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Conversion of squid pen by *Pseudomonas aeruginosa* K187 fermentation for the production of *N*-acetyl chitooligosaccharides and biofertilizers

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ABSTRACT

Pseudomonas aeruginosa K187, a protease- and chitinase-producing bacterium, exhibited protease and chitinase activity after three and five days of incubation, respectively. The protease and chitinase were both produced by using 1% squid pen powder (SPP) (w/v) as sole carbon and nitrogen source. After fermentation, the deproteinization rate of the recovered squid pen gradually increased up to 68% on the fourth day. After five days of fermentation, the production of GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$ and $(GlcNAc)_5$ were 1.18 mg/mL, 0.76 mg/mL, 1.02 mg/mL, 0.93 mg/mL and 0.90 mg/mL, respectively. The culture supernatant of K187 also exhibited activity of enhancing vegetable growth. For *Brassica chinensis* Linn treated with the fifth day culture supernatant, the total weight and total length increased up to 529% and 148%, respectively, compared to the control group. With this method, the production of protease, chitinase, *N*-acetyl chitooligosaccharides and biofertilizers may be useful for biological applications.

1. Introduction

Bioconversion of chitinous materials has been proposed as a waste treatment alternative to the disposal of shellfish wastes. However, both SCS and SP contain chitin, protein, and inorganic compounds such as calcium carbonate. Conventionally, preparation of chitin from such shellfish chitin wastes involves deproteinization and demineralization with strong bases and acids. However, the use of these chemicals may cause partial deacetylation of the chitin and hydrolysis of the polymer, resulting in final inconsistent physiological properties.[1,2] The chemical treatments also create waste disposal problems because neutralization and detoxification of the discharged wastewater are necessary. Furthermore, the value of the deproteinization liquid is diminished because of the presence of sodium hydroxide. To overcome the defects of chemical treatments, alternative methods of using microorganisms or proteolytic enzymes for the deproteinization of shellfish chitin wastes have been cited.[1–4]

Recent studies on chitin have attracted interest for converting them into oligosaccharides because these compounds are not only water-soluble but also possess versatile functional properties such as antitumor activity and antimicrobial activity.[5–8] Traditionally, chitin oligosaccharides have been processed by chemical methods in industries. There are many problems existing in chemical processes, such as a large amount of short-chain oligosaccharides produced, low yields of oligosaccharides, high cost in separation, and also environmental pollution. Alternatively, with its advantages in environmental compatibility, low cost, and reproducibility, chitinase hydrolysis has become more and more popular in recent years.[9] Recently, production of chitin oligomers by enzymatic hydrolysis has been reported, and GlcNAc is produced as a major chitinolytic product.[10–16] However, these enzymes are not yet available for large-scale commercial application. In our work, the chitin oligomers were produced by *Pseudomonas aeruginosa* K187 fermentation, and $(GlcNAc)_n$, $n = 1–6$ were produced as major chitinolytic products. In addition, the culture supernatant of K187 also exhibited activity of enhancing vegetable growth. In this study, we have shown that squid pen wastes can be utilized for the production of β-chitin, enzymes (both protease and chitinase), *N*-acetyl chitooligosaccharides, and biofertilizers by *P. aeruginosa* K187 fermentation. These technologies facilitate its potential use in industrial applications and functional foods.

2. Materials and methods

2.1. Materials

The squid pen powder (SPP) used in these experiments was prepared as described earlier.[17] Squid pens were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). Bromelain and papain were purchased from Challenge Bioproducts Co., Ltd (Tou-Liu, Taiwan). In the preparation of the squid pens, they were washed thoroughly

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with tap water and then dried. The dried materials were then milled to powders for using as the carbon/nitrogen source for the production of *N*-acetyl chitooligosaccharides and biofertilizers. All other reagents used were of the highest grade available.

2.2. Squid pen biowaste fermentation

P. aeruginosa K187, isolated from the soil in Taiwan, exhibited strong protease and chitinase activity. The squid pen waste was fermented under optimized conditions as reported previously.[2] After fermentation, the culture broth was centrifuged (4 °C and 12,000*g* for 20 min), and the supernatant and the residual SPP were collected. The residual SPP was washed with distilled water, followed by the removal of cells and the dissolved materials by filtration, and the process was repeated twice. Then, after lyophilizing, the dry weight of remaining squid pens was calculated.

2.3. Deproteinization of squid pens by alkali

SPP was mixed with 2 N NaOH solutions at a ratio of 3:8 (w/v). The mixture was allowed to react at 100 °C for 30 min. The ratio of chitinous materials to solvent, 3:40 (w/v), was the same as the deproteinization method for the preparation of crustacean chitin.[4] After centrifugation, the solid residues were washed with de-ionized water until these became a neutral pH, and then dried at 65 °C in an oven. The dried residues were used for the analysis of deproteinization. The resultant supernatant was also used for analysis of protein concentration by the method of Bradford[18] using Bio-Rad dye reagent concentrate and bovine serum albumin as the standard.

2.4. Measurement of enzymes activity

For measuring protease activity, a diluted enzyme solution (0.2 mL) was mixed with 1.25 mL of 1.25% casein in phosphate buffer (pH 7) and incubated for 30 min at 37 °C. The reaction was terminated by adding 5 mL of 0.19 M trichloroacetic acid (TCA). The reaction mixture was centrifuged and the soluble peptide in the supernatant fraction was measured by the method of Todd with tyrosine as the reference compound.[17] One unit of protease activity was defined as the amount of enzyme required to release 1 μmol of tyrosine per min.

Colloidal chitin (1.3% in 50 mM phosphate buffer) was used as the substrate for the measurement of chitinase activity. The mixture of enzyme solution (0.5 mL) and substrate (1 mL) was incubated at 37 °C for 30 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by the method of Imoto and Yagishita[19] with *N*-acetylglucosamine as a reference compound. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per min.

2.5. Preparation of the chitooligosaccharides by *P. aeruginosa* K187 fermentation

The culture supernatant was concentrated to about 20% of the original volume on a rotary evaporator under diminished pressure. The pH of the sample was adjusted to pH 9 with 10% NaOH and followed by adding ethanol. Yellow agglomerates were formed in the solution. The agglomerates were concentrated with a rotary evaporator under diminished pressure and were collected after drying in vacuum. The supernatant was then concentrated to about 10% of the original volume on a rotary evaporator under diminished pressure. Then, it was precipitated by adding acetone. The precipitates were collected after drying under vacuum.

2.6. HPLC analysis

HPLC analysis of K187 chitooligosaccharides was performed on a Hitachi L-7000 apparatus (column, Nucleosil 5 NH_2 4.6 × 250 mm; mobile phase, acetonitrile–water = 70:30, v/v; flow rate = 1.0 mL/min; detection, RI). After fermentation and filtration, the sample was analyzed to measure the amount of $(GlcNAc)_n$, n = 1–6 in the culture supernatant by HPLC. The amounts of $(GlcNAc)_n$, n = 1–6 were estimated with the calibration curve of standard 3.75 mg/mL, $(GlcNAc)_n$, n = 1–6. The yield of $(GlcNAc)_n$, n = 1–6 was calculated by the following equation.

The concentration of sample (mg/mL) = 3.75 × the area of sample/the area of standard.

2.7. MALDI-TOF MS analysis

An amount of 0.5 μL of the sample solution (2 mg/mL) was mixed on the target with 2 μL of a solution of 2,5-dihydroxybenzoic acid as a matrix (15 mg/mL) in 30% aqueous ethanol. Positive ion MALDI mass spectra were acquired with MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser emitting at 337 nm operating in linear mode. Each mass spectrum was the accumulating data of approximately 30–50 laser shots. External three-point calibration was used for mass assignment.

2.8. Enhancing effect on the growth of *Brassica chinensis* Linn

Culture soil without added nutrients was placed in nursery plates. After evenly inseminating a certain amount of *B. chinensis* Linn seeds and watering, the plates were placed in plant incubator for three days. Seedlings of uniform size were transplanted to pots for further study.

For the *B. chinensis* Linn seedling growth enhancing test, the seedlings of the same size were divided into six groups with nine seedlings each. K187 was inoculated to the culture medium using 1% SPP, and incubated at 37 °C for 0–5 days. The concentration of each resultant culture supernatant was adjusted with RO (Reverse Osmosis) water to 10%. RO water provided control (0%). The solutions were used to irrigate *B. chinensis* Linn to investigate the effect on plant growth. During the experiment, each pot was irrigated daily with preparations. After four weeks of culture, the weight and the height of the whole plant including above and beneath the earth, were measured.[20]

3. Results and discussion

3.1. Production of enzymes and deproteinization of squid pen

K187 was isolated from the soil in Taiwan and maintained on nutrient agar plates at 37 °C as reported previously.[2] K187 was identified as *P. aeruginosa* by the Bioresource Collection and Research Center (Shin-Chu, Taiwan). The biological utilization of squid pen was less investigated and reported. The squid pen is rich in protein and chitin. The carbon and nitrogen sources with both protein and chitin were more suitable as an inducer for protease and chitinase production by microorganism than carbon and nitrogen sources with only protein but no chitin.[4,21] *P. aeruginosa* K187 exhibited strong protease and chitinase activity. The medium containing squid pen and small amounts of minerals was more suitable for the production of protease and chitinase by *P. aeruginosa* K187. *P. aeruginosa* K187 was grown in liquid medium containing 1% squid pen powder, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ in an shaking incubator at 37 °C. During five days fermentation, the squid pen powder decreased gradually in the medium. As to degra-