Journal of Metals, Materials and Minerals, Vol.15 No.1 pp. 73-81, 2005

Chitin and Chitosan: Production and Application Research Asian Institute of Technology 1994 – 2004

Willem F. STEVENS

JF Kennedy Professor of Bioprocess Technology (BPT)
Asian Institute of Technology
POBox 4, Klong Luang, Pathumthani 12120 Thailand.

Abstract

As a tribute to the late Prof. Hirano and in acknowledgement of his strong support, an overview is presented of 10 years chitin and chitosan research program at the Asian Institute of Technology (AIT) in Bangkok. The research has been focused on the improvement of the extraction of chitin and chitosan from various sources and on quality assessment and applications of these marine biopolymers.

Chitosan of very high quality can be produced chemically by applying biowaste pretreatment and by using new insights in the mechanism of the chemical deacetylation. A biocatalytic method using chitin deacetylase from *Absidia coerulia* has been established for the complete deacetylation of chitin. A new method for large-scale production and purification of the chitin deacetylase has been developed. For quality assessment of chitin and chitosan a standardized system has been proposed.

In addition, a wide range of chitosan applications in agriculture, biomedicals, bioremediation, biotechnology and papermaking has been investigated. Decrystallized chitosan has been shown to be enhanced in binding capacity of textile dye in waste water. Chit-in-osan, a product of enzymatic surface-deacetylation, has potential in protein chromatography. Chitosan is very suitable to size paper and to improve its properties, to design matrices for controlled drug release both for gastric passage and transdermal uptake, to enhance bone repair and wound healing and to promote orchid embryo morphogenesis in tissue culture.

Introduction

The processing and fractionation of chitin and chitosan is an interesting combination of environmental engineering and product valorization in S. E. Asia. High quality shrimp is produced in large quantities under controlled conditions of aquaculture (about 500.000 MT); the bio-waste that remains after peeling is of very high quality as well. About 10 years ago an intensive research program has been started in Chitosan Bioprocess Technology at AIT to give the region more benefit from the valorization of this bio-waste. The Second Asia Pacific Chitin and Chitosan Symposium, organized at AIT upon instigation by Prof. Hirano, marked the start of this program (Stevens *et al.*, 1996a).

Production of Chitosan

Chemical production

The main focus of research at AIT has been the production of chitosan from shrimp, crab, squid, cuttlefish and fungi. The basic philosophy in chitin production is to remove all material that cannot resist treatment with alkali (removes in the case of shrimp and crab mainly protein) and diluted acid (removes minerals mainly calcium carbonate). After extensive washing of non-reacted chemicals, the residue is chitin. The conversion of chitin into chitosan is achieved by treatment with alkali 50%.

E-mail: wfstevens@gmail.com fax: 662 524 6111

This concentration is quite critical. The rate of deacetylation of natural chitin falls steeply in case the actual alkali concentration drops below 40% due to the dilution by the moisture in the chitin material.

A considerable part of the protein can be collected to produce food supplement protein powder by application of pretreatments that avoid the use of alkali. Pretreatment also allows for milder process conditions and a reduction in the overall amount of chemicals needed for the subsequent chemical extraction (Aye and Stevens, 2004). High quality chitin can be produced at 30°C, whereas chitosan deacetylation needs a temperature of 50-60°C. In industry, much higher temperatures are used commonly; however the higher process temperature causes the presence of insoluble materials and turbidity in the final chitosan in solution. This can be explained on the basis of the mechanism of the chemical deacetylation.

Mechanism of the chemical deacetylation

Knowledge on the mechanism of the chemical deacetylation has been obtained by a systematic study of the chemical deacetylation at various temperatures, at various alkali concentrations and at various durations. By comparing different experimental conditions that led to the same degree of deacetylation Ng *et al.* (2000), it was concluded that depending on alkali concentration and reaction temperature the deacetylation can proceed predominantly from sites in the chitin molecule that have been deacetylated already or by starting preferably at new sites for deacetylation. The distribution of the deacetylated sites along the partially deacetylated chitin molecule can be analyzed after nitrous acid treatment, breaking the glycosidic C-1 bond at deacetylated sites, followed by HPLC of the oligo-chitin molecules. In the first case there are predominantly mono-glucosamines and fewer rather long oligochitins, in the second case there are predominantly short oligo-chitins. Playing with pretreatment and with the deacetylation conditions makes it possible to produce very high qualities of completely soluble and transparent chitosan with any desired degree of deacetylation and, upon dissolution in diluted acetic acid, with a viscosity of 7000 cps, (Stevens, 2002).

Chitosan from fungi

Chitosan from fungi has been isolated by submerged and solid state fermentation (Nwe *et al.*, 2002; collaboration with Dr Khor, National University Singapore). The covalent binding to β-glucan created major problems in its purification. Treatment with β-glucanase was not very effective. But it was discovered Nwe and Stevens, (2002) that the β-glycosidic chitosan and β-glucan are connected by an α-(1-4) glycosidic bond which can be split by α-amylase (Figure 1). This has brought the isolation of chitosan a big step ahead because now β-glucan and chitosan can be separated and purified individually.

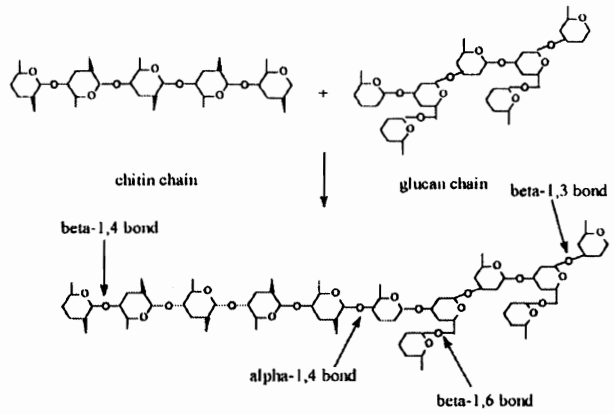

Figure 1. Proposed α-glucosidic binding between β-chitosan and β-glucan in fungal cell wall

Methods for Chitosan Analysis

Degree of deacetylation and assay of protein content

Since the beginning, AIT has worked on the issue of product quality assurance for chitin and chitosan realizing that without a non-ambivalent and broadly accepted quality assessment system, chitosan never could reach the status of a product of world trade. A generally applicable assessment system has been proposed (Stevens, 1996b; and Hein *et al.*, 2001). Many analytical methods have been critically evaluated. (Hein *et al.*, 2004). A very useful method for the determination of the degree of deacetylation of a chitin/chitosan sample is the acid hydrolysis-HPLC method (Ng *et al.*, 2005). The method can be applied irrespective of the solubility of the sample and compares very favorably with all generally known methods to assay. Another simple but intriguing problem is the estimation of the protein content in a chitosan sample. Both protein and chitin/chitosan contain Nitrogen but only a very limited number of methods are able to distinguish between nitrogen from protein and chitosan. The micro-biuret method has been shown to be a reliable method for residual protein determination from chitin/chitosan (Hein *et al.*, 2004).

Assay insolubles

A third problem is the assay of insolubles when a chitosan sample in diluted acetic acid has a high viscosity but the chitosan appears not to dissolve completely. Dilution followed by filtration is very impractical, reducing the viscosity by nitrous acid treatment breaks down also the insolubles. An attractive alternative is to treat the viscous solution with food-grade transglucosidase L-500 (EC 2.4.1.24, Genencor, USA) solution that has sufficient chitinolytic acivity but does not degrade the insolubles to a great extent. Therefore, the viscosity of chitosan solution goes down and the amount of the insolubles in the chitosan solution can be easily quantified after filtration (Hein *et al.*, 2003).

Biochemical Extraction

Lactobacillus assisted extraction

The harsh chemical conditions used for the extraction of chitin and chitosan is a direct invitation to try more subtle and environmental friendly biotechnological techniques, based on lactobacillus fermentation to convert the biowaste into chitin, followed by enzymatic deacetylation using chitin deacetylase (CDA) to produce chitosan.

Conditions have been worked out for a larger scale fermentative extraction of protein and minerals (Rao *et al.*, 2000; collaboration with Dr. Hall, University of Loughborough.). The system has many advantages: the fermentation soup, containing most of the shrimp protein, is composed of only food grade shrimp biowaste and food grade bacteria. The material is excellent to prepare high value protein powder with a net economical value that even may exceed that of the chitosan. The fermentation leads also to a large reduction in volume of the treated shrimp solids. However, 10-20% protein and minerals still remain in the waste after fermentation. These materials can be removed in the conventional chemical way but due to the volume reduction and the small amounts of protein and minerals left, this additional treatment requires only a small amount of the chemical agents.

Chitin Deacetylase, CDA

The enzymatic deacetylation of chitin has been investigated using the CDA isolated from the fungus *Absidia coerulia*. Data on CDA activity are usually obtained with partially deacetylated chitosan (degree of deacetylation 60-80%). Significant enzymatic deacetylation of natural chitin without prior treatment with strong alkali had so far not been accomplished. From a large number of experiments it was concluded that the catalytic activity of the enzyme was sufficient, but that the crystalline nature of the chitin substrate was the problem. Small particle size chitin was a better substrate. Dissolving chitin in dimetyl acetamide/ LiCl did not produce a better substrate. As soon as the aqueous enzyme solution was added, the chitosan precipitated in crystalline form. Better results were obtained using the solvent methanol/$CaCl_2$ (communication Dr. Tokura, Osaka). A system was designed to produce superfine chitin based on dissolution and controlled precipitation. Figure 2. This superfine chitin was treated in 20% formic acid to weaken the intra-crystalline forces. After pH adjustment to pH 4 this chitin could be deacetylated enzymatically over the full range from 15% to 85% degree of deacetylation. (Win and Stevens, 2001). These observations show the way in which natural chitin can be deacetylated avoiding the use of strong alkali.

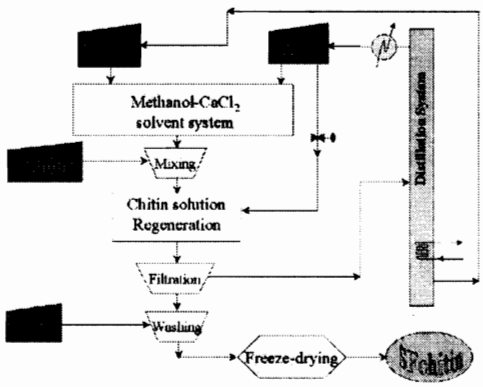

Figure 2. Production of superfine chitin by precipitation from methanol-calcium chloride

Other sources CDA

The next step was to find new fungal enzymes that can handle the crystalline chitin and deacetylate it into chitosan. Whereas the *Absidia* enzyme did not show any activity on 50-mesh chitin powder, the *Colletotrichum lindemuthianum* CDA was found to show a limited activity. This fungus has the disadvantage of slow growth. Since in a parallel search for fungi that can penetrate through the chitinous cuticle of insects (collaboration with BIOTEC, Thailand) no better candidates were found for enzyme acting on natural chitin, it was

decided to clone the enzyme from *Colletotrichum lindemuthianum.*

Cloning and purification CDA

In cloning experiments using the bacterium *E. coli* as intermediate host system it has been possible to transform the gene for CDA from the fungus to the fast growing and high protein producing yeast *Pichia pastoris* (Shrestha *et al.*, 2004; collaboration with University of Paris). Before transformation, the CDA gene was extended at the C terminal end with the DNA codons for a tag of 6 histidines. This histidine tag in the final CDA product allowed for an extremely efficient isolation procedure using a chelating nickel agarose column (Table 1). The enzyme could be isolated in milligrams amounts with a one-step purification of over 350 times. GCMS data show that the transcript was the exact product of the CDA gene minus the signaling sequences that control intracellular transport (Figure 3).

Table 1. Single step purification of *C. lindemuthanum* CDA from *P. pastoris supernatant*

Step	Total activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification factor	Purification yield
Supernatant	570	2890	0.197	1	100
Ni-agarose	516	7.2	71.7	364	90

Chit-in-osan

Enzymatic treatment can be explored in various ways. It can be applied to treat chitin with the intention to generate deacetylated groups predominantly at the outside of the chitin particle. The product was called chit-in-osan (Stevens *et al.*, 2004; and Aye *et al.*, 2005). As the name implies the product has a dual property of chitin and chitosan. It has the reactivity of chitosan with the acid insoluble property of chitin. The material has been tested for chromatography of the protein ovalbumine at a pH value were normal chitosan would quickly form gel. Chit-in-osan remained sturdy and did not show a loss of flow rate. Therefore, the new product, chit-in-osan, has potential in biotechnological separation processes.

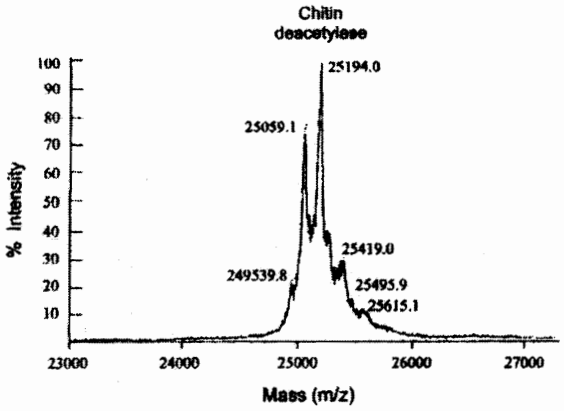

Figure 3. Maldi-Tof GC-MS of purified CDA. The value for the molecular weight found exactly corresponds to the value calculated from the DNA sequence minus the signal sequences.

Application Research

Dye absorption by decrystallized chitosan

Crystallinity is a limitation when chitosan is used as an absorbent. A simple way to decrystallize chitosan was found Trung *et al.* (2003), using a principle of dissolving and precipitation earlier published by Prof. Hirano. The decrystallized chitosan has been found to be superior in dye removal from the wastewater from a local textile-dyeing factory. Activated carbon, chitosan and the decrystallized chitosan were compared in their efficiency to bind textile dye. The absorption to activated carbon and chitosan is slow and incomplete compared to the decrystallized material. A system was developed to reuse the decrystallized chitosan. The absorbed dye is first desorbed in acid, later the very concentrated pigment in the acid is effectively bound to a small amount of activated carbon that can be burned. (Figure 4). After the acid desorption, the decrystallized chitosan can be reused for at least 10 times without significant loss of dye binding capacity. The binding to chitosan is actually a concentration step needed to make the process economically feasible.

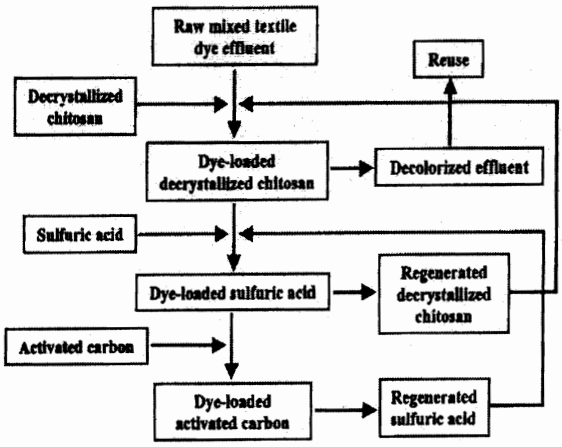

Figure 4. Scheme for large scale use of decrystallized chitosan for waste water dye absorption. The very diluted dye in the waste water is concentrated on chitosan, extracted from chitosan by H_2SO_4, absorbed from H_2SO_4 by a very small amount of activated coal that can be burned. Chitosan and H_2SO_4 can be reused many times.

Improved quality of chitosan treated paper

Chitosan is very effective to improve paper quality, both in wet end addition and in sizing operations. Applied at the wet end, paper strength is enhanced and wetability is decreased (Lertsutthiwong *et al.*, 2002). In sizing operations a combination of chitosan with 10% oxidized starch much more effective as oxidized starch only. By binding to cellulose fibers, chitosan reinforces the surface structure of the paper and reduces the release of fibers into the process water, (Table 2) (Lersutthiwong *et al.*, 2004).

Chitosan in agriculture and horticulture

Chitosan has found major applications in agriculture and horticulture. Many data have been collected for the effect of chitosan on rice, vegetables and horticulture especially on orchid cultivation (Chandrkrachang, this issue). The effect of chitosan has been proven statistically in a study on orchid protocorms and orchid embryonic plants in tissue culture, Figure 5 (Nge *et al.*, 2004). The best results were obtained with chitosan from fungi with a molecular weight of about 10 kD.

Table 2. Effect of chitosan on paper sizing

Chitosan 2% at least equivalent with sizing by 10% oxidized starch or polyvinyl alcohol
Lower water absorption, important in offset printing
Enhanced fiber binding, reduction COD load mill effluent
Sizing effect of 10% oxydized starch is enhanced in formulation with chitosan 0.01%

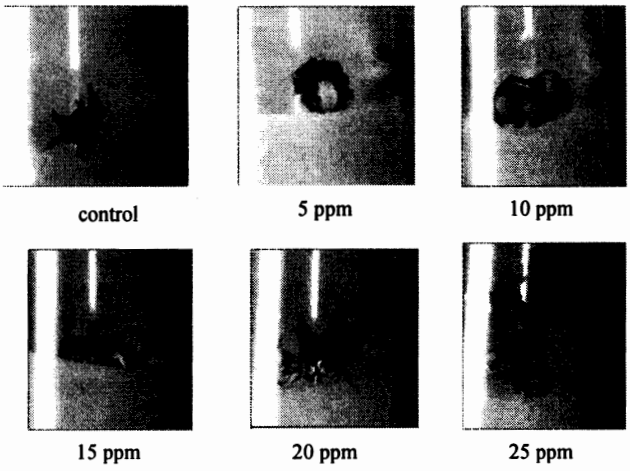

Figure 5. Effect of chitosan on the development of orchid embyo plant in tissue culture.

Biomedical Applications

Beads for controlled drug release

Chitosan as a biocompatible and biodegradable biopolymer is a very attractive candidate for the development of matrices for controlled drug release. Drug containing chitosan beads can be prepared by dropping a viscous drug-chitosan mixture in a solution of alginate. The chitosan droplet does not mix immediately with the alginate solution but becomes coated as a bead by the formation of a coat of chitosan- alginate complex. The majority of the drug is still inside. The beads are quite strong and release their drug depending on the properties of the chitosan and release conditions. Chitosan-alginate beads have been proven to resist the pH and pepsin concentration in the human stomach. Transferred to the condition of the intestine, the beads lose their drug content mainly through a total disintegration of the bead Anal *et al.* (2003), collaboration Kansai University, Japan). The system has been set up with BSA as a model release compound. Recent experiments show that it can be used equally for the small molecular drug ampicillin

in a chitosan bead reinforced by tripolyphosphate. In both systems, the release kinetics can be controlled by using chitosan with different molecular properties (Anal *et al.*, 2004). Very small drug containing chitosan beads can be generated by dispersion in organic fluids (collaboration University Santiago de la Compostella Spain). Using alginate-chitosan beads reinforced with a mixture of barium and calcium ions, beads can be obtained that release model proteins linearly with time during 24 hours (Bhopatkar *et al.*, 2005).

Chitosan for bone repair

Early experiments of Dr. Muzzarrelli (University of Ancona) show that chitosan has an accelerating effect on the regeneration of bone tissue. This has been confirmed in experiments in dogs suffering from a complicated bone fracture. Chitosan was applied as 5-methyl pyrrolidone chitosan powder during the surgery, deposited at the site of the bone prosthesis. Figure 6 (Khanal *et al.*, 2000; collaboration Dept. Veterinary Surgery, Kasetsart University, Bangkok). Bone recovery was assessed by X-ray about 6 weeks after surgery.

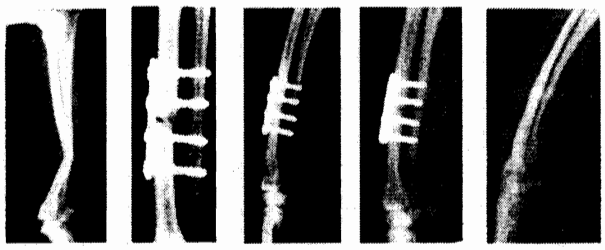

Figure 6. 5-Methyl pyrrolidone chitosan assisted bone repair in dogs. Chitosan was applied as the site of the fracture, indicated by the arrow.

Chitosan patch for controlled transdermal uptake

An investigation on the control of drug release through a chitosan membrane for use in a transdermal patch system was successfully completed. As a model drug, the anesthetic lidocaine HCl was chosen. The rate of membrane permeation by the drug was dependent on the degree of deacetylation and on the membrane thickness. A transdermal patch was developed, which contained a release rate limiting chitosan membrane attached to a chitosan gel that served as a reservoir that could be loaded with the drug of choice.

In experiments with human volunteers a study was carried out, using the anesthetic effect of lidocaine as a parameter. Patches consisting of a chitosan reservoir and a chitosan rate limiting membrane with known release properties were used. The sensations cold, warm and 2 types of tactile sensation were applied on the skin of the volunteers. This was done as reproducibly as possible. The volunteers could not see or hear what sensation was applied. Their vocal responses were recorded, quantified and expressed on a scale of 0 – 10. It was demonstrated that the time course of the release of Lidocaine coincided with the generation of the anesthetic effect Figure 7 (Thein Han and Stevens, 2004; collaboration Pharmacology, Mahidol University, Bangkok). The experiment shows that the release in vitro gives a good prediction of the transdermal release by the patch and that the degree of anesthesia can be controlled by the release properties of the chitosan membrane in the patch.

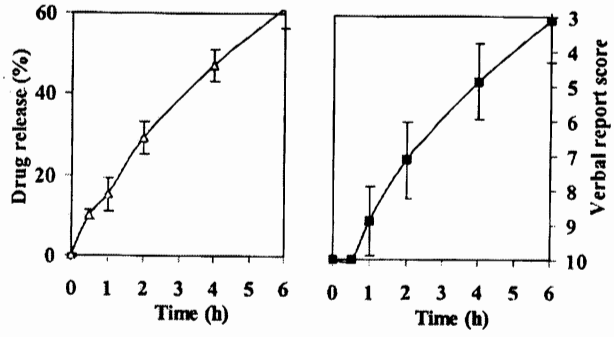

Figure 7. Correlation between the in vitro release of Lidocaine from a patch with a rate limiting chitosan membrane on the skin of human volunteers and the onset of its analgesic effect.

Human study in chitosan wound healing

The most classical application of chitosan membrane in the biomedical area is in wound healing, very popular in the Japanese veterinary sector and applied with excellent results by many medical institutes on human burn wounds. Since wounds in humans are highly variable in their anatomic pathology, the wounds themselves are not amenable for a study design including control groups. In a study on the wound healing, the effect of 2 bandages, the standard bactigras and a chitosan membrane were compared by investigating the healing of the donor site in human homo-transplantation treatment. The study also included the histopathology of the wound healing. At the donor site, usually an arm or leg, a piece of skin

Hein, S., Ng, C.H., Chandrkrachang, S. and Stevens, W.F. 2001. A Systematic Approach to Quality Assessment System of Chitosan. In: *Chitin and Chitosan, Chitin and Chitosan in Life Science. Uragami, T., Kurita, K. and Fukamizo, T.(eds.)* Tokyo, Kodansha Scientific Ltd: 332-335.

Hein, S., Ng, C.H. and Stevens, W.F. 2003. Quantification and Characterization of Insoluble Chitinous Materials in Viscous Chitosan Solutions. *Biotechnol Lett.* **25**: 863-868.

Hein, S., Ng, C.H. and Stevens, W.F. 2004. Development of analytical protocols for quality assessment of chitin and chitosan: Protein content, crystallinity and assay of insolubles. In: *Proc. 9th Int. Conf. on Chitin and Chitosan* (eds: Boucher, I., Jamieson, K. and Retnakaran, A.): 13-18.

Khanal, D.R., Choontanom, P., Okamoto, Y., Minami, S., Rakshit, S., Chandrakrachang, S. and Stevens, W.F. 2000. Management of Fracture with Chitosan in Dogs. *Indian Vet. J.* **77**: 1085-1089.

Lertsutthiwong, P., Chandrkrachang, S., Mousa, M.N. and Stevens W.F. 2002. Chitosan as a Dry Strength Agent for Paper. *Appita J.* **55(3)**: 208-212.

Lertsutthiwong, P., Mousa, M.N., Chandrkrachang, S. and Stevens, W.F. 2004. Chitosan as a surface sizing agent for offset printing paper. *Appita J.* **57(4)**: 274-280.

Ng, C.H., Chandrkrachang, S. and Stevens, W.F. 2000. Effect of the Rate of Deacetylation Conditions on the Physico-Chemical Properties of Cuttlefish Chitin. *Adv. Chiti Sci.* **4**: 50-54.

Ng, C.H., Hein, S., Chandrkrachang, S. and Stevens, W.F. 2005. Validated acid hydrolysis-HPLC method for the acetyl content measurement in chitin and chitosan. *J. Biomed. Mater. Res.*: accepted for publication.

Nge, K.L., Nwe, N., Chandrkrachang, S. and Stevens, W.F. 2004. Chitosan as a growth stimulator in orchid tissue culture. *Plant Sci.*: submitted for publication.

Nwe, N., Chandrkrachang, S., Stevens, W.F., Maw, T., Tan, T.K., Khor, E. and Wong, S.M. 2002. Production of Fungal Chitosan by Solid State and Submerged Fermentation. *Carbohydr. Polym.* **49(2)**: 235-237.

Nwe, N., Stevens, W.F. 2002. Chitosan Isolation from the Chitosan-Glucan Complex of Fungal Cell Wall using Amylolytic Enzymes. *Biotechnol. Lett.* **24(18)**: 1461-1464.

Rao, M.S., Muñoz, J. and Stevens, W.F. 2000. Critical Factors in Chitin Production by Fermentation of Shrimp Biowaste. *Appl. Microbiol. Biotechnol.* **54**: 808-813.

Shrestha, B., Blondeau, K., Stevens, W.F. Le Hegarat, F. 2004. Expression of Chitin Deacetylase from *Colletotrichum lindemutheanum* in *Pichia pastoris*: Purification and Characterization. *Protein Expr. Purif.* **38(2)**: 196-204.

Stevens, W.F., Rao, M.S. and Chandrkrachang, S. 1996a. *The Proceedings of 2nd Asia Pacific Symposium on Chitin and Chitosan: Environmental Friendly and Versatile Biomaterials,* Asian Institute of Technology, Bangkok, Thailand.

Stevens, W.F. 1996b. Towards a System for Product and Manufacturing Quality for Chitin and Chitosan. *In: Proceedings 2nd Asian Pacific Symposium on Chitin and Chitosan,* Bangkok: 295-301.

Stevens, W.F. 2002. Production and Storage of High Quality Chitosan from Shrimp, Crab and Fungus. In: *Advances in Chitin Science Vol 5 Proceeding 5th Asia Pacific Chitin and Chitosan Symposium, Suchiva, K., Chandrkachang, S., Methacanon, P. and Peter, M.G. (eds)*, Bangkok 6-11.

Stevens, W.F., Toan, N.V., Trung, T.S. and Aye, K.N. 2004. Advances in Isolation of Highly Functional Chitin and Chitosan. *Proceedings 6th Asia Pacific Chitin and Chitosan Symposium*, (eds.) Khor E., Hutmacher, D. W. and Lim, L. Y. National University of Singapore.

Thein-Han, W.W. and Stevens, W.F. 2004. Transdermal Delivery by a Chitosan Membrane. *Drug Dev. Ind. Pharm.* **30(4)**: 397-404.

Trung, T.S., Ng, C.H. and Stevens, W.F. 2003. Characterization of Decrystallized Chitosan and its Application in Biosorption of Textile Dyes *Biotechnol. Lett.* **25**: 1185-1190.

Win, N.N. and Stevens, W.F. 2001. Shrimp Chitin as Substrate for Fungal Chitin Deacetylase. *Appl. Microbiol. Biotechnol.* **57**: 334-341.