行政院國家科學委員會專題研究計畫成果報告 5714

以嗜酸性酵母菌 *Candida parapsilosis* CCRC 20515
發酵蝦殼以分離幾丁質及其發酵廢液之利用

Isolation of chitin from fermentation of shrimp shell by acidophilus yeast *Candida parapailosis* CCRC 20515 and the utilization of waste liquid after deproteinization

計畫編號：NSC-89-2313-B-019-014
執行期限： 88 年 08 月 01 日 至 89 年 07 月 31 日
主持人： 陳幸臣 國立台灣海洋大學食品科學系
E-mail：hcchen@ntou66.ntou.edu.tw

一、 中文摘要

本研究利用一種嗜酸酵母菌 (*Candida parapailosis CCRC 20515*) 去除去鈣蝦殼(DDS)之蛋白質，以分離幾丁質。發酵液含 0.5% (w/v)的酵母萃及 2% (w/v)的葡萄糖，與 DSS 混合後調 pH 3.0，無需殺菌。種入該酵母菌於 30℃(120 rpm) 發酵 6 天，完成去除蛋白質的操作。發酵作業能去除 DSS 上 86.9% 的蛋白質；殘留物含 97.0% 的幾丁質，乙醯度及分子量分別為 71.8% 及 2.2 x 10^5 Da。以反應曲面法(RSM)求得最適起始 pH 為 2.8 及葡萄糖濃度為 1.1% ；而酵母萃濃度維持不變，可得到最高的蛋白質去除效果；以此最適混合條件可獲得殘留物含 97.6% 的幾丁質。在去蛋白質的發酵液中，含游離氨基酸的濃度為 73mg/100mL，比營養肉汁(nutrient broth，Difco)略低，但以此廢液培養 *Cellulomonas flavigena* NTOU 1，其最終菌數可達 10^9CFU/mL。因此利用此廢液為微生物培養基可降低分離幾丁質的成本。

關鍵詞：去蛋白質、蝦殼、*Candida parapailosis*、廢液利用

Abstract

Candida parapsilosis CCRC 20515, an acidophilic yeast, was employed for the deproteinization of decalcified shrimp shell (DSS) to isolate chitin. A fermentation medium containing 0.5% (w/v) yeast extract and 2% (w/v) glucose was mixed with DSS, and initial pH adjusted to 3.0. The mixture was inoculated with the yeast without sterilization and incubated (30℃, 120 rpm) for six days. After fermentation, the percent deproteinization of DSS was 86.9%. The chitin content of the residue was 97.0% (dry base); degree of acetylation and molecular weight were 71.8% and 2.2 x 10^5 Da, respectively. Response surface methodology revealed, the optimized initial pH (2.8) and

glucose concentration (1.1 %) for maximum protein removal from the DSS in the fermentation medium, while the concentration of yeast extract remained unchanged. Using this optimal combination, chitin content of the residue was 97.6% (dry base). Total concentration of free amino acids in the waste liquid after deproteinization was 73 mg/100 mL lower than that in the nutrient broth (Difco). This difference did not, however, obviously affect the growth yield of the bacterial strain (*Cellulomonas flavigena* NTOU 1). The utilization of waste liquid as a culture medium may reduce the cost of chitin isolation from crustacean shells using biodeproteinization and reduce environmental impact.

Keywords: deproteinization; shrimp shells; *Candida parapsilosis*; waste liquid utilization

二、 Objective and background of the research

Crab shells and shrimp shells are the main raw materials for chitin preparation. The general procedures for isolating chitin are decalcification (or demineralization), deproteinization and decoloration. Chemically, minerals in the shells are typically dissolved using hydrochloric acid while the proteins are hydrolyzed by alkalis and the residues are decolorized using solvents and/or oxidants.[1)] The procedure for removal of minerals and proteins can be altered according to the intended use of the waste liquid, however, the treatment of chemicals, especially by alkalis, may lead to a partial deacetylation and depolymerization of the chitin, [2, 3)] which may be detrimental to the preparation of a biomedical material with specific physical and chemical properties. By contrast, deproteinization of crustacean shells can be achieved using processes involving proteolytic enzymes [4)] or microorganisms. [5- 9)] Since this reaction is relatively mild and more specific, the process of chitin isolation will not lead to depolymerization. This processed chitin is then suitable for controllable processes using enzymatic deacetylation to prepare chitosan for biomedical applications.[2)]

For the deproteinization of crustacean shells using microorganisms which grow well at a neutral pH, culture media have to be sterilized together with the shells prior to seeding with starter organisms. For this study, the sterilization process was omitted, to save energy. The proteolytic and acidophilic microorganism, *Candida parapsilosis,* was chosen for this purpose. In addition, the waste liquid byproduct of

deproteinization was also tested as a bacterial culture medium to improve waste utilization.

三、 Results and discussion

Fermentation of *C. parapsilosis* CCRC 20515 revealed an 87 % deproteinization from DSS (Table 1). Yang *et al.*[9] removed 76% proteins from acid treated (or decalcified) shrimp shells using *Bacillus subtilis* Y-108, obtaining an 88% deproteinization from untreated (not decalcified) shrimp shells. For their study, the mixture of culture medium and shell samples was sterilized prior to *B. subtilis* inoculation. Although type of shrimp shell may affect the efficiency of deproteinization by microorganisms, the species of shrimp used for their study was not reported.

Deproteinization of shrimp shells can precede decalcification. From our preliminary study, however, the waste liquid after deproteinization without prior decalcification was adjusted to pH 8.5, salinity was around 2.6% and not suitable for the growth of *C. flavigena* NTOU 1. Thus, for this study, deproteinization was carried out after decalcification during the chitin isolation process, since the salinity of WLD was only 0.6% (data not shown).

To achieve maximum DSS protein extraction, the optimal initial pH and glucose concentration for the fermentation medium was 2.8 and 1.1% , respectively, as estimated by RSM (Fig. 1). Under these conditions, chitin content in the residues after deproteinization was 97.6 %, and degree of acetylation and molecular weight were 71.2 % and 2.1×10^5 Da, respectively (data not shown). These results are almost identical to those obtained by Chen and Hsu[5] using *Pseudomonas maltophilia* 1-1 for biodeproteinization.

During decalcification, shrimp shells were soaked in 2 N HCl for two hours. This procedure was effective for destruction of putrefactive microorganisms (data not shown). Further, the medium at pH 3.0 is not suitable for the proliferation of most microorganisms, except acidophiles like *C. parapsilosis*. Since the proteases produced by *C. parapsilosis* CCRC 20515 are acidic proteinases, the proteolytic activity is reduced to 50% at pH 6.8 and inactivated at a pH higher than 7.5.[10] During fermentation, the yeast displayed a higher proteolytic activity at pH 4.8 on day 4, with the viable yeast count the highest on the same day. Therefore, a six-day fermentation may be sufficient for the

hydrolyzation of shrimp-shell proteins. For the mass production of chitin, controlling pH within a given range (e.g. 3~4) is suggested.

The yields of *C. flavigena* NTOU 1 in NB and WLD are slightly different (Fig. 2). The lower yield revealed for WLD may be the result of the lower nutrient value of WLD in comparison to NB (Table 2). The free amino-acid profile determined by this study is intended to indicate part of the nutrient source in these media. Yeast extract is basically a mixture of amino acids and peptides, water soluble vitamins and carbohydrates, however, the amino nitrogen in yeast extract consists of 3.4~4.8% , while in meat extract and peptone, it consists of 1.5 and 1.7~3.8% , respectively.[11] This could be the reason that the level of total free amino acids for the fermentation medium (FM; 157.50 mg/100ml) was 1.8 times higher than that for NB (Table 2). The proliferation of yeast cells renders the reduction of nutrient source in FM for microbial growth. The hydrolysis of proteins in DSS could, however, contribute amino acids to the WLD. This can be proved by the detection of tryptophan (0.95 mg/100ml) in WLD. Nevertheless, the application of WLD to cultivate *C. flavigena* NTOU 1 is practical for waste utilization.

C. flavigena NTOU 1 has been employed to process shrimp sauce, [12] and to produce chitinases[13] for preparing chito-oligosaccharides. The utilization of WLD as culture medium for *C. flavigena* NTOU 1, may reduce the cost of chitin isolation from shrimp shells. Furthermore, biodeproteinization may reduce the production overhead of chemical treatment where discharged wastewater must be neutralized and detoxified.

Since we believe the requirement for chitin and chitosan will escalate with the gradual introduction of new applications, we suggest it will become increasingly important to develop and improve methods for isolating chitin from crustacean shells, or other raw materials, through cost reduction and minimization of environmental impact.

四、References

1) No, H. K., and Meyers, S. P., Preparation of chitin and chitosn. In" Chitin Handbook", eds. Muzzarelli, R. A. A., and Peter, M. G., Atec, Grottammare, Italy, pp. 475-489 (1997).

2) Martinou, A., Tsigos, I., and Bouriotis, V., Preparation of chitosan by enzymatic deacetylation. In"

Chitin Handbook", eds. Muzzarelli, R. A. A., and Peter, M. G., Atec, Grottammare, Italy, pp. 501-505 (1997).

3) Chang, K. L. B., Tsai, G., Lee, J., and Fu, W. R., Hetreogeneous *N*-dacetylation of chitin in alkaline solution. *Carbohydr. Res.*, **303**, 327-332 (1997).

4) Gagne, N., and Simpson, B. K., Use of proteolytic enzyme to facilitate the recovery of chitin from shrimp waste. *Food Biotechnol.*, **7**, 253-263 (1993).

5) Chen, H. C., and Hsu, C. C., Production of chitin and its partial deacetylation from shrimp shell by microbial degradation. *J. Chin. Agric. Chem. Soc.*, **35**, 342-353 (1997).

6) Roa, M. S., Munoz, J., Aye, K. N., Nuchnoi, P., and Stevens, W., Use of lactic acid bacteria and protease enzymes in the deproteination and demineralization of shrimp biowaste for production of chitin. In" Advances in Chitin Science "(Vol. III) , eds. Chen, R. H. and Chen, H. C. Rita Adevertising Co., Taipei, pp. 206-214 (1999).

7) Wang, S. L., and Chio, S. H., Deproteinization of shrimp and crab shell with the protease of *Pseudomonas aeruginosa* K-187. *Enzyme Microbial Technol.*, **22**, 629-633 (1998).

8) Shimahara, K., Ohkouchi, K., and Ikeda, M., In" Chitin and Chitosan" , eds. Hirano, S., Tokura, S. The Japenese Society of Chitin and Chitosan, Sapporo, Japan, pp. 10-14 (1982).

9) Yang, J. K., Shih, I. L., Tzeng, Y. M., and Wang, S. L., Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enzyme Microbial Technol.*, **26**, 406-413 (2000).

10) Fusek, M., Smith, E. A., Monod, M., Dunn, M. M., and Founding, S., Extracellular aspartic proteinases from *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis* yeasts differ substantially in their specificities. *Biochem.*, **33**, 9791-9799 (1994).

11) Bridson, E. Y., and Brecker, A., Design and formulation of microbial culture media. In" *Methods in Microbiology* " (Vol. 3A) , eds. Norris, J. R., and Ribbons, D. W., Academic Press, Inc., New York, pp. 229-295 (1970).

12) Chen, H. C., Karaibrahimoglu, Y., Moody, M. W., and Jiang, S. T., Shrimp-shell hydrolysation by

Cellulomonas flavigena NTOU 1B and its application. *J. Fish. Soc. Taiwan*, , **20**, 73- 81 (1993)

13) Chen, H. C., Hsu, M. F., and Jiang, S. T., Purification and characterization of an exo-*N*, *N'*–diacetylchitobiohydrolase-like enzyme from *Cellulomonas flavigena* NTOU 1. *Enzyme Microbial Technol.*, **20**, 191-197 (1997).

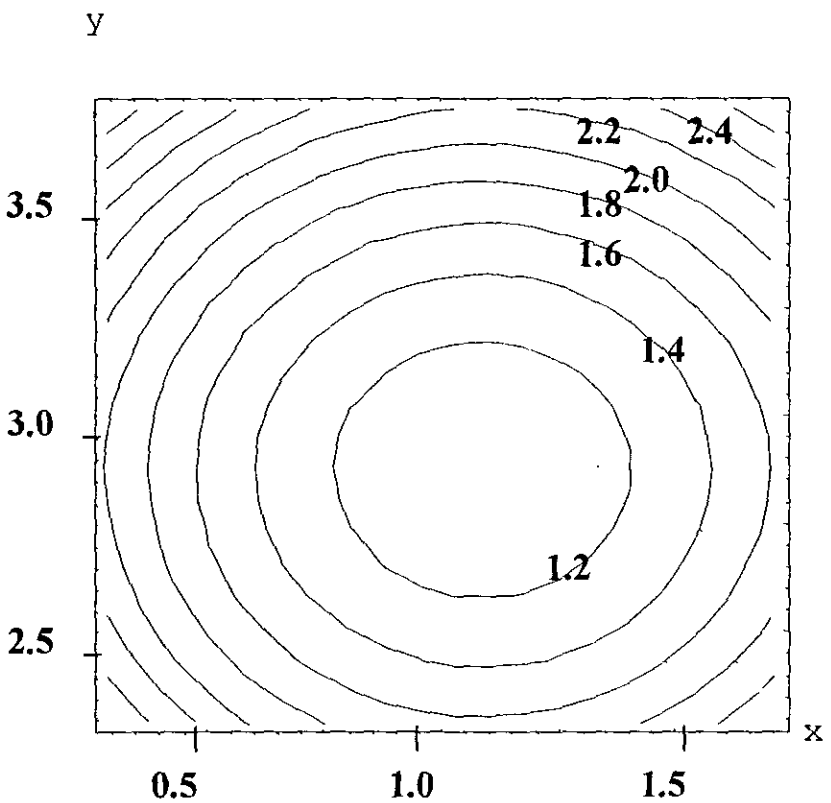

Figure 1.Changes of viable cell counts, pHs and relative protease activities in non-sterilized fermentation medium [0.5% (w/v) yeast extract, 2% (w/v) glucose, pH 3.0] during incubation (30°C, 120 rpm) with *Candida parapsilosis* CCRC 20515.

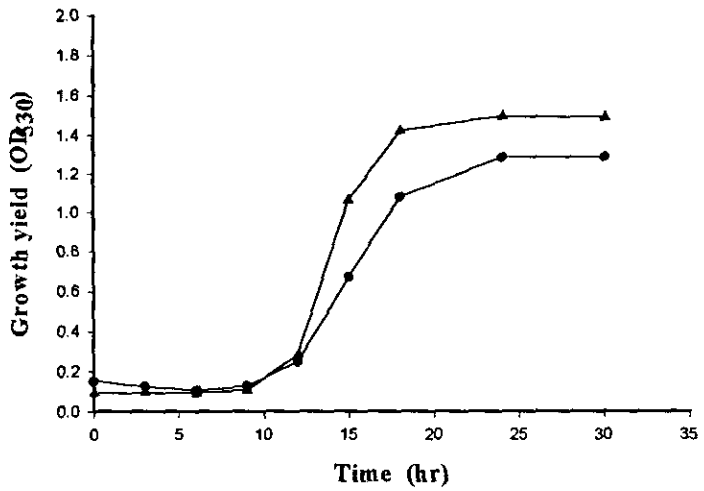

Figure 2. Growth yields of *Cellulomonas flavigena* NTOU 1 in nutrient broth (▲) and in waste liquid after deproteinization (●), at 30℃.

Table 1. Changes of proximate compositions of decalcified shrimp (*Solenocera prominentis*) -shell residues before and after deproteinization by *Canidia parapsilosis* CCRC 20515 in fermentation medium (pH 3.0) containing 2 % glucose (w/v) and 0.5% (w/v) yeast extract.

Treatment	Proximate composition (%)				Degree of acetylation (%)	Molecular weight (Da)
	Moisture	Protein	Chitin	Ash		
Before deproteinization	4.0	13.0 (13.7)[1]	81.1 (85.4)	0.9 (0.9)		
After deproteinization	5.0	1.7 (1.8)	91.2 (97.0)	1.1 (1.2)	71.8	2.2×10^5

[1] Dry weight base.

Table 2. Free amino acid profiles (mg/100ml) of nutrient broth (NB, Difco) ,fermentation medium (FM) and waste liquid after deproteinization (WLD) of decalcified shrimp shell by *Candida parapsilosis* CCRC 20515.

Amino acid	NB	FM	WLD
Ala	10.26	16.27	11.34
Arg	17.53	5.70	2.71
Asn	0.85	5.29	0.83
Asp	2.65	8.69	5.76
Gln	—[1]	0.69	0.15
Glu	6.82	35.07	1.13
Gly	6.84	4.87	4.87
His	0.54	2.04	1.45
Ile	5.09	8.89	6.20
Leu	8.78	15.98	10.89
Lys	6.68	6.56	6.31
Met	0.45	3.67	1.16
Phe	5.94	9.09	6.64
Pro	2.51	3.68	0.28
Ser	2.73	8.59	0.71
Thr	3.05	7.25	1.36
Trp	—	—	0.95
Tyr	2.50	4.40	4.40
Val	4.54	10.77	5.88
Total	87.76	157.50	73.02

[1] Not detected.