ISSN 1330-9862
(FTB-1498)

*preliminary communication*

# The Use of Crude Shrimp Shell Powder for Chitinase Production by *Serratia marcescens* WF

*Jesús E. Mejía-Saulés*[1], *Krzysztof N. Waliszewski*[2*], *Miguel A. Garcia*[2] *and Ramon Cruz-Camarillo*[1]

[1]Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México DF, México

[2]Instituto Tecnológico de Veracruz, M.A. de Quevedo 2779, P.O. Box 1380, Veracruz, 91860 México

Received: April 4, 2005
Revised version: June 23, 2005
Accepted: October 6, 2005

**Summary**

From 102 *Serratia marcescens* strains screened, 57 strains showed chitinase activity and *Serratia marcescens* WF showed the highest chitinolytic activity so this strain was selected for further study on the use of crude shrimp waste for chitinase production. The concentration of crude shrimp shell content at 10–70 g/L, incubation temperature of 28–37 °C, pH=6–9, and time 24–96 h on kinetics of chitinase production by *S. marcescens* WF were evaluated. The maximal chitinase production related to process variables was obtained with the second order polynomial model: dry shrimp shell powder at 6 %, pH=6.5, temperature of 28 °C during fermentation for up to 72 h.

*Key words:* crude shrimp waste, chitinase, *Serratia marcescens* WF, modeling

## Introduction

Chitin is the second most abundant polysaccharide in nature, after cellulose, and it largely exists in wastes from the processing of marine food products (crab, shrimp and krill shells). About $10^{11}$ t of chitin is produced annually in the aquatic biosphere alone (*1*). The waste generated from the worldwide production and processing of shellfish is a serious problem of growing magnitude. This abundant waste may pose environmental hazard due to the easy deterioration. Many bacteria and fungi produce extracellular chitinolytic enzymes, known as chitinases (E.C. 3.2.1.14), able to convert chitin into compounds that can be of industrial interest, mainly *N*–acetyl–D–glucosamine. There is an increasing interest in the use of chitinases for the control of moulds, insects, nematodes, and production of different chitin oligomers (*2*). The ability of chitinase to hydrolyze chitin makes it very useful for the production of value added products such as sweeteners, growth factors, and single cell protein (*3–6*).

The main drawback is the high cost of chitinase production and this enhances the necessity to search for highly enzyme-productive strains and inexpensive cultivation media (*7,8*). Several species of bacteria such as *Bacillus pabuli* (*9*), *Bacillus licheniformis* (*4*), and mainly *Serratia marcescens* have shown a chitinase producing ability. Monreal and Reese (*10*) isolated *Serratia marcescens* QMB 1466 from 100 tested microorganisms, and the optimal conditions for chitinase production were found: swollen pure chitin at 1.5 %, 30 °C and pH=7.5.

Preparation of pure chitin from shellfish waste involves a costly process of harsh chemical treatments of deproteinization at pH=11 and demineralization with 8 % HCl (*11*). Chitin of less than 5 % of protein was isolated from shrimp shell waste by three proteolytic *Aspergillus niger* strains (*12*). The most important chitinase producing strains utilize pure chitin, mainly in the colloidal form, as enzyme inducement and a major carbon source (*6*).

*Corresponding author; Phone: ++52 229 93 45 701 ext. 108; Fax: ++52 229 93 45 701 ext. 201; E-mail: kw@itver.edu.mx

The objective of this study was to select from 102 *Serratia marcescens* strains an appropriate microorganism of the highest chitinolytic activity ratio. The novelty of this work is to use the selected *Serratia marcescens* WF strain of the highest chitinolytic activity for modeling the effect of milled crude shrimp shell powder at the concentration of 10–70 g/L, incubation temperature of 28–37 °C, pH=6–9, and time 24–96 h for microbial chitinase production.

## Material and Methods

### *Screening study*

A total of 102 *Serratia marcescens* strains were obtained from the Microbial Enzymes Laboratory, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México DF, México. Strains were cultivated on Petri dishes for 72 h composed of the synthetic medium (SM) which contained (in g/L): ammonium citrate 0.625, NaCl 0.250, $KH_2PO_4$ 0.375, $Na_2CO_3$ 0.375, $MgSO_4$ 0.275, including 23 g/L of bacteriological agar (Bioxon) and 10 g/L of colloidized chitin hydrated before use (Sigma Chemical Co.). From our previous study, a culture pH=6.5 and temperature of 28 °C were considered as the best for the chitinase production screening test. Activity ratio was defined as the ratio of the diameter of the chitinolytic zone to the diameter of the bacterial colony.

### *Liquid medium culture conditions*

The strain *Serratia marcescens* WF, previously selected due to its highest activity ratio of chitin hydrolysis, was used to determine optimal conditions for enzyme production using liquid SM. Instead of chitin as a major carbon source, dry crude shrimp shells were prepared by treating waste with boiling water, then crushed, dried in an oven at 60 °C, and fine milled and sieved in the range of 100–140 mesh and added to the liquid medium at 10, 20, 30, 40, 50, 60 and 70 g/L. In addition, incubation temperature (28, 31, 34 and 37 °C) and pH (6, 6.5, 7, 7.5, 8 and 9) were analyzed for enzyme production in a 250-mL Erlenmeyer flask. The culture was grown with agitation (180 rpm) and at 24, 36, 48, 72 and 96 h samples were taken for bacteria count, reducing sugar, soluble protein and chitinase activity analyses.

### *Microbiological and chemical analyses*

The bacteria colony count was determined according to the Official Method of the American Society for Microbiology (*13*). Chemical analyses were performed in the supernatant after liquid culture centrifugation at 20 000 rpm for 15 min. The protein concentration was measured using the Folin method (*14*). The amount of reducing sugar was quantified using the modified dinitrosalicylic acid (DNS) method expressed as *N*-acetyl-D--glucosamine (*15*). Chitinase activity was determined according to the Monreal and Reese (*10*) method using swollen chitin as substrate. One unit of enzyme activity (UC) was defined as the amount of enzyme required to liberate 1 μmol of *N*-acetyl-D-glucosamine during 1 min at 28 °C and pH=6.5.

### *Experimental design and the results of modeling*

A surface response model was used to evaluate numerically the effect of dry crude shrimp shell content (CSS), incubation temperature, and pH on *S. marcescens* WF chitinase production. The chitinase activity (Y) was represented by a second-order polynomial equation of the codified variables of dry crude shrimp shells, temperature, and pH named $x_1$, $x_2$ and $x_3$, respectively.

The general model was as follows:

$$y_i = \beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2} + \beta_3 x_{i3} + \beta_4 x_{i1} x_{i2} + \beta_5 x_{i1} x_{i3} + \\ + \beta_6 x_{i2} x_{i3} + \beta_7 x_{i1}^2 + \beta_8 x_{i2}^2 + \beta_9 x_{i3}^2$$

## Results and Discussion

Only 57 strains showed chitinase activity of the total 102 *S. marcescens* strains screened. It means that not all *S. marcescens* strains can be considered as a potential chitinase producer. Results of the highest activity ratio of some *S. marcescens* strains are shown in Table 1. The activity ratio of *S. marcescens* QMB1466 strain, considered by some authors as the most productive chitinase enzymes, was only 3.2, 15 % higher than that found in another screening study (*16*). In another similar screening study of 100 different microorganisms, *Serratia marcescens* QMB 1466 was found to be the most active organism (*10*) when yeast extract was added at 0.5 g/L. In our study, colloidal chitin was used at the same concentration as by these authors, but cheaper ammonium citrate substituted yeast extract. From 102 *S. marcescens* strains, seven strains presented in Table 1 were more active in the enzyme production than high productive *S. marcescens* QMB 1466. By analysis of variance, only one strain (*S. marcescens* WF) showed the highest enzyme production and it was taken into consideration for liquid medium culture study for chitinase production.

Table 1. Activity ratio of high chitinolytic *S. marcescens* strains

| *S. marcescens* strain | Mean activity ratio |
|---|---|
| QMB 1466 | 3.2[a] |
| 69 | 4.5[b] |
| 103 | 4.7[b] |
| MBS9 | 4.7[b] |
| 96 | 5.3[c] |
| NIMA | 5.5[d] |
| 63 | 6.0[e] |
| WF | 6.5[f] |

Different letters indicate the difference between activity ratio results (α=0.5)

Mean proximate analysis of five randomly selected dried CSS samples was as follows (in g/kg): moisture 43.5, protein 263.2, ether extract 17.4, chitin 327.2 and ash 348.8. Compared to another study, CSS was richer in cultivation nutrients, almost 50 % higher in chitin and protein and 15 % lower in ash (*11*).

*Effect of process variables on S. marcescens WF growth*

The effect of a crude shrimp shell powder concentration in liquid medium on bacterial count during 96 h of culture growth at 28 °C and pH=6.5 is shown in Fig. 1. The highest bacterial growth was observed when *S. marcescens* WF was grown on 6 % of CSS at 72 h. Analyzing whole growth tendency with other conditions than on 6 % of CSS, the highest bacterial growth was found up to 36 h. After this time, bacterial counts showed a tendency to decrease. However, when the strain was grown on 6 % of CSS, bacterial count did not stop at 36 h and grew up to 72 h. After this time, it showed the tendency to decrease.

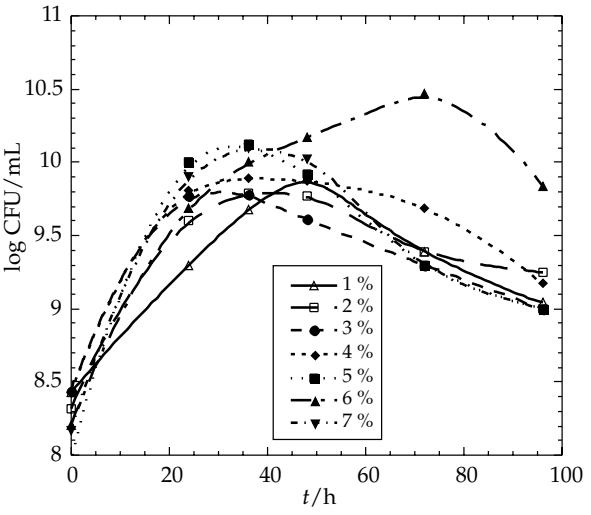


**Fig. 1.** Effect of CSS concentration on *Serratia marcescens* WF count during 96 h of fermentation at pH=6.5 and 28 °C. The symbols indicate different concentrations of CSS

When the study was conducted on only 6 % of CSS at 28 °C, considering the effect of pH on the cell growth (6, 6.5 and 7), the results of bacteria count were not different at 24 and 36 h. Beginning from 48 and 72 h of growing, counts were different (higher) at pH=6.5. When compared with the treatment at pH=6.5, the growing strain curves were lower at pH=6 and 7, but of the same tendency. Analyzing the tendency of whole growth performance, culture growth at 28 °C was different (higher) when compared to the growth performance at 31, 34 and 37 °C. The microorganism was intolerant of the high temperature level of above 37 °C, decreasing substantially chitinolytic activity.

The above mentioned conditions of cultivation were considered as reference for limiting the process variables. Considering that at any time maximal activity of chitinase may appear, maximal enzymatic activity ($a_{max}$) at any time ($t_p$) was taken as response variable. The most adequate conditions for chitinase production by *S. marcescens* WF were considered: dry shrimp shell powder at 6 %, pH=6.5, and temperature 28 °C. Therefore, the relation between these variables was expressed in the following equations:

$x_{i1} = (c_S - 4)/3$
$x_{i2} = (t - 32.5)/4.5$
$x_{i3} = (\text{pH} - 7.5)/1.5$

These equations were stated in a manner that the limits for codified variables $x_1$, $x_2$ and $x_3$ were between –1 and 1.

The complete experimental design is shown in Table 2 and the parameter values obtained by linear regression are listed in Table 3.

Table 2. Experimental design for the evaluation of $a_{max}$ and $t_p$ on chitinase production (each treatment was repeated at least twice)

| CSS content/% | Temperature/°C | pH |
|---|---|---|
| 1 | 28 | 6.5 |
| 2 | 28 | 6.5 |
| 3 | 28 | 6.5 |
| 4 | 28 | 6.5 |
| 5 | 28 | 6.5 |
| 6 | 28 | 6.5 |
| 7 | 28 | 6.5 |
| 6 | 28 | 6.0 |
| 6 | 28 | 7.0 |
| 6 | 28 | 8.0 |
| 6 | 28 | 9.0 |
| 6 | 31 | 6.5 |
| 6 | 34 | 6.5 |
| 6 | 37 | 6.5 |
| 5 | 28 | 6 |
| 5 | 28 | 7 |
| 7 | 28 | 6 |
| 7 | 28 | 7 |
| 5 | 34 | 6 |
| 7 | 34 | 6 |
| 5 | 34 | 7 |
| 7 | 34 | 7 |
| 6 | 31 | 6.5 |
| 7 | 31 | 6.5 |
| 5 | 31 | 6.5 |
| 6 | 31 | 7 |
| 6 | 31 | 6 |

Table 3. Estimated parameter results applied to the general model fitted by linear regression at maximal enzyme activity and time

| Parameter | Estimated value for $a_{max}$ | Estimated value for $t_p$ |
|---|---|---|
| $\beta_0$ | –0.74812E+01 | 0.55067E+02 |
| $\beta_1$ | 0.15073E+02 | 0.30786E+01 |
| $\beta_2$ | –0.45874E+01 | –0.92753E+01 |
| $\beta_3$ | –0.21774E+02 | –0.38218E+01 |
| $\beta_4$ | –0.46598E+01 | 0.11404E+02 |
| $\beta_5$ | 0.17952E+02 | –0.18680E+01 |
| $\beta_6$ | 0.28560E+01 | 0.10300E+02 |
| $\beta_7$ | –0.29425E+01 | 0.11948E+01 |
| $\beta_8$ | 0.10253E+02 | –0.97668E+01 |
| $\beta_9$ | –0.69238E+01 | –0.30036E+01 |

Parameters β and the estimated values of enzymatic activity ($a_{max}$) at any time ($t_p$) were considered in the estimation of the following process variables for chitinase production.

### *Effect of CSS content on chitinase production by S. marcescens WF*

Specific chitinase activity

When $H_0$ (probability of null hypothesis): $\beta_j=0$ for j=1, 4, 5, 7 *etc.*, the probability of null hypothesis was only 0.80436E-02, which means that the concentration of substrate affects enzyme production expressed as specific activity.

The effect of CSS on chitinase specific activity is shown in Fig. 2.

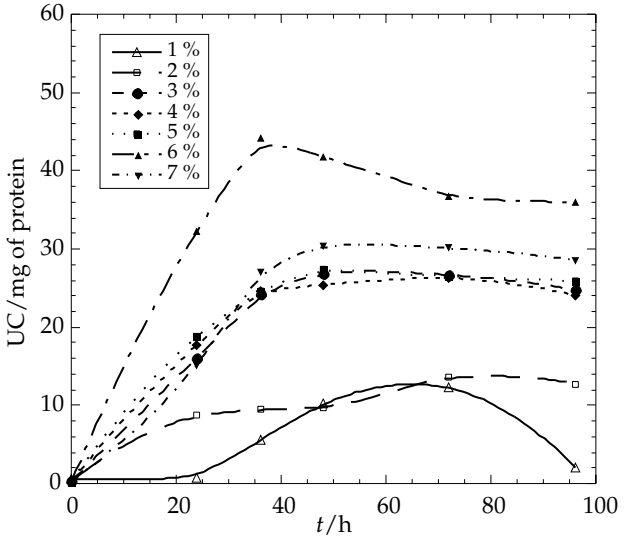


**Fig. 2.** Effect of CSS concentration on *Serratia marcescens* WF extracellular specific units (UC) of chitinolytic activity during 96 h of fermentation at pH=6.5 and 28 °C. The symbols indicate different concentrations of CSS

At 1 % of CSS, the highest enzyme specific activity was found at 72 h, but at 96 h enzymatic activity dropped. At 2 % of CSS enzymatic activity was not different when compared with 1 % of CSS at 72 h, but at 96 h chitinase activity was maintained. At 3, 4 and 5 % of CSS, enzymatic activity was doubled at 72 h when compared with 2 % of CSS, and a small decrease of chitinase activity was also observed at 96 h. By the analysis of the results of chitinase activity at 3, 4 and 5 % of CSS concentration, no difference in results during 36 to 96 h of cultivation was found and the peak of enzyme production was found earlier, at 48 h. The highest enzymatic activity was found at 6 % of CSS at 36 h and only a small drop during the rest of the time of cultivation was observed. At 7 % of CSS, inhibiting effect of CSS on chitinase production was found and enzymatic activity was lower when compared to the activity at 6 % of CSS. It means that about 2 % of chitin from CSS as cultivation medium is the most adequate concentration for chitinase production by *Serratia marcescens* WF strain.

Chitinase activity in liquid medium

When $H_0$: $\beta_j=0$ for j=1, 4, 5, 7 *etc.*, the probability of null hypothesis was only 0.7248E-02, which means that the concentration of substrate affects enzyme production in liquid medium. The effect of CSS on chitinase activity is shown in Fig. 3.

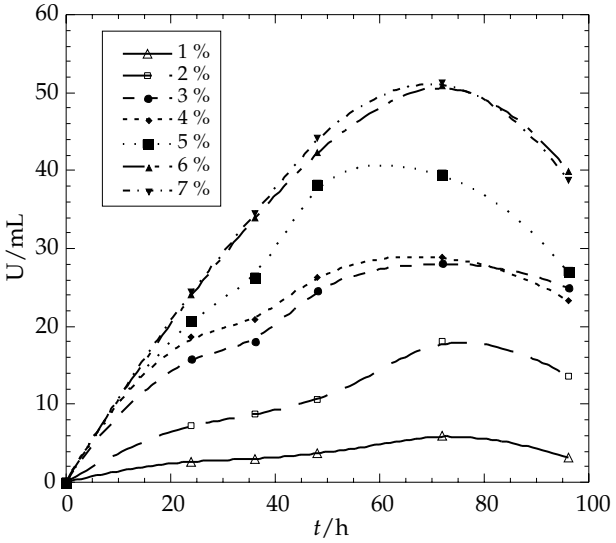


**Fig. 3.** Effect of CSS concentration on *Serratia marcescens* WF extracellular units (U) of chitinolytic activity during 96 h of fermentation at pH=6.5 and 28 °C. The symbols indicate different concentrations of CSS

Increasing CSS concentration from 1 to 7 %, which means from 0.33 to 2.29 % of chitin present in CSS up to 72 h, resulted in the increase in chitinolytic activity. After this time decrease in the enzyme activity was found. The highest chitinase activity in liquid medium was found at 6 and 7 % of CSS at 72 h (50.9 and 51.2 U/mL), respectively. After this time, the enzyme activity decreased at all substrate concentrations. Recent study of four forms of chitin (colloidal chitin, processed chitin, prawn shell chitin, and chemically isolated chitin) on chitinase production by *Serratia marcescens* QMB 1466 has shown that processed chitin isolated by lactic acid fermentation of prawn shell induced a higher level of enzyme activity (*17*). When prawn shell substrate was used, the highest enzyme activity (25 U/mL) was found at pH=7.0, 25 °C and 1 % mass per volume ratio of chitin. These results are in close agreement with those of *Serratia marcescens* QMB 1466 with respect to pH and temperature (*10,18*). Increasing the substrate concentration from 0.5 to 1 % generally resulted in an approximately twofold increase in enzyme activity. Further increase of substrate concentration above 1 % inhibited the enzyme activity. In our study with *Serratia marcescens* WF, the highest activity (50.6–51.2 U/mL) was found at 6–7 % of CSS chitin concentration. The reason for twofold enzymatic activity in liquid medium is that the strain WF grows at higher (1.9–2.3 %) chitin concentration and is of higher enzymatic productivity.

Effect of pH on chitinase production by *S. marcescens* WF

When $H_0$: $\beta_j=0$ for j=3, 5, 6, 9 *etc.*, the probability of null hypothesis was only 0.21937E-02, which means that

pH also affects enzyme production. When our study was conducted at pH=6, 6.5 and 7 at 6 % of CSS, the difference in enzymatic activity was found during 96 h of fermentation and the highest enzymatic activity was found at 36 h and pH=6.5 (Fig. 4). This result is different from those obtained in other studies with *Serratia marcescens* QMB 1466 at pH=7.5 (*10*) and pH=7.0 (*17*).

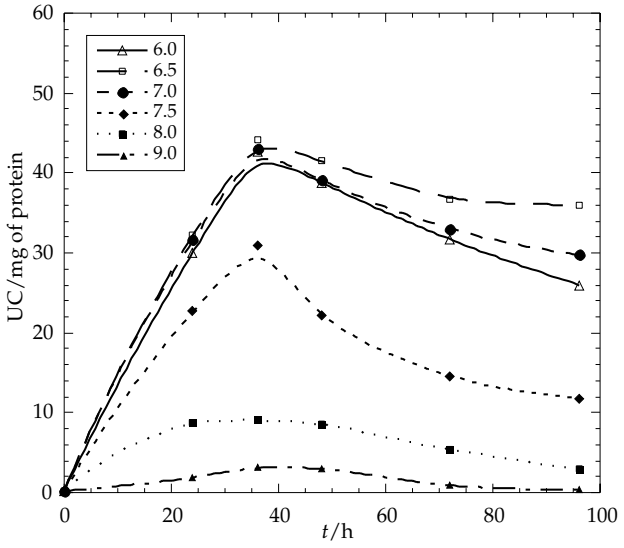


**Fig. 4.** Effect of pH on *Serratia marcescens* WF extracellular specific units (UC) of chitinolytic activity during 96 h of fermentation at 6 % of CSS and pH=6.5. The symbols indicate different pH

Effect of temperature on chitinase production by *S. marcescens* WF

When $H_0$: $\beta_j$=0 for j=7, 8, 9 *etc.*, the probability of null hypothesis was only 0.53121E-02, which means that temperature also affects enzyme production. Analyzing enzyme activity changes due to temperature, the highest chitinase activity was found at 28 °C and the strain produced smaller enzyme quantity when grown at 31, 34, and 37 °C (Fig. 5). This result is different from those obtained in other studies with *Serratia marcescens* QMB 1466 at 30 °C (*10*), and 32.5 °C (*17*).

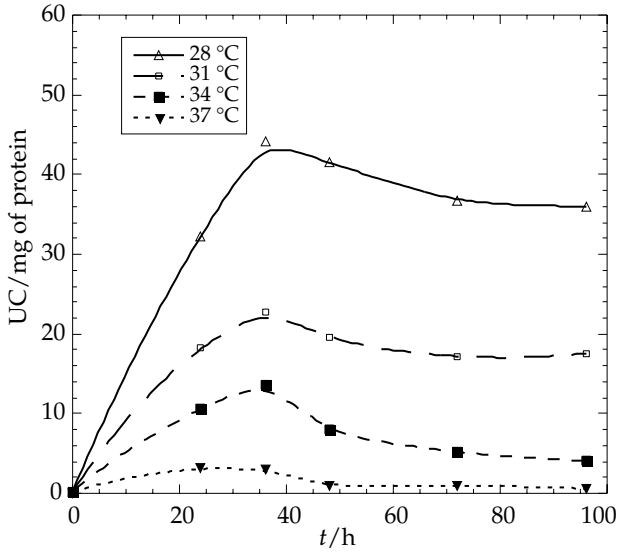


**Fig. 5.** Effect of temperature on *Serratia marcescens* WF extracellular specific units of chitinolytic activity during 96 h of fermentation at 6 % of CSS and pH=6.5. The symbols indicate different temperature

## Conclusion

In conclusion, a new strain *Serratia marcescens* WF with higher production of chitinolytic enzyme than *Serratia marcescens* QMB 1466 was found. All three factors (CSS, pH, and temperature) considered in experimental design had an effect on the chitinolytic activity induced by *Serratia marcescens* WF. This investigation has shown that crude shrimp shells at 6 % can be used for optimal high chitinase production by *Serratia marcescens* WF at 28 °C, pH=6.5, and 72 h of fermentation. This study has demonstrated that crude dry shrimp shell powder can be considered as the adequate cultivation medium for *S. marcescens* WF growth and excellent inducer for chitinase production, which may enhance the process and improve the final cost of enzyme production. The production of inexpensive chitinolytic enzymes is an important element in the utilization of shrimp shell waste that not only solves environmental problems but also decreases the production cost of microbial chitinases.

## References

1. S.L. Wang, S.H. Chio, Deproteinization of shrimp and crab shell with the protease of *Pseudomonas aeruginosa* K-187, *Enzyme Microb. Technol. 22* (1998) 629–633.
2. S. Revah-Moiseev, A. Carroad, Conversion of the enzymatic hydrolysate of shellfish waste chitin to single-cell protein, *Biotechnol. Bioeng. 28* (1981) 1067–1078.
3. K.T. Sakai, Y. Uchiyama, Y. Matahira, F. Nanjo, Immobilisation of chitinolytic enzymes and continuos production of *N*-acetylglucosamine with immobilised enzymes, *J. Ferment. Bioeng. 72* (1991) 168–172.
4. T. Takayanga, K. Ajisaka, Y. Takiguchi, K. Shimahara, Isolation and characterization of thermostable chitinase from *Bacillus licheniformis* X-7U, *Biochem. Biophys. Acta, 1078* (1991) 404–410.
5. J. Ferrer, G. Perez, Z. Marmol, E. Ramones, H. Garcia, C.F. Forster, Acid hydrolysis of shrimp shell waste and the production of single cell protein from the hydrolysate, *Bioresour. Technol. 57* (1996) 55–60.
6. P.A. Felse, T. Panda, Production of microbial chitinases, A revisit, *Bioprocess Eng. 23* (2000) 127–134.
7. S. Hirano, Chitin biotechnology applications, *Biotechnol. Annu. Rev. 2* (1996) 237–258.
8. J.K. Yang, I.L. Shih, Y.M. Tzeng, S.L. Wang, Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes, *Enzyme Microb. Technol. 26* (2000) 406–413.
9. E. Frandberg, J. Schnurer, Chitinolytic properties of *Bacillus pabuli* K1, *J. Appl. Bacteriol. 76* (1994) 361–367.
10. J. Monreal, E.T. Reese, The chitinase of *Serratia marcescens*, *Can. J. Microbiol. 15* (1969) 689–696.
11. I.G. Cosio, R.A. Fisher, P.A. Carroad, Bioconversion of shell fish chitin waste: Waste pretreatment, enzyme production, process design, and economic analysis, *J. Food Sci. 47* (1982) 901–905.

*12.* W.L. Teng, E. Khor, T.K. Tan, L.Y. Lim, S.C. Tan, Concurrent production of chitin from shrimp shells and fungi, *Carbohydr. Res. 332* (2001) 305–316.

*13.* *Manual of Methods for General Bacteriology*, Chapter 4, American Society for Microbiology, Washington DC (1984).

*14.* O.H. Lowry, N.J. Rosenbrough, A.L. Fan, R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem. 193* (1951) 265–271.

*15.* L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem. 31* (1987) 426–429.

*16.* P.A. Carroad, R.A. Tom, Bioconversion of shellfish chitin wastes: Process conception and selection of microorganisms, *J. Food Sci. 43* (1978) 1158–1161.

*17.* A.T. Green, M.G. Healy, A. Healy, Production of chitinolytic enzymes by *Serratia marcescens* QMB 1466 using various chitinous substrates, *J. Chem. Technol. Biotechnol. 80* (2005) 28–34.

*18.* J.D. Reid, D.M. Ogrydziak, Chitinase-overproducing mutant of *Serratia marcescens*, *Appl. Environ. Microbiol. 41* (1981) 664–669.

# Korištenje smrvljenih ljuštura morskih račića za proizvodnju hitinaze s pomoću soja *Serratia marcescens* WF

**Sažetak**

Od 102 ispitana soja *Serratia marcescens*, 57 sojeva imalo je hitinaznu aktivnost, a *Serratia marcescens* WF imao je najveću hitinolitsku aktivnost, pa je odabran za daljnja ispitivanja proizvodnje hitinaze na smrvljenim ljušturama morskih račića. Kinetika proizvodnje hitinaze sa sojem *Serratia marcescens* ispitana je korištenjem 10–70 g/L smrvljenih ljuštura račića uz temperaturu inkubacije od 28 do 37 °C, pH=6–9 tijekom 24–96 h. Maksimalna proizvodnja hitinaze postignuta je primjenom polinomskoga modela drugoga reda za varijable procesa: 6 % suhih smrvljenih ljuštura, pH=6,5, temperatura 28 °C tijekom fermentacije do 72 h.