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Effective Production of N-Acetyl-β-D-glucosamine by *Serratia marcescens* Using Chitinaceous Waste

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The strain of *Serratia marcescens* QM B1466 produces selectively large amount of chitinolytic enzymes (about 1mg/L medium). Enzymatic hydrolysis of chitin to N-acetyl-β-D-glucosamine (NAG) was performed with a system consisting of two hydrolases(chitinase and chitobiase) produced by optimization of a microbial host consuming chitin particles. For the development of Large-scale biological process for the production of NAG from chitinaceous waste, the selection and optimization of a microbial host, particle size of chitin and pretreatment of chitin source were investigated. Also, the effect of crab/shrimp chitin sources and initial induction time using chitin as a sole carbon source on chitinase/chitobiase production and NAG production were examined. Crab-shell chitin(1.5%) treated by dilute acid and, ball-milled with a nominal diameter less than 250m gave the highest chitinase activity over a 7 days culture. Crude chitinase/chitobiase solution obtained in a 10 L fed-batch fermentation showed a maximum activities of 23.6 U/mL and 5.1 U/mL, respectively with a feeding time of 3 hrs, near pH 8.5 at 30℃.

Key words : *Serratia marcescens* QM B1466, chitinase/chitobiase, N-acetyl-β-D-glucosamine chitinolytic hydrolysis, feeding strategy, enzyme induction, chitin pretreatment

INTRODUCTION

This paper focuses on the production of N-acetyl-β-D-glucosamine (NAG) by enzymatic hydrolysis of chitin using chitinase (chitinglycanohydrolase, EC 3.2.1.14) and chitobiase (acetylaminodeoxyglucohydrolase, EC 3.2.1.29) secreted from the bacterial cell line *Serratia marcescens* QM B1466.

Development of a NAG production technology follows from the abundance and low cost of chitin and new evidence for the efficacy of NAG in the treatment of ulcerative colitis and other gastrointestinal inflammation disorders [1, 2]. Chitin is a β-1,4-linked unbranched polymer composed primarily of NAG. It is the second most abundant polysaccharide polymer (after cellulose) occurring in nature [3]. Natural sources of chitin include the exoskeletons of arthropods and the cell walls of many fungi [4]. The primary commercial source of chitin is crab and shrimp shells ; global estimates of the total chitin content in shellfish waste are in the range of 1.2×10^5 metric tons per annum [5]. However, much of this waste from the North American seafood packing industry is currently dumped inland or hauled out to sea(In contrast, Japan is annually producing and processing about 10^6 kg of chitin for industrial use [6].). Here, we propose an economically viable alternative to current environmentally-unattractive chitinaceous-waste disposal procedures

Because of its inherently mild operating conditions, enzymatic hydrolysis of chitin is an attractive method for producing NAG from chitinaceous waste. Microorganism use a combination of lytic enzymes to convert chitin to NAG. The dimer, chitobiose, can be obtained by the action of the enzyme chitinase on chitin in the presence of a factor (CH_1); chitobiose is then degraded to NAG by the enzyme chitobiase [7]. The specificity of these enzymes insures that NAG is the ultimate hydrolysis product, making the biodegradation of chitin a cost effective NAG-production process compared with chemical synthesis or acid hydrolysis. Chitinases are currently being studied as biocontrol agents against plant pathogenic fungi and as biopesticides which attack and degrade the exoskeletons of insects [8]. Natural sources of chitinases include snails, crustacea, insects, vertebrates, and bean seeds. However, the most convenient sources of chitinases, from a processing point of view, are microorganisms. The process utilizes chitinaceous waste material from the seafood processing industry for the production of NAG. Minimal-media bacterial cell fermentations are used to produce and secret the chitinolytic enzymes necessary for chitin hydrolysis.

Recent studies have identified bacteria, including *Serratia marcescens* [9] and *Streptomyces lividans* [10], and some chitinase producing fungi, including

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Trichoderma harzianum [11] and *Myrothecium verrucaria* [12], which express chitinase and chitobiase at high levels. All of these organisms secrete chitinase and chitobiase to the extracellular production and thereby provide an easy route for continuous recovery of the lytic enzyme ensemble. However, enzyme production levels and the efficacy of the enzymes in degrading chitin vary among chitinase-producing bacteria. Thus, care must be taken in choosing an appropriate microorganism for our NAG-production process. Although data are limited, *S. marcescens*, specially strain QM B1466, appears to produce relatively high levels of chitinolytic enzymes (*ca.* 1 mg/L) in the presence of chitin. Moreover, the chitinase produced by *S. marcescens* are active in hydrolyzing both amorphous and crystalline chitin; the latter activity is particularly attractive since it may decrease the chitin-pretreatment requirements of this process. In contrast, purified chitinase from *S. lividans* and *T. harzianum* hydrolyze powdered chitin slowly and therefore require substantial chitin pretreatment.

Limited information is available on enzymology of the chitinase system of *S. marcescens* [9]. Thus, one objective of this studies is to determine the effects of substrate composition, enzyme inhibition, temperature, and pH on overall enzymatic activities of the chitinase and chitobiase from *S. marcescens*. From this data, fermentation conditions and media compositions will be optimized for high density cultivation of the chitinase-producing bacteria. Rates of chitin degradation will be determined and chitin-digestor conditions will be optimized by measurements of enzymatic activities and enzyme stabilities as a function of reactor configuration and enzyme and substrate concentrations for maximum NAG production.

MATERIALS AND METHODS

Process Scheme

NAG production scheme is shown below in Fig. 1. Powdered chitin (particle size of 180~210 μm) is fed as a carbon source to a fermenter containing an inoculum of the selected chitinase-producing organ ism in lag-phase growth. The introduction of chitin to the cell suspension induces production of chitinase and chitobiase, which are secreted by the growing cells to the extracellular fluid [13]. The chitin-degrading enzymes are continuously recovered from the fermented by drawing a fraction of the fermentation broth containing cells, chitin particles, chitinase, chitobiase and oligomers of NAG, and then passing it through a membrane separator to yield a solution of chitinase and chitobiase in the filtrate [14-16]. The residual cells and chitin is recycled to the fermenter for further cell growth, chitin processing, and chitinase production.

The filtrate solution is mixed with a concentrated aqueous chitin solution and fed to a solid-liquid separator fixed at a temperature low enough such that the free enzymes will bind to chitin but not hydrolyze it to any appreciable extent. The crystalline chitin/enzyme complex is settled, separated from the residual nutrient solution, washed, and then fed to the fermenter where the chitin is enzymatically

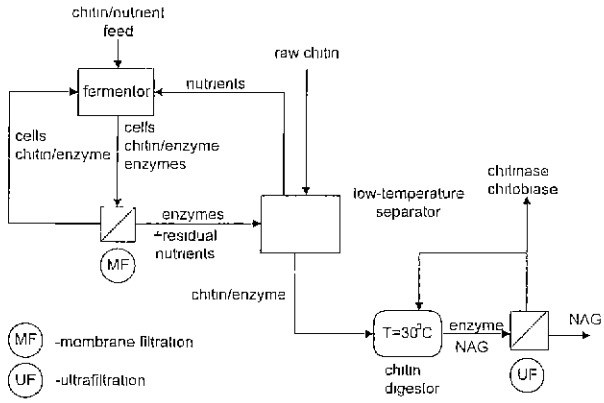

Fig. 1. N-acetyl-β-D-glucosamine production scheme.

hydrolyzed by chitinase and chitobiase to produce NAG [17, 18]. Final separation of NAG from chitinase, chitobiase, and remaining chitin oligomers is achieved by membrane ultrafiltration. To facilitate further production of NAG, a portion of the crude enzyme extract is recycled to the chitin digestion unit.

Serratia marcescens QM B1466 Cultures

Stock cultures of *S. marcescens* QM B1466 were maintained on nutrient agar (DIFCO) at 4℃ and transferred every 2 weeks. Seed agar cultures were prepared by streaking from glycerol stock and incubating at 30℃ for 24 hours.

The strain was grown overnight at 30℃ and pH 7.0 in 25 mL YEPD medium (DIFCO), consisting of 1 %(w/v) yeast extract, 2 %(w/v) peptone, and 2 % (w/v) glucose. This culture was grown at 30℃ and pH 7.0 for 3 hours, at which time the culture is in full exponential growth.

Batch culture experiments were performed in 125 mL shake flasks containing 15 g/L practical grade crab-shell chitin (Sigma Chemicals), 0.5 g/L yeast extract (Merck Chemicals), 1.0 g/L $(NH_4)_2SO_4$, 0.3 g/L $MgSO_4$, and 1.36 g/L KH_2PO_4. The pH of the medium was adjusted to between 8.5 and 9.0 with 1 M NaOH before steam sterilization and then readjusted to between pH 7.5 and 8.5 before addition of the inoculum.

Fed-batch fermentation was carried out in 10 L jar type fermenter(working volume: 7 L) at 30℃. The composition of culture medium is same that of batch fermentation and pH was maintained 8.5 with 5M NaOH. The dissolved oxygen(DO) was maintained 20~30% by air flow rate and agitation speed, 7.5 L/min and 200 rpm, respectively. Foam was suppressed by the addition of sterilized 10% SIGMA antifoam C as an antifoamer.

Chitin Sources and Pretreatment

Chitin and chitinaceous-waste materials are available from a variety of sources [19]. For initial studies, production of NAG from semi-crystalline shrimp-shell or crab-shell chitin was attempted. One of the advantages of the organism *S. marcescens* is that the chitinases it produces are capable of hydrolyzing both amorphous and crystalline chitin. This substantially diminishes chitin pretreatment requirements

and suggests that an economically viable process could eventually be developed from a shellfish-waste feed.

Substrate Preparation

Crab-shell and shrimp-shell based substrates containing chitin were prepared in a number of way with the aim of developing an inexpensive pretreatment procedure which yields a suitable chitin-containing substrate for *S. marcescens* growth, chitinase production, and NAG production. Raw shrimp shells, crab shells, and lobster shells were all explored as potential substrates, as were hammer-milled and ball-milled preparations of crab and shrimp shells.

Various forms of colloidal chitin were also prepared according to the method of Reynolds [20]. Crab or shrimp shells were ball-milled for 3 hrs and then soaked in a 2 %(w/v) $KMnO_4$ solution at room temperature for 20 hours. The remaining powder was then washed with a 1 %(w/v) oxalic acid solution to reduce the residual $KMnO_4$ and MnO_2 contents and to remove the oxidized proteins.

Colloidal chitin (used primarily as a reference for enzymatic assays) was prepared by washing the foregoing chitin with acetone to form a chitin paste, and then slowly adding the paste to 7 to 9 volumes of concentrated HCl cooled in an ice bath to *ca.* 4℃ to arrest hydrolysis. The syrupy liquid was filtered in a sintered glass plate with 10~15 μm of pore size. The chitin-containing filtrate was dropped into a vigorously stirred aqueous 50% ethanol solution to precipitate the chitin in a highly colloidal state. The colloidal residue was centrifuged at 8000 rpm for 20 minutes and resuspended in water three to five times, then sedimented by gravity and washed several times with 100 mM potassium phosphate buffer containing 1 mM $CaCl_2$ (pH 6.0) to remove excess acid and alcohol. Finally, the colloidal chitin solution was dialyzed against 100 mM potassium phosphate buffer until pH of 5 to 6 was maintained.

Methods of Assay

Chitinolytic enzymes secreted by *S. marcescens* include chitinase, which degrades polymers of NAG to short chains, and chitobiase, which hydrolyzes the dimer chitobiose to NAG. *S. marcescens* also secretes a number of other proteins which do not exhibit chitinolytic activity. Thus, determination of specific enzyme activities (units of activity/mg protein) requires measurement of total protein concentrations as well as chitinase and chitobiase activities.

Total Protein Assay

Total protein concentrations were determined by the Bio-Rad protein assay which follows the colorimetric procedure of Bradford [21]. For our studies, the assay was modified to accommodate the multi-sample format of an ELISA 96 well plate reader. All samples and standards were assayed in triplicate at an absorbance wavelength of 595 nm using a Molecular Devices Vmax Kinetic Microplate Reader and Corning flat-bottom disposable ELISA plates.

Chitinase Activity Assay

Chitinase activity was measured by the colorimetric method of Ressig *et al.* [22] with some modification to allow for multi-sample analysis using an ELISA 96 well plate reader. The reaction mixture contained 0.8 mL of a 1.25% (w/v) suspension of colloidal chitin, 0.1 mL of 100 mM potassium phosphate buffer at pH 6.0, and 1 mM calcium chloride. The reaction mixture was mixed with 0.1 mL of enzyme (supernatant or cell lysate) solution diluted 5 to 200 fold in the same phosphate buffer and incubated at 37℃ for one hour, at which time the reaction was stopped by boiling it for 5 minutes. A chitin blank and the samples were then centrifuged at 5000 rpm for 5min and 275 μL of each supernatant was collected and assayed for NAG concentration.

NAG concentration were determined by mixing 55 μL mL of 0.8M potassium tetraborate (pH 9.9) with the 275 μL supernatant sample and boiling this mixture for 3 minutes. The mixture is then cooled in an ice bath. A 0.1 mL of 112 mM p-dimethylaminobenzaldehyde (Ehrlich's reagent) in analytical grade glacial acetic acid, which contains 12.5% (w/v) 10N HCl, is then added and, immediately after mixing, 100 mL of the mixture is deposited in an ELISA plate maintained at 37℃. NAG standard and blank solutions are also added to the plate and, after precisely 20 minutes of reaction time, the plates are cooled to 4℃ for 3 minutes and the absorbance of each well is measured at 545 nm in an ELISA analyzer. One unit of activity was defined as the amount of enzyme able to liberate 1 mg of NAG per hour.

Chitobiase Activity Assay

Chitobiase activity [23] was determined by meas uring the amount of p-nitrophenol released when an aliquot of the enzyme solution is incubated with an aqueous solution of p-nitrophenyl-N-acetyl-β-D-glucosamine(pNP-NAG). A 50 μL of the 5 mM pNP-NAG solution containing 20 μL of the enzyme solution appropriately diluted with Tris (hydroxymethylaminomethane)-malate buffer at pH 7.0. After 10 minutes incubation at 37℃, the reaction was stopped by adding 100 μL of 0.25 M Na_2CO_3. The liberated p-nitrophenol was measured at 405 nm in an ELISA plate reader. One unit of chitobiase activity is equal to the amount of enzyme necessary to liberate 1 μmol of p-nitrophenol per minute.

RESULTS AND DISCUSSION

Selection of Bacterial Strain

A variety of research groups have performed shake flask culture experiments at 30℃ to screen the microorganism for maximum chitinase production and secretion. In most cases, the culture medium consisted of 15 g/L chitin, 0.5 g/L yeast extract, 1.0 g/L $(NH_4)_2SO_4$, 0.3g/L $MgSO_4$, and 1.36 g/L KH_2PO_4. All substrate and salt solutions were sterilized separately and mixed together just before inoculation. After 7 to 8 days culture, chitinolytic activity was measured to determine the best host for NAG production.

Selection of the microorganisms was primarily based on the studies by Reynolds [20], Berger and Reynolds [24], and Monreal and Reese [9]. Although certainly not exhaustive, *S. marcescens* turned out to be one of the most active microorganisms to degrade raw ball-milled chitin. Growth rates for *Serratia marcescens* QM B1466 raised on chitin were at least 30% higher than those for *Enterobacter liquefaciens* 3354-ICPB, the microbe showing the next highest chitinolytic activity. Thus, *Serratia marcescens* QM B1466 [25] was chosen for this work.

High-Density Fermentation of *S. marcescens*

Fed-batch cultures, where nutrients are supplied during cultivation but culture broth is not removed until the end of the fermentation, often provide an efficient method for increasing host biomass and product yields by an order of magnitude and more. Effective application of fed-batch fermentations to the production of proteins requires optimization of the culture conditions and feeding strategy such that essential nutrients are supplied at concentrations well below inhibitory or insolubility levels. Of central importance is the minimization of production of organic acids(particularly acetate), which are thought to inhibit biomass production. Here, we have applied fed-batch fermentation methodologies to the development of a high-density culture system for *Serratia marcescens* QM B1466.

A series of factorial shake flask experiments have been performed to determine those media components which are essential for growth of both *E. coli* and *S. marcescens*, which is similar to *E. coli* in glucose metabolism. Potential growth-limiting nutrients tested included amino acids, vitamins, phosphate, sulfur, and nitrogen sources, trace metal nutrients (*e.g.*, Mg, Co, Fe, etc.), and the carbon source. Based on results from this study and those of Veron [26], the concentrations of the essential media components were established relative to the concentration of the carbon source (either glucose or crab shell chitin) for both the inoculum and feed solutions in both our batch and fed-batch cultures. Table 1 presents the composition of the optimized media which was used for both the batch and fed-batch fermentation media.

Fig. 2 shows the growth curve and feeding strategy for *S. marcescens* in a 10-L fed-batch fermentation. Final cell mass is reached OD of ca. 22 at 660 nm, representing a substantial increase in cell density over conventional batch cultures of *S. marcescens*

Table 1. Medium composition for high-density cultures of *S. marcescens*

Component	Concentration [g/L]
Glucose	2.00
KH_2PO_4 (Buffer component)	6.80
K_2HPO_4 (Buffer component)	2.61
NaCl	10.00
$(NH_4)_2SO_4$	1.00
$MgSO_4(7H_2O)$	0.123
$CaCl_2$	0.0147
Metal solution	1.0 % [v/v]

*(in 1 L H_2O) : 3.84g critic acid, 55.6 mg $FeSO_4 \cdot 7H_2O$, 28.7 mg $ZnSO_4 \cdot 7H_2O$, 16.9 mg $MnSO_4 \cdot H_2O$, 2.5 mg $CuSO_4 \cdot 5H_2O$, 2.5 mg $CoCl_2$, 6.2 mg H_3BO_3

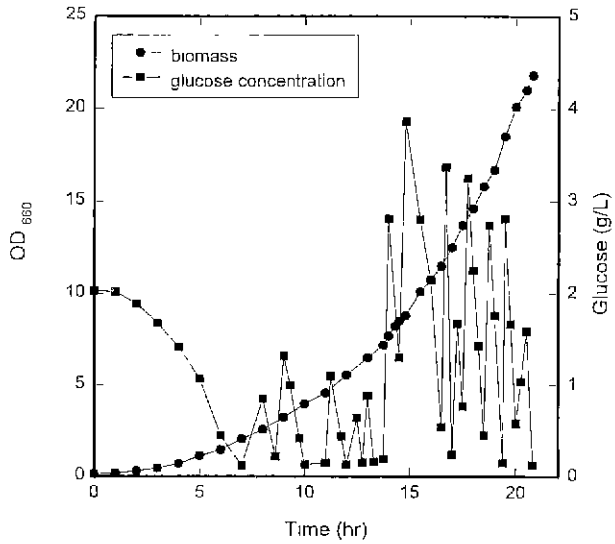

Fig. 2. Growth of *Serratia marcescens* QM B1466 in a fed-batch fermentation with glucose. Culture conditions · minimal media, Temp. 30℃, pH

grown on glucose. Assuming that the chitinolytic-enzyme production rate per cell is constant, increase of cell mass would dramatically decrease size requirements of the fermenter system.

Induction of Chitinolytic-Enzymes Production

Factorial shake flask experiments indicated that induction of chitinolytic-enzymes production by raw ball-milled crab-shell chitin severely inhibited *S. marcescens* growth. This repression of cell growth in the presence of chitin points to the development of a fed-batch strategy where *S. marcescens* is initially grown on glucose and then induced chitinolytic-enzyme with chitin after all added glucose has been consumed. Two induction strategies have been examined as shown in Fig. 3: (1) induction with chitin at late logarithmic growth phase(12-24 hrs), and (2) induction with chitin during early logarithmic growth

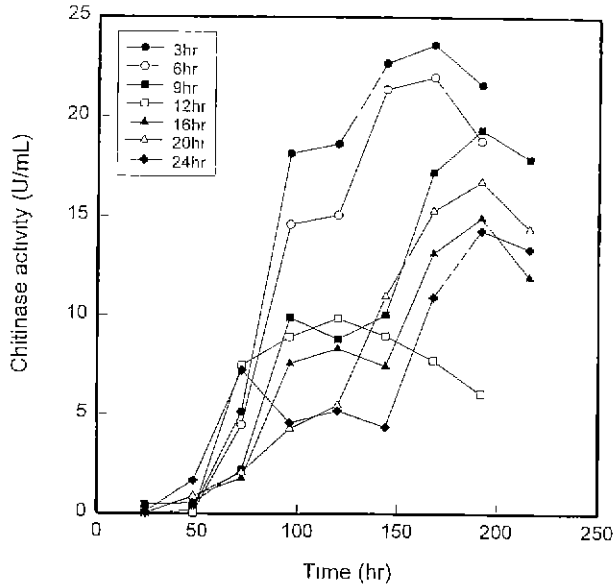

Fig. 3. Time course of the chitinase production in a batch fermentation at varying chitinolytic-enzyme induction times. Chitin (1.5%) was used as a carbon source.

phase(3-9hrs). Induction with chitin at early logarithmic growth phase resulted in higher secretion of chitinolytic enzymes at 3 hrs and 6 hrs than that of late logarithmic growth phase between 12hrs and 24 hrs.

The other parameters, such as the amount of chitin in the medium, is also being explored. The optimum temperature was 30℃ and the optimum pH for enzyme production by *S. marcescens* seemed to be 8.5.

Chitin Sources and Pretreatment

Natural sources such as shrimp, crab, lobster shells, insect exoskeletons, and the cell walls of many fungi include chitin. However, the content and structure of chitin in these various exoskeletons are not identical. Owing to the exquisite selectivity of enzymes, these differences in chitin content and structure may influence both the induction of chitinolytic enzymes and the susceptibility of the chitin to enzymatic hydrolysis.

Since the primary chitinolytic enzyme, chitinase, can only hydrolyze solvent-accessible β-1,4-linkages, the effective surface area and the morphology of the crab-shell waste are important to both chitinase production and catalytic activity. To address this issue, we have explored a number of inexpensive crab-shell pretreatment procedures on the purpose of increasing the production of chitinase. They included ball-milling and hammer-milling to increase the external surface area, autoclaving and steam exploding (Parbombing) to swell the crab-shell particles and thereby increase the intraparticle area, and treatment with dimethyl-acetimide and weak acid cosolvents to swell the crab-shell particles through disruption of the interchain hydrogen-bond structure.

Fig. 4 shows the effect of crab-shell pretreatment on the production of chitinase in shake-flask cultures of *S. marcescens* QM B1466. The remarkable differences in chitinase production were found between pretreatments. Pretreatment with dilute acid and, ball-milled chitin with a nominal diameter less than

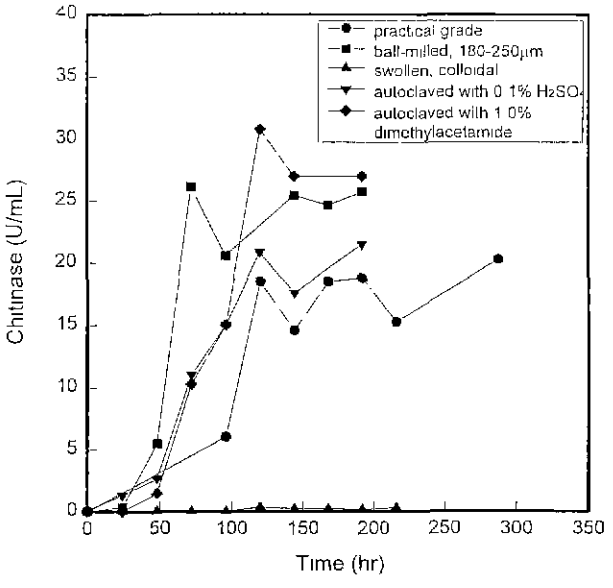

Fig. 4. Effect of crab-shell chitin pretreatment on chitinase production in a batch culture, 1.5% chitin, 30℃

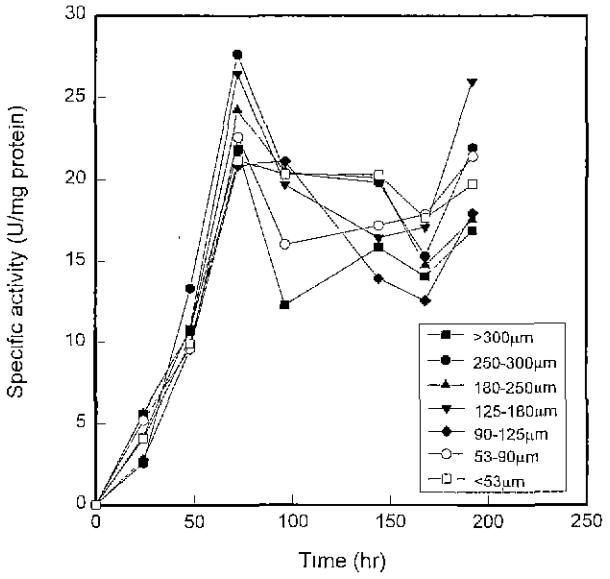

Fig. 5. Effect of chitin particle size on chitinase production. Batch cultures of *Serratia marcescens*, 1.5% chitin, 30℃

250 mm gave the highest chitinase activity. However, the particle size below 125 μm or above 300 mm seemed to decrease the rate of hydrolysis as shown in Fig. 5.

Optimum Conditions for Chitinolytic Activity: The Chitin Digestor

Fig. 6 shows the influence of substrate pretreatment on NAG production rates when contacted with chitinolytic-enzyme solution fermented from ball-milled chitin with a nominal diameter between 180 and 250 μm. Again, substrate pretreatment resulted in higher NAG production rates in the case of swollen crab-shell chitin.

Optimization of NAG production in the chitin digestor also requires knowledge of the conditions where the chitinolytic-enzyme ensemble shows

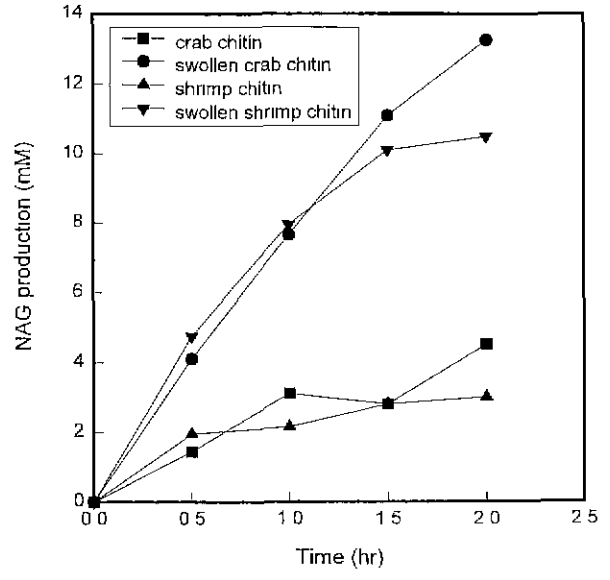

Fig. 6 Effects of chitin sources and pretreatment on NAG production. Chitinase from batch culture of *Serratia marcescens*. 1.5% crab chitin (180-250 μm particle size), 30℃.

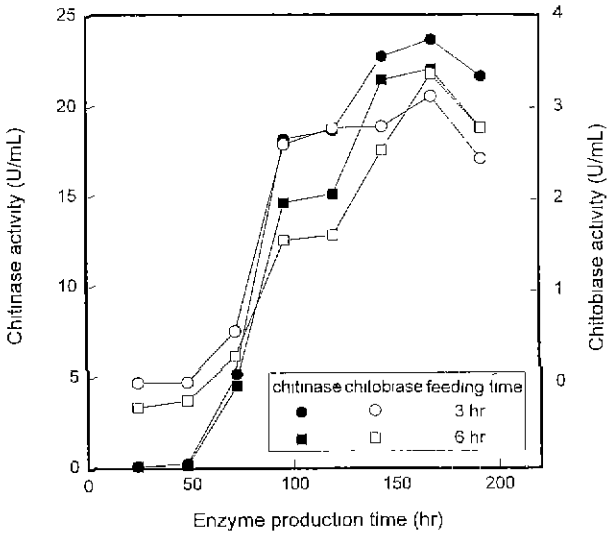

Fig. 7 The optimum feeding time to produce the chitinolytic enzyme and NAG in a fed-batch fermentation using 1.5% chitin as a sole carbon source.

maximum activity. As shown in Fig. 7, the optimum feeding time for the induction of the chitinolytic enzyme and the production of NAG with chitin in a fed-batch fermentation was early logarithmic phase(3-9 hrs). In this case, chitinase and chitobiase activities reached to 23.6 U/mL and 5.1 U/mL, respectively. As a result, chitinolytic enzyme in the crude solution was higher at which induction time was 3hrs, while chitobiase activity was lower value than that at 6hrs.

Chitinolytic-enzyme solution from batch cultures of *S. marcescens* showed a maximum activity near pH 7. Action of the crude enzyme solution on chitin appears to yield only N-acetyl-D-glucosamine at all stages of hydrolysis. Enzyme fraction, however, have been obtained which yield mostly the dimer of NAG. Thus, the system appears to involve both an endoglycanase (chitinase) and an N-acetyl glucosaminidase (chitobiase).

REFERENCES

[1] Freidman, S.J. and P. Skehan (1980) Membrane-active drugs potentiate the killing of tumor cells by D-glucosamine. *Proc. Natl. Acad. Sci. USA.* 77: 1172-1176.

[2] Prudden, J. F. (1977) Method and agent for treating inflammatory disorders of the gastrointestinal tract. U.S. Patent, 4,006,224.

[3] Cosio, I.G., R. A. Fisher, and P. A. Carroad (1982) Bioconversion of shellfish chitin waste : waste pretreatment, enzyme production, process design, and economic analysis. *J. Food Sci.* 47: 901-905.

[4] Zikakis, J.P. (1984) *Chitin, chitosan, and related enzymes*, Academic Press, Orlando, Florida.

[5] Knorr, D. (1991) Recovery and utilization of chitin and chitosan in food processing waste management. *Food Tech.* 45: 114-122.

[6] Cody, R. M., N. D. Davis, J. Lin, and D. Shaw (1990) Screening microorganism for chitin hydrolysis and production of ethanol from amino sugars. *Biomass.* 21: 285-295.

[7] Johnson, L.N. and D. C. Phillips (1964) Crystal structure of N-acetylglucosamine. *Nature.* 202: 588.

[8] Schlumbaum, A., F. Mauch, U. Vogeli, and T. Boller (1986) Plant chitinase are potent inhibitors of fungal growth . *Nature.* 324: 365-367.

[9] Monreal, J. and E. T. Reese (1969) The chitinase of *Serratia marcescens. Can. J. Microbiol.* 15: 689-696.

[10] Neugebauer, E., G. Gamache, C. V. Dery, and R. Brzezinski (1991) Chitinolytic properties of *Streptomyces lividans. Arch. Mocrobiol.* 156: 192-197.

[11] Ulhoa, C.J. and J. F. Peberdy (1992) Purification and some properties of the extracellular chitinase produced by *Trichoderma harzianum. Enzyme Microb. Technol.* 14: 236-240.

[12] Vyas, P. and M. V. Deshpande (1989) Chitinase production by *Myrothecium verrucaria* and its significance for fungal mycelia degradation. *J. Gen. Appl. Microbiol.* 35: 343-350.

[13] Gilkes, N. R., E. Jervis, B. Henrissat, B. Tekanr, R. C. Miller Jr, R. A. J. Warren, and D. G. Kilburn (1992) The adsorption of a bacterial cellulose and its two isolated domains to crystalline cellulose. *J. Biol. Chem.* 267: 6743.

[14] Gilkes, N. R., R. A. J. Warren, R. C. Miller Jr, and D. G. Kilburn (1988) Precise excision of the cellulose binding domains from two *Cellulomonas fimi* cellulases by a homologous protease and the effect on catalysis. *J. Biol. Chem.* 263: 10401.

[15] Gilkes, N. R., B. Henrissat, D. G. Kilburn, R. C. Miller Jr, and R. A. J. Warren (1991) Domains in microbial b-1,4-glysanases: sequence conservation, function, and enzyme families. *Microbiol, Rev.* 55: 503.

[16] Joshi, S., M. Kozlowski, S. Richens, and D. M. Comberbach (1989) Chitinase and chitobiase production. *Enzyme Microb. Technol.* 11: 289-296.

[17] Tom, R.A. and P. A. Carroad (1981) Effect of reaction conditions on hydrolysis of chitin by *Serratia marcescens* QMB 1466 chitinase. *J. Food Sci.* 46: 646-647.

[18] Ong, E., N. R. Gilkes, R. C. Miller Jr., R. A. J. Warren, and D. G. Kilburn (1991) Enzyme immobilization using a cellulose-binding domain: properties of a β-glucosidase fusion protein. *Enzyme Microb. Technol.* 13: 59-65.

[19] Sabry, S.A. (1992) Microbial degradation of shrimp-shell waste. *J. Basic Microbiol.* 32: 107-111.

[20] Reynold, D.M. (1954) Exocelluar chitinase from a *Streptomyces* sp. *J. Gen. Microbiol.* 11: 150-1 59.

[21] Bradford, M. (1976) A rapid and sensitive method for the quantitation of microorganism quantities of protein utilizing the protein-dye binding. *Anal. Biochem.* 72: 248-254.

[22] Ressig, K.L., J. L. Strominger, and L. F. Leloir (1955) A modified colorimetric method for the estimation of N-acetylamino sugars. *J. Biol. Chem.* 27: 959.

[23] Sigma Chemical Co. (1994) Sigma quality control test procedure enzymatic assay of b-N-

acetylglucosaminidase(EC 3.2.1.30) Sigma Prod. No. A-3189. St. Louis MO. U.S.A.
[24] Berger, L.R. and D. M. Reynolds (1958) The chitinase system of a strain of *Streptomyces griseus*. *Biochem. Biophys. Acta.* 29: 522-534.
[25] Reid, J. D. and D. M. Ogrydziak (1981) Chitinase-overproducing mutant of *Serratia marcescens*. *Appl. Environ. Microbiol.* 41: 664-669.
[26] Veron, M. (1975) Annales de microbiologie. *Ann. Microbiol. Inst. Past.* 126A: 267-274.