophilia*, *Bacillus subtilis*, *Streptococcus faecium*, *Pediococcus pentosaseus* and *Aspergillus oryzae*, had higher molecular weights compared to chemically prepared shellfish chitin.[8]

The objective of this work was to investigate the concurrent production of chitin from shrimp shells and fungi by placing shrimp shells in direct contact with the fermentation of filamentous fungi. Proteolytic enzymes released from the fungi were anticipated to deproteinize and demineralize the shrimp shell releasing amino acids in the process that, in turn, would act as a nitrogen source for fungal growth. Two distinct sources of chitin would thus be obtained.

2. Results and discussion

Chitin in the exoskeleton of shrimp shells is associated intimately with proteins; therefore, deproteinization in the chitin extraction process is crucial. A potentially cost-effective method would be to utilize the proteolytic enzymes present in certain chitin-containing fungal strains to deproteinize the shrimp shells and simultaneously use the hydrolyzed proteins from the shrimp shells for the cultivation of fungi, accumulating fungal mycelia, which are an additional source of chitin, distinct from the shrimp shell waste. Towards this goal, an initial systematic screening among filamentous fungal strains, reported to contain significant quantities of chitin for proteolytic activities was performed that would be optimized for fungal fermentation of shrimp shells.[9]

Fungal screening.—A total of 34 fungal strains of deuteromycetes and zygomycetes were screened for protease activity, as both groups of fungi have been reported to possess sufficiently high levels of chitin in their cell walls.[9–12] The screening was initially performed on solid media, yeast extract agar (YEA) with skim milk as the protein substrate, and corn-meal agar (CMA) with gelatin as the protein substrate, to provide semiquantitative measurements of protease activity. A clearing in the agar medium would indicate degradation of the protein substrate incorporated in the solid media, and the size of the clear zone would be a measure of the extent of protease activity, as summarized in Table 1.

A. carbonarius 0431, *Cunninghamella* sp. 0004 and all 17 strains of *A. niger* surveyed showed clearing zones around the fungal colony on YEA with skim milk but not on CMA with gelatin. The reverse was observed with three strains of *A. oryzae* (strains 0207, 0416 and 0510), *A. flavusoryzae* 0607 and *C. echinulata* 0601. Only two fungal strains, *Gongronella butleri* (strains 0201and 0489) showed protein degradation on both solid media, while eight fungal strains, *A. oryzae* (strains 0486 and 0512), *M. hiemalis* 0452, *M. hiemalis* +0566, *Rhizopus* sp. 0477, *R. arrhizus* 0261, *R. microsporus* 0264 and *R. oligosporus* 0260, did not show clearing on

either solid media. Since some protease activity was detected for all 34 fungal strains, a third screening method utilizing a casein-based substrate liquid assay was performed. Casein was readily hydrolyzed to varying extents by all 34 fungal strains with this liquid assay as compiled in Table 1, indicating substrate specificity of the extracellular protease produced by different fungal strains.

Most of the *A. niger* strains were found to give a higher protease activity compared to the other strains. This is consistent with the fact that *A. niger* strains have been and are used to produce commercial protease enzymes for industrial applications in the leather industry, as meat tenderizers and for proteolysis of milk and soy proteins to provide emulsifiers.[13] From the results of the solid media and liquid assay, *A. niger* 0307 had the highest protease activity (0.6790 unit) followed by *A. niger* 0576 (0.6260 unit) and *A. niger* 0474 (0.5695 unit). Therefore these three strains, *A. niger* strains 0576, 0307 and 0474 were selected for concurrent fermentation with shrimp shells.

Table 1
Protease activity of 34 fungal strains screened by yeast extract agar (YEA), corn meal assay (CMA) and protease assay

Fungus	YEA (1.5% skim milk) [a]	CMA (gelatin) [a]	Protease assay [b]
A. oryzae 0416	[c]	2.3 ± 0.5	4.9 ± 0.3
A. oryzae 0510		1.7 ± 0.4	4.3 ± 0.2
A. flavus-oryzae 0607		1.7 ± 0.4	2.3 ± 0.2
A. oryzae 0207		1.4 ± 0.3	4.0 ± 0.1
A. oryzae 0512		[c]	3.1 ± 0.3
A. oryzae 0486			0.2 ± 0.0
A. carbonarius 0431	12.4 ± 0.2		5.4 ± 0.2
A. niger 0576	14.6 ± 0.2		6.3 ± 0.2
A. niger 0474	12.2 ± 0.2		5.7 ± 0.3
A. niger 0577	11.9 ± 0.2		4.2 ± 0.6
A. niger 0238	11.5 ± 0.1		5.0 ± 0.1
A. niger 0290	11.4 ± 0.1		4.2 ± 0.2
A. niger 0412	11.1 ± 0.2		3.6 ± 0.7
A. niger 0237	11.0 ± 0.1		3.4 ± 0.2
A. niger 0307	9.9 ± 0.1		6.8 ± 0.4
A. niger 0386	9.9 ± 0.1		5.6 ± 0.2
A. niger 0398	9.5 ± 0.1		4.1 ± 0.2
A. niger 0397	9.3 ± 0.2		4.6 ± 0.2
A. niger 0825	9.2 ± 0.1		5.2 ± 0.3
A. niger 0243	8.6 ± 0.1		4.7 ± 0.1
A. niger 0206	8.2 ± 0.1		5.2 ± 0.2
A. niger 0384	7.1 ± 0.1		3.9 ± 0.3
A. niger 0241	5.9 ± 1.2		5.4 ± 0.2
A. niger 0236	4.7 ± 0.8		0.8 ± 0.3
G. butleri 0489	2.0 ± 0.4	1.2 ± 0.3	0.9 ± 0.1
G. butleri 0201	1.8 ± 0.3	1.1 ± 0.2	1.4 ± 0.1
Cunninghamella sp. 0004	1.8 ± 0.3		1.0 ± 0.0
C. echinulata 0601		3.3 ± 0.4	0.5 ± 0.0
M. hiemalis 0452			3.0 ± 0.3
R. oligosporus 0260			1.8 ± 0.2
Rhizopus sp. 0477			1.4 ± 0.1
M. hiemalis + 0566			1.1 ± 0.2
R. arrhizus 0261			1.0 ± 0.1
R. microsporus 0264			0.7 ± 0.1

[a] Protease activity is expressed as width of clear zone in mm. Values reported are the average of five replicates ± standard deviation.

[b] One unit activity of protease is defined as 1 μM of tyrosine produced in 1 min. Values reported are the average of five replicates ± standard deviation.

[c] Signifies no clearing zone was observed.

Fungal fermentation.—In fungal fermentation in the presence of shrimp shells, the contribution of washed (WS) and demineralized (DS) shrimp shells as a source of carbon, nitrogen and minerals was evaluated to determine the minimal medium composition. In the growth profile for the three *A. niger* strains 0307, 0474 and 0576 with WS or DS as the nitrogen source with minerals supplement added, fungal growth was better in the presence of WS compared to DS. This result may be attributed to WS shrimp shells retaining a rich nutrient source that promotes fungal growth. In the case of the DS shrimp shells, most of the minerals and trace nutrients were probably removed by the demineralization treatment. Therefore, WS shrimp shells could possibly supply the minerals and other trace nutrients in addition to being a nitrogen source, for fungal growth. This deduction was supported by the results of growing the three *A. niger* strains in a medium containing 1% (g/mL) D-glucose and 1% (g/mL) WS with or without mineral supplements (MS). In the medium containing only D-glucose and WS, fungal growth was found to be good and was only slightly improved in the presence of MS. Therefore, this experiment substantiates that it is possible to culture proteolytic fungi using WS only as the nitrogen, mineral and trace nutrient source.

A further conclusion from the results of the experiments was that the WS and DS shrimp shells were not able to participate as a carbon source as no growth was observed for the three *A. niger* strains in phosphate-buffered saline (pH 7.5) containing only WS or DS. This is in contrast to the report by Bustos in which commercial inoculants comprising various bacteria strains and one *A. oryzae* were able to grow in DS suspended in 0.2% phosphate buffer.[8] A possible explanation for the dissimilarity is that in the case of Bustos, the mixture of microorganisms, especially bacteria that are fast growing, facilitates more efficient hydrolysis of the proteins in the shells to support microbial growth compared to a single strain inoculation in the case of the three *A. niger* strains 0576, 0307 and 0474. Therefore, when these strains of fungi are used for single-strained inoculation, the minimal medium for fungal fermentation should consist of D-glucose (or a suitable carbon source) and WS or DS shrimp shells participating as nitrogen and mineral sources.

Another consequence of the fermentation of WS shrimp shells with the three *A. niger* strains was the lowering of the pH of the medium to between pH 3 and 6. A lower pH augments the proteolytic hydrolysis of proteins by the secreted extracellular protease as well as promotes the demineralization of the WS.[7] This demonstrates that fungal fermentation, may be a better alternative to commercial protease enzymes that are not only more costly and can only deproteinize but not demineralize shrimp shells, as the pH of the fermentation medium is above pH 8.

Finally, in preliminary experiments, it was observed that autoclaving shrimp shells caused deproteinization ($56.06 \pm 3.14\%$), releasing hydrolyzed proteins into the medium as corroborated by a high protein content in its supernatant ($62.16 \pm 4.32\%$). This relatively high degree of deproteinization of WS by autoclaving must be noted as it would give a false deproteinization efficiency by the proteolytic microorganisms used to ferment crustacean shells if not corrected prior to inoculation with various proteolytic microorganisms when their degree of deproteinization of crustacean shells were measured.

Chitin extraction.—Chitin is usually extracted from crustacean shells by digestion with a strong alkali and acid.[14] The use of strong alkali and acid can lead to deacetylation of chitin or chain degradation of the isolated chitin. In this study, chitin from non-fermented WS, fermented WS and fungal mycelia were directly extracted with 5% lithium chloride–*N*,*N*-dimethylacetamide (5% LiCl in DMAC), a non-degradative solvent for chitin.[15] Fig. 1 summarizes the chitin yield from WS shrimp shells after various concurrent fermentation experiments. Direct extraction gave reasonably good amounts of chitin isolates as a percentage of the shrimp-shell powder or mycelial biomass. In all instances, the chitin yield was higher compared to the non-fermented WS shrimp shells of 19%.

For *A. oryzae* commercial protease, the chitin yield increases linearly, from 26 to 30%,

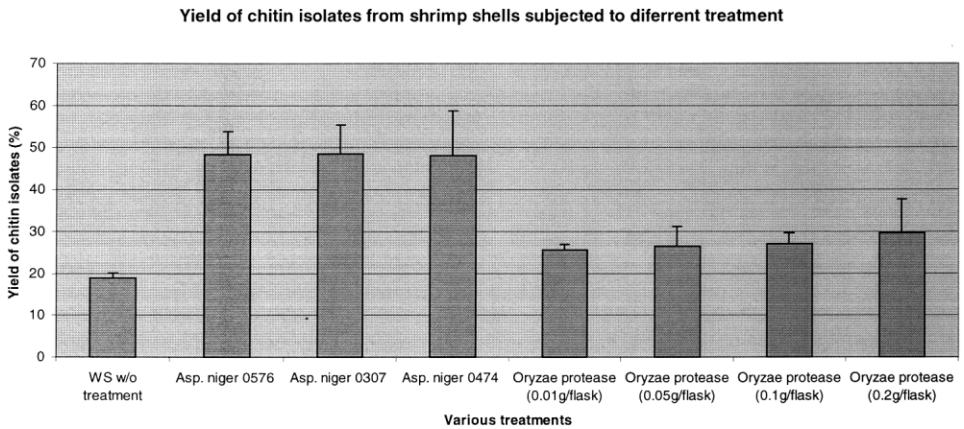

Fig. 1. Yield of chitin isolates from shrimp shells subjected to different treatment.

Table 2
Characteristics of chitin isolates from shrimp shells after treatment with fungal strains

Treatment	Degree of N-acetylation (%) [a]	Residual protein (%) [b]	Relative molecular weight ($\times 10^5$) [c]
WS w/o treatment	63 ± 1	3.9 ± 0.7	1.9 ± 1.0
WS in *A. oryzae* protease (0.01 g/150 mL medium)	56 ± 3	3.0 ± 0.1	2.0 ± 0.2
WS in *A. oryzae* protease (0.05 g/150 mL medium)	56 ± 3	2.6 ± 0.3	1.8 ± 0.1
WS in *A. oryzae* protease (0.1 g/150 mL medium)	55 ± 3	2.5 ± 0.1	1.9 ± 0.1
WS in *A. oryzae* protease (0.2 g/150 mL l medium)	54 ± 3	1.6 ± 0.1	1.7 ± 0.2
WS in *A. niger* 0576	72 ± 1	3.3 ± 0.3	1.5 ± 0.3
WS in *A. niger* 0307	60 ± 3	2.8 ± 0.5	1.6 ± 0.1
WS in *A. niger* 0474	62 ± 3	2.9 ± 0.3	2.06 [d]

[a] Values reported are the mean of three replicates $\pm$ standard deviation.
[b] Values reported are the mean of five replicates $\pm$ standard deviation.
[c] Values reported are the mean of two replicates $\pm$ standard deviation.
[d] One value determined.

as the protease concentration increased from 0.01 g/150 mL to 0.2 g/150 mL in the medium. However, statistical analysis shows that the increase in yield for the four concentrations were not significantly different ($P < 0.05$) compared to the non-fermented WS shrimp shell. In contrast, the chitin yield from WS shrimp shells exposed to the three *A. niger* strains 0576 (48.32%), 0307 (48.53%) and 0474 (48.15%) in the concurrent fermentation process were as high as 50%. A possible explanation is that the protease enzyme cannot perform as efficiently as the fungi strains. This is reasonable because the presence of fungi in the reactor provides a constant and gradual increase of protease throughout the fermentation period that is not possible by placing a fixed amount of enzyme in the reactor. Furthermore, the environment provided by the fungi as explained in the previous section is a complete process where demineralization as well as deproteinization activity occurs leading to the higher amount of chitin extractable.

Chitin characterization.—Good quality chitin isolates were obtained from WS shrimp shells (Table 2). The percentage of residual protein in the chitin isolates was below 5%. The protein content was even lower when WS was subjected to either fungal fermentation or deproteinization by the *A. oryzae* commercial protease prior to the extraction. The small quantity of residual protein is in good agreement with those reported using microbial enzymes and is attributed to regions of

inaccessibility in the shrimp shells by microbial protease.[16] Table 3 summarizes the characterization results for the fungal chitins. The protein content in the fungal chitin isolates was higher ranging between 10 and 15%. This suggests that a final deproteinization purification step is necessary for fungal chitin. In this work, a final purification of fungal chitin at room temperature (~ 25 °C) for 24 h with 1 M NaOH reduced the protein content to less than 1% with no extensive deacetylation of the chitin.

WS shrimp shell chitin from fungal fermentation was found to have a better degree of N-acetylation compared to untreated WS shrimp shell chitin. This again demonstrates that the concurrent process advocated here facilitates the recovery of good-quality shrimp chitin. The relative average molecular weight estimated from GPC showed that all the chitin isolates from WS shrimp shell as well as from fungal mycelia were in the 10^5 dalton range. This indicates that no extensive chitin chain degradation occurred in the processes used.

A comparison of the FTIR spectra of the various chitin isolates is presented in Fig. 2. The amide I (1650 cm^{-1}) and amide II (1560 cm^{-1}) bands were present in the WS powder spectra, but a peak at approximately 1423 cm^{-1} was very prominent compared to the others. However, when untreated WS was extracted with 5% LiCl/DMAC, the resultant spectrum did not show this feature and was identical to that of commercial chitin. All chitin isolates from WS after fungal fermentation and the fungal mycelia showed similar FTIR spectra with that of the commercial chitin. NMR analysis of the chitin isolates from WS after fungal fermentation and fungal mycelia gave similar peak patterns to that of the commercial chitin (Fig. 3). The C-1–C-6 carbons of the main *N*-acetylglucosamine are found between 50 and 100 ppm, the carbonyl peak (acetyl group) is around 170 ppm and the methyl peak of the acetyl group is around 20 ppm. The separation in the C-1–C-6 and methyl peaks was less distinct for fungal chitin although the essential peaks used for chitin identification are essentially present.

In biomedical applications, the absence of acute cytotoxicity is crucial. Chitin extracted from shrimp shells and fungal mycelia would represent the raw chitin material for further chemical derivatization and modification that can be developed into useful biomedical products. Therefore, the evaluation of the presence of any cytotoxicity in the extracted chitin is desirable. This can be readily obtained by the quantitative evaluation of the survival and proliferation of fibroblast cell lines from mouse (CCL 1) and human (CCL 186) and osteoblast cell line (CRL 1427) after 4 days of exposure to samples of the various chitin isolates using the tetrazolium colorimetric based (MTT) and Neutral Red uptake assays.

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells.[17] Neutral Red is a vital dye, and its uptake assay is based on the endocytosis of the dye, which will be internalized inside the lysosomes. The dye uptake indicates lysosome integrity and indirectly cell integrity. It is also well correlated to the number of viable cells.[18] The viability of cells is represented by the

Table 3
Characteristics of chitin isolates from fungal mycelia

Fungi	Yield (%) [a]	Degree of N-acetylation (%) [a]	Residual protein (%) [b]	Relative molecular weight ($\times 10^5$) [c]
A. niger 0576	22 ± 2	76 ± 6	13.8 ± 0.5	1.5 ± 0.1
A. niger 0307	27 ± 3	75 ± 4	15.1 ± 0.6	1.6 [d]
A. niger 0474	17 ± 3	82 ± 3	11.1 ± 0.4	1.3 ± 0.1

[a] Values reported are the mean of five replicates $\pm$ standard deviation.
[b] Values reported are the mean of three replicates $\pm$ standard deviation.
[c] Values reported are the mean of two replicates $\pm$ standard deviation.
[d] One value determined.

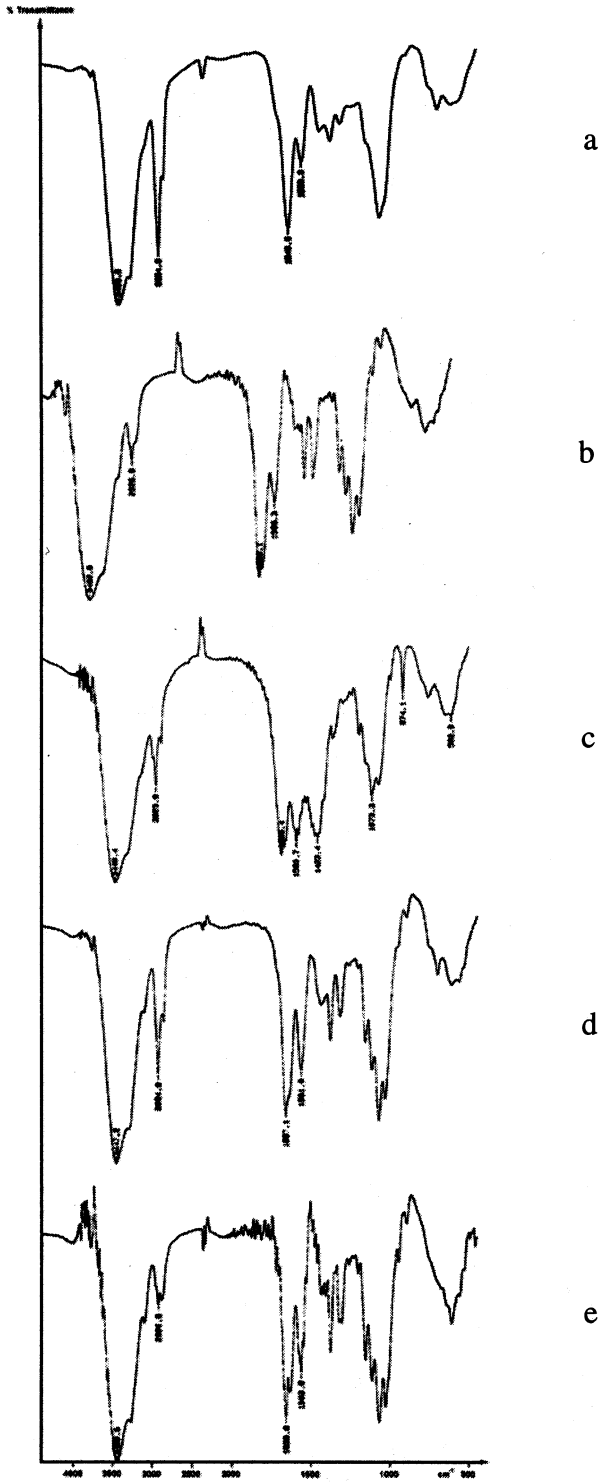

Fig. 2. FTIR spectra of chitin isolates from shrimp shells and fungal mycelia. (a) Chitin extracted from *A. niger* 0576. (b) Chitin extracted from shells treated with *A. niger* 0576. (c) Washed shrimp shells. (d) Chitin extracted directly from shrimp shells with 5% LiCl/DMAC. (e) Commercial chitin from Polyscience.

percentage of control. The percentage of control is defined as the percentage of the number of viable cells after exposure to the chitin samples relative to the control not exposed to any of the chitin samples.

The percentage of control for fibroblast cells (CCL 1 and CCL 186) and osteoblast cells (CRL 1427) exposed to the negative control were either near to or above 100% and either near to 0% or below 20% for the positive control. Table 4 shows the effect of chitin isolates from WS and fungal mycelia on the viability of the three cell lines over 4 days of exposure. Generally the effects of chitin extracted from WS and fungal mycelia were similar to that of the negative control (high-density polyethylene), near to 100%. This shows that no acute cytotoxicty and no pro-proliferation effects were observed on the mouse and human fibroblast cells and human osteoblast cells over the 4 days of exposure by all chitin isolates. This was similar to that reported by Mori and co-workers who studied the effects of chitin and its derivatives on the same mouse fibroblast cells.[19] Chung and co-workers however, observed a pro-proliferation effect on human F1000 fibroblasts by chitin-containing materials extracted from *Mucor mucedo*, *A. oryzae* and *Phycomyces blakesleeanus*.[11] The different responses observed may be due to the different sensitivity of cell lines to different sources of chitin samples.

The percentage of control quantitated by MTT assay gave lower values than the Neutral Red uptake assay. Again, this may be attributed to the different sensitivity of different assay methods. The three different cell lines gave similar effects after exposure to the same chitin isolates from WS and fungal mycelia. In this study, chitin isolates from shrimp shells and fungal mycelia showed neither distinct acute cytotoxicity nor distinct pro-proliferation of the mouse, human fibroblast and human osteoblast cells. The chitin samples did not affect the attachment of cells to surfaces and growth, since the cells proliferate even though samples were added at 0 h of incubation and did not cause cell death after prolonged (>24 h and <30 days) exposure.

In conclusion, concurrent deproteinization of shrimp shells by three proteolytic *Aspergillus niger* (strains 0576, 0307 and 0474) has

been demonstrated. The results also suggest that demineralization occurs under these conditions. Fungal fermentation is a cost-effective method compared to commercial protease enzymes that only deproteinize but not demineralize shrimp shells. FTIR and solid-state NMR studies support the identity of chitin of all the isolates from shrimp shells and fungal mycelia. The absence of acute cytotoxicitiy with mouse (CCL 1) and human (CCL 186) fibroblast cells and human (CRL1427) osteoblast cells was found for all chitin isolated from shrimp shells and fungal mycelium. Therefore, proteolytic fungal fermentation of shrimp shells is a simple, effective and inexpensive approach to concurrently recovered

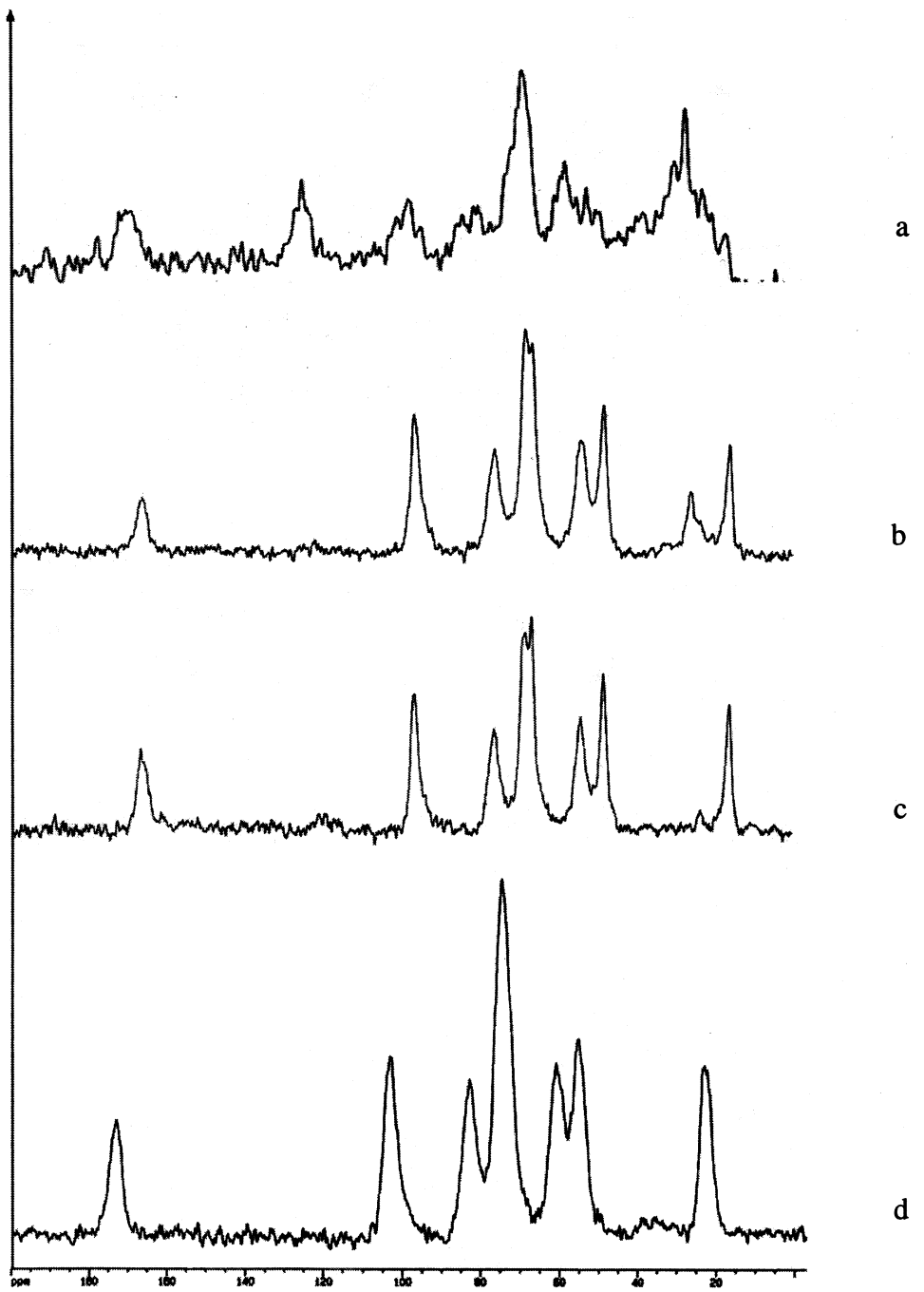

Fig. 3. NMR spectra of chitin isolates from shrimp shells and fungal mycelia. (a) Chitin extracted from *A. niger* 0576. (b) Chitin extracted from shrimp shells treated with commercial protease from *A. oryzae*. (c) Chitin extracted from shells treated with *A. niger* 0576. (d) Commercial chitin from Polyscience.

Table 4
Average of the percentage of absorbance over 4 days for MTT and Neutral Red assay

Sources of chitin samples	Percentage of absorbance (%) [a]					
	MTT assay			Neutral Red assay		
	CCL1	CCL186	CRL1427	CCL1	CCL186	CRL1427
A. niger 0576 treated WS	73.69	110.57	87.45	105.6	110.2	104.6
A. niger 0307 treated WS	96.60	86.00	83.21	109.4	112.1	139.0
A. niger 0474 treated WS	86.26	71.03	73.74	102.9	118.7	154.0
A. niger 0576 mycelia	96.60	84.85	95.16	101.1	103.2	107.6
A. niger 0307 mycelia	98.99	90.42	96.47	103.6	104.2	113.6
A. niger 0474 mycelia	101.25	101.42	97.43	97.7	101.7	118.2

[a] Values reported are the mean of eight replicates ± standard deviation.

relatively good quality of chitin from shrimp shell and fungal mycelia.

3. Experimental

Fungal culture.—A total of 24 deuteromycetes, i.e., *A. oryzae* (strains 0207, 0416, 0486, 0510, 0512), *A. flavus-oryzae* 0607, *A. carbonarius* 0431, *A. niger* (strains 0206, 0236, 0237, 0238, 0241, 0243, 0290, 0307, 0384, 0386, 0397, 0398, 0412, 0474, 0576, 0577, 0825) and 10 zygomycetes, i.e., *Mucor hiemalis* 0452, *M. hiemalis* + 0566, *Cunninghamella* sp. 0004, *C. echinulata* 0601, *Rhizopus* sp. 0477, *R. oligosporus* 0260, *R. arrhizus* 0261, *R. microsporus* 0264, *G. butleri* (strains 0201 and 0489), were obtained from the collection of the Department of Biological Sciences, National University of Singapore. *A. oryzae* 0510 and *A. oryzae* 0512 were maintained on 5% (w/v) malt extract agar (MEA, Oxoid) slant at 5 °C. The other *Aspergillus* strains were maintained on 4.54% (w/v) Czapek Dox Agar (CDA, Oxoid) slant at 5 °C. *M. hiemalis* 0452, *M. hiemalis* + 0566 and *C. echinulata* 0601 were maintained on 3.3% (w/v) oat meal agar (OMA) slant at 5 °C. The remaining cultures were maintained on 3.9% (w/v) potato dextrose agar (PDA, Oxoid) slant at 5 °C.

Protease activity screening.—The 34 fungi strains were screened for their protease activity using the following three assay methods.

Yeast extract agar (YEA) with 1.5% skim milk.—Yeast extract agar (YEA, Oxoid) plates with 1.5% (w/v) skim milk were prepared, inoculated with all 34 fungal strains and incubated at 25 °C for 4 days. The plates were observed daily for signs of clearing (representing protein degradation) of the agar around the fungal colony. When clearing was observed, the width of the clear zone was measured as protease activity. Five replicates were performed for all experiments.

Corn meal agar (CMA) with gelatin.—Corn meal agar (CMA, Oxoid) plates with 1.8% (w/v) gelatin were prepared, inoculated with all 34 fungal strains and incubated at 25 °C for 4 days. The culture was flooded with satd $(NH_4)_2SO_4$. The clear zone around the fungal colony after flooding represents protein degradation. The width of the clear zone was measured as protease activity. Five replicates were performed for all experiments.

Liquid assay using casein as substrate.—A 1 mL spore suspension in distilled water (5×10^4 spores/mL) of each of the 34 fungal strains was inoculated into 25 mL of a medium comprising 1.0% casein, 0.4% D-glucose, 0.4% yeast extract, 1.0 g/L $K_2HPO_4 \cdot 3\ H_2O$, 0.5 g/L $MgSO_4 \cdot 7\ H_2O$ and 0.1 mL/L Vogel trace element solution, adjusted to pH 7.4 with 1 M NaOH. After incubating for 5 days at 25 °C and 200 rpm, the mycelia were filtered. The clear culture filtrate (diluted if necessary), containing the crude protease, was analyzed for protease activity according to the method described by Walter.[20] The clear culture filtrate (0.2 mL) was added to 2.5 mL of buffered casein solution and incubated at 37 °C for 10 min. Trichloroacetic acid (5 mL of a 0.3 M

solution) and 0.2 mL of 0.05 M HCl were added to the mixture successively. After standing at 25 °C for 10 min, the solution was filtered, and the absorbance of the clear filtrate at 280 nm was read. A series of varying concentrations of tyrosine solutions (2.5 mL) were used to plot the standard curve. One unit of protease activity is defined as 1 μM of tyrosine produced per min at pH 7.5 and 37 °C.

Growth profile.—Dried shrimp shells were a gift from the Asian Institute of Technology, Bioprocess Technology Program, Thailand. Washed shrimp shells (WS) were prepared by washing the dried shrimp shell waste thoroughly with water, oven drying at 90 °C and grinding to a powder. Demineralized shrimp shells (DS) were prepared by continuous stirring of WS in 2 M HCl (1 g/100 mL) for 1 h at rt (25 °C), filtered, washed thoroughly with water, oven drying at 90 °C and grinding to a powder.

A. niger (strains 0576, 0307 and 0474) were grown up to 7 days at a final spore concentration of 1×10^4 spores/mL in various medium comprising of D-glucose (10.0 g/L) with either WS powders (0.5 g/50 mL), DS powders (0.5 g/50 mL) or $NaNO_3$ as the nitrogen source with or without minerals supplement (MS). The MS consists of 0.52 g/L $MgSO_4 \cdot 7\ H_2O$, 0.52 g/L KCl, 1.52 g/L KH_2PO_4, 0.001 g/L $FeSO_4 \cdot 7\ H_2O$ and 0.001 g/L $ZnSO_4 \cdot 7\ H_2O$. The pH of the media was adjusted to 6.5 with 1 M NaOH. After an initial incubation of 3 days at 30 °C and 200 rpm, the mycelia were harvested daily and oven dried until constant weight, before the final weight was recorded. For media containing shells, the used shells were separated from the mycelia at harvest. Five replicates were performed for all experiments.

Extraction of chitin from shrimp shells and fungal mycelia.—*A. niger* (strains 0576, 0307 and 0474) were grown for 4 days at a final spore concentration of 6.7×10^3 spores/mL in the medium consisting of WS (1 g/150 mL) and D-glucose (10 g/L), adjusted to pH 6.5 with 1 M NaOH. A 1-mL aliquot of four different concentrations (0.01, 0.05, 0.1 and 0.2 g/mL) of *A. oryzae* commercial protease enzyme type XXIII (Sigma) was also inoculated per flask containing 150 mL of the medium. After 4 days of incubation at 30 °C and 200 rpm, the WS were separated from the fungal mycelia. In this process, the white fungal mycelia tend to float while the brownish WS tends to sink. The fungal mycelia are carefully decanted leaving the WS containing some entrapped fungal mycelia in the container. Hot water is added to the container, and this releases some of the entrapped fungal mycelia. The released fungal mycelia are again decanted. After several repeated sedimentations and decantations, clean shells and mycelia were obtained. Non-fermented WS was also extracted and used as the control. These shells and mycelia were dried and placed in 5% LiCl/DMAC solvent at a ratio of 1 g/150 mL with constant stirring at rt for 48 h. The viscous suspension was centrifuged at 4000 rpm for 5 min, the supernatant containing dissolved chitin was collected, and distilled water (1:1) was added to the supernatant to precipitate the chitin over 24 h. The precipitate was recovered as chitin pellets by centrifugation. The chitin pellets were washed with distilled water to remove remaining DMAC, freeze-dried, weighed, and characterized.

Protein content determination.—Chitin samples were hydrolyzed with 2 M NaOH (0.5 g/50 mL) at 121 °C/1 psi for 15 min. The supernatant was retained by filtration and used for protein assay according to the modified Lowry method.[21] A 1-mL aliquot of alkaline copper sulfate reagent was added to 1 mL of the supernatant (diluted when necessary) and allowed to stand for 10 min at rt. A 3-mL aliquot of diluted Folin–Ciocalteu reagent was added next, and incubated for 30 min, and the absorbance was read at 750 nm. Various concentrations of bovine serum albumin (BSA, Sigma) were used for the standard curve. The percentage of proteins in chitin isolates was expressed as the percentage of the measured protein content in μg of albumin relative to the weight of chitin samples obtained from the precipitate from 5% LiCl/DMAC.

Gel-permeation chromatography (GPC).—The GPC columns (phenogel: 10 μm, 300 × 7.8 mm, s/no. 42635, 42636 and 79586) and guard column (phenogel: 10 μm, 50 × 7.8 mm,

s/no. 649286) were from Phenomenex. The pump (600E) and differential refractometer detector (410) were from Waters Associates. Dextran standards (broad molecular weight kit from Phenomenex) of 535,000, 74,300, 9900 molecular weights were used. A total of 0.2% of the standards were dissolved in 5% LiCl/DMAC with continuous stirring for 3 days, and 0.5% of the samples were dissolved in 5% LiCl/DMAC. The samples were filtered through a 0.45-μm nylon filter before 150 μL was injected into the pump. The flow rate was set at 0.8 mL/min, while the temperature of the columns was set at 60 °C and that of the detector at 30 °C. A calibration curve was plotted from elution time versus the absolute molecular weight of the standards. The relative average molecular weights of the chitin samples were extrapolated from the standard curve.

Fourier-transform infrared spectroscopy (FTIR).—All chitin samples were freeze-dried overnight, made into KBr discs, and the FTIR spectra obtained with a Perkin–Elmer 1600 FTIR spectrophotometer. The average number of scans taken per sample was 16 in the spectral region between 400 and 4000 cm^{-1}. The degree of N-acetylation of the samples was calculated based on Baxter method using the following equation:[22]

$$\% \text{ N-acetylation} = (A_{1655}/A_{3450}) \times 115$$

Solid-state nuclear magnetic resonance (SS-NMR).—The NMR spectra of chitin samples were obtained by the ^{13}C cross polarization magic angle spinning (CPMAS) spectroscopy method using a Bruker Varian 400 MHz spectrometer with a contact time of 2 ms and rotor frequency at 10 kHz. The proton cross polarization and decoupling frequency was adjusted to the pure water resonance at 4.65 ppm. The ^{13}C-transmitter frequency was adjusted to 100 ppm using adamantine as the reference at 38.5 ppm for the CH_2 peak, downfield. A total of 1500 scans were acquired for each run, and measurements were taken at room temperature.

Cell lines.—NCTC Clone 929 (ATCC CCL-1) mouse fibroblast, IMR-90 (ATCC CCL-186) human fibroblast and MG-63 (ATCC CRL1427) human osteoblast cell lines were obtained from American Type Culture Collection (ATCC) and maintained in Eagle's Minimum Essential Medium (EMEM) with 1.5 g/L $NaHCO_3$ and Earle's Balance Salt Solution (BSS) adjusted to contain 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% fetal bovine serum, at 37 °C in 5% CO_2 atmosphere. All medium supplements were from Gibco. A total of 1×10^4 cells in 100 μL culture medium were seeded into each well of a flat-bottomed 96-well microplate (Costar) for the following MTT and Neutral Red uptake assay. Latex rubber and high-density polyethylene were used as the positive and negative controls, respectively, as recommended control materials for cell culture methods for cytotoxicity according to ASTM F813-83 (Reapproved 1992).

MTT assay.—This assay was performed with slight modifications to the referenced procedure.[17] A finely powdered chitin sample (10 μL) suspended uniformly in the culture medium was added to each well and incubated for 24, 48, 72 and 96 h. After the specified exposure time, 20 μL of 3-[4,5-dimethylthiazolyl-2]-2,-diphenyl tetrazolium bromide (MTT) obtained from BDH (5 mg/mL in phosphate-buffered saline, PBS; pH 7.4) was added to each well and incubated for 3 h at 37 °C. After incubation, the medium was removed, and the cells were washed gently with PBS to remove any untransformed MTT and residues of chitin samples. Dimethyl sulfoxide (DMSO, GC grade from Fluka, 150 μL) was added per well to dissolve the intracellular formazan purple crystals. Absorbance at 540 nm was read using a microplate spectrophotometer (CERES UV 900C, Bio-Tek Instruments, INC.). Results are expressed as percentage of absorbance relative to a control without chitin samples.

Neutral Red uptake assay.—This assay was carried out with slight modifications to the referenced procedure.[18] A finely powdered chitin sample (10 μL) suspended uniformly at 3 mg/mL in the culture medium was added to each well and incubated for 24, 48, 72 and 96 h. After the specified exposure time, the medium was discarded and replaced with 0.1 mL per well of 0.4% Neutral Red (Gibco) diluted 1:80 in the culture medium. After incubation for 2 h at 37 °C, the cells were washed

gently with PBS to remove residues of chitin samples and 0.1 mL of extractant solution (50% EtOH in AcOH, 1%) was added. The extracted dye absorbance at 540 nm was read using a microplate spectrophotometer (CERES UV 900C, Bio-Tek Instruments, INC.). Results are expressed as percentage of absorbance relative to a control without chitin samples.

Statistical analysis.—The Tukey test was used to compare the means using SIGMASTAT 2.0® software. The Tukey test is the preferred all pairwise comparison test. It uses a table of critical values that is computed based on a mathematical model of the probability structure of the multiple comparisons.

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