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Comparative evaluation of pectolytic and proteolytic enzyme production by free and immobilized cells of some strains of the phytopathogenic *Erwinia chrysanthemi*

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Whole cells of the phytopathogenic *Erwinia chrysanthemi* strains were immobilized in k-carrageenan and grown in high-calcium *Xanthomonas campestris* medium containing sodium polypectate as carbon source. All the strains used survived immobilization into k-carrageenan beads. Immobilized *E. chrysanthemi* strains displayed higher pectolytic and proteolytic enzyme activities than free cells in liquid suspension. Carrageenan immobilization techniques could provide a system to mimic the conditions of *E. chrysanthemi* cells in the infected plant tissue. This could prompt a thorough study of the factors governing the biosynthesis of virulence factors by this bacterium. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 215–219.

Keywords: immobilized *Erwinia chrysanthemi* cells; pectolytic enzyme; proteolytic enzyme

Introduction

Erwinia chrysanthemi is a phytopathogenic bacterium that causes soft rot disease in various plants and in maize particularly [4,22]. The bacterium produces several extracellular enzymes including cellulolytic, pectolytic, and proteolytic enzymes [2]. Study of the role of these extracellular enzymes in the pathogenicity of *E. chrysanthemi* could be promoted by defining the environmental conditions that would support high production of the enzymes by the microbe [10,13,22].

Development of soft rot disease involves the entrance of bacteria into the plant through dispersal by water. The bacteria may enter the plant host through wounds or normal plant openings on the outside of the stem or leaves, such as hydathodes and stomata, and end up in intercellular spaces in the tissue of the plant stem [10,13]. When the bacteria are in the intercellular spaces of the stem tissue, they may multiply and produce several kinds of extracellular enzymes that cause maceration of the plant host tissue by degrading plant cell wall components [14,20].

The plant cell wall is a polymeric mesh consisting primarily of cellulose, hemicellulose, pectic substances, phospholipids, and proteins. The stem tissue of the plant could be described as a mesh-like structure intercalating intercellular spaces with plant cells. This suggests that the cells of the phytopathogenic *E. chrysanthemi* in the host plant tissue would behave differently from cells grown free in solution. Unfortunately, our current knowledge on extracellular enzyme production by *E. chrysanthemi* comes from studies performed using cultures grown in liquid suspensions. These conditions imposed on the microorganisms in the laboratory differ vastly from the conditions in the "natural" environment where their mobility, for instance, may be restricted by the mesh structure of the stem tissue [1].

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The structural detail of alginate bead matrix architecture [16] suggested that the immobilization technique could provide us with a unique tool to mimic the conditions of *E. chrysanthemi* in the host plant tissue. It has been stated that immobilized microorganisms produce larger quantities of enzymes than the free-living organisms growing in liquid suspension [9]. Observation of immobilized cells has indicated that these cells behave differently from cells free in solution [12]. Immobilization could affect microbial viability and/or growth [7]. Higher specific rates of substrate utilization for immobilized cells have been demonstrated [18], while conditions for optimal growth have been reported to differ from those for suspended cells [7,15].

There are many immobilization methods available. Matrix entrapment methods are, however, the methods of choice for immobilization of whole cells. Polymer matrices are generally nontoxic and the methods for their gelation are very mild [19]. Entrapment methods have many advantages for the study of extracellular enzyme production. Mesh diameters of the natural polymers used are usually too large to retain single enzyme molecules. This makes it easy for the extracellular enzymes produced by immobilized cells to cross the bead wall into the medium without being denatured. Various immobilization matrices, such as agar, alginate, pectin, polyacrylamide, and carrageenan have been used [9,23]. However, its stability in the culture medium has resulted in k-carrageenan being the material of choice for this study.

The purpose of this study was to compare pectolytic and proteolytic enzyme production by free and immobilized cells of four different strains of the phytopathogenic *E. chrysanthemi*. Immobilization of *E. chrysanthemi* was intended to simulate the conditions of this bacterium in the host plant tissue.

Materials and methods

E. chrysanthemi strains

Four strains of *E. chrysanthemi* were used in this study: *E. chrysanthemi* SR120, SR237, Ech6/2 S^+, and ECS. The strains

were isolated in South Africa and have been deposited in the collection of Prof. A. Kelman (Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin). SR in SR120 and SR237 stands for soft rot disease in maize, while 120 and 237 are numbers assigned to differentiate these strains. Ech6/2 S^+ is a streptomycin-resistant strain isolated from potato. ECS is a slimy variant, found to constantly give rise to slimy colony morphology in 24 h of growth on high-yeast agar [8]. Stock cultures were maintained on nutrient agar at 20°C and subcultured weekly.

Culture medium used for enzymatic activity studies

High-calcium *Xanthomonas campestris* (HXC) medium containing 1.0 g K_2HPO_4, 05 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4{\cdot}7H_2O$, 1.1 g $CaCl_2{\cdot}2H_2O$, 4.0 g yeast extract, 10.0 g sodium polypectate (NaPP) as carbon source, and 1 l distilled water, at pH 7.0, was used to assess pectolytic and proteolytic enzyme production by *E. chrysanthemi* strains. $CaCl_2{\cdot}2H_2O$ and $MgSO_4{\cdot}7H_2O$ were prepared and autoclaved separately. K_2HPO_4, $(NH_4)_2SO_4$, yeast extract, and NaPP were also prepared and autoclaved as a basal salt solution. The $CaCl_2{\cdot}2H_2O$ and $MgSO_4{\cdot}7H_2O$ sterile solutions were added aseptically to a cool, sterile basal salt solution, such that their addition did not dilute the overall concentration of each constituent.

Inoculum preparation

A loopful of *E. chrysanthemi* cells from the stock culture maintained on nutrient agar was inoculated into 50 ml HXC broth medium and incubated at 30°C, 180 rpm for 12 h. The OD_{600} of the culture was adjusted to 0.6 using sterile HXC medium. The inoculum was then ready for immobilization as well as for inoculation of the free cell reaction systems.

Immobilization technique

Entrapment of *E. chrysanthemi* cells within k-carrageenan beads was carried out according to an adaptation of the method of Chibata *et al* [5]. A 4% k-carrageenan solution in a saline buffer (0.85% NaCl in distilled water) was used. The solution was sterilized by autoclaving it for 15 min at 121°C. One milliliter of the 16-h culture diluted to OD_{600} 0.6 using the basal medium was mixed with 20 ml sterile k-carrageenan solution at about 40°C on a magnetic stirrer (Fried Electric, Haifa, Israel) at medium speed. The k-carrageenan and bacteria mixture was then added dropwise using a sterile 20-ml glass syringe with an 18-gauge LB needle (i.e., 1.2×40 mm) into a sterile 0.3 M KCl solution at about 40°C. Beads were immediately formed into the KCl solution entrapping *E. chrysanthemi* cells. Beads were allowed to harden in the KCl solution for about 1 h. They were then washed three times with sterile distilled water before use.

Experimental setup for pectolytic and proteolytic enzyme production studies

Two sets of reaction flasks were prepared. One set was prepared using k-carrageenan beads entrapping *E. chrysanthemi* cells, and the other one with free cells of the same strains. Both immobilized and free cells were from the same inoculum (i.e., a 16-h *E. chrysanthemi* culture). About 18 g of k-carrageenan beads entrapping *E. chrysanthemi* cells was used to inoculate 150 ml sterile HXC medium with NaPP as a carbon source contained in a 300-ml Erlenmeyer flask. The volume of the same 16-h *E. chrysanthemi* culture containing theoretically an equal density of cells as that entrapped in the beads was used to inoculate the other reaction flasks. Both sets of reaction flasks, namely immobilized and free cells, were incubated at 30°C and shaken at 180 rpm for 24 h on an orbital incubator S150 (Stuart Scientific, Redhill, Surrey, UK). Starting from time 0, then every 6 h, 10 ml of bulk medium was removed from each reaction flask to monitor pectate lyase and protease activities. The experiment was replicated four times.

Viability determination of immobilized and free *E. chrysanthemi* cells

Simultaneous with the sampling for enzyme activity, 1 g beads and 1 ml bulk medium were removed from the immobilized cell reaction system for viability and leakage determination, respectively. The carrageenan beads entrapping *E. chrysanthemi* cells were washed three times with sterile distilled water. To recover the *E. chrysanthemi* cells for viable counts, the beads were immersed in 9 ml saline buffer and dissolved by vigorous vortexing. Counts of viable cells were performed on nutrient agar medium incubated at 30°C for 24 h. Simultaneous with the immobilized cell viability determination, 1 ml of free cells in suspension was removed from the reaction flask for viable cell counts. Viable cell counts were performed under the same conditions as described for immobilized cells.

Leakage of immobilized *E. chrysanthemi* cells

Every 6 h, simultaneous with the immobilized cell viability monitoring, serial dilutions and viable counts were also performed using the bulk HXC medium to determine whether any *E. chrysanthemi* cells had leaked out of the k-carrageenan beads into the medium.

Preparation of the crude enzyme extracts

The samples for pectate lyase and protease activity (i.e., 10 ml bulk medium samples) were centrifuged at 27,200×*g* (Centrifuge Model J2-21, Beckman, Palo Alto, CA) at 4°C for 10 min. The supernatants were collected and used as crude enzymes in the pectate lyase and protease assays.

Pectate lyase assay used

The reaction was initiated by mixing 10 μl of crude enzyme solution with 990 μl of 0.4% (w/v) polygalacturonic acid in 50 mM Tris–HCl buffer, pH 8.5, containing 1 mM $CaCl_2{\cdot}2H_2O$ at 30°C. The increase in absorbancy was monitored continuously at 230 nm in a recording spectrophotometer (Spectronic 1201, Milton Roy, Rochester, NY) for 1 min. The slope of the absorbancy curve at 230 nm was converted into enzyme activity in units per milliliter. One unit of the enzyme is defined as the amount of the enzyme that catalyzed the production of 1 μmol of unsaturated uronide per minute. A molar coefficient of 4600 M^{-1} cm^{-1} was used to calculate enzyme activity [17]. A conversion factor of 10.869 was used to convert the slope to units per milliliter [6].

Protease assay used

A volume of 0.25 ml azocasein (1.5% w/v in water; Sigma Chemical, St. Louis, MO) was added to 0.25 ml of imidazole–HCl buffer (0.1 M imidazole–HCl, pH 6.2) and the mixture was maintained at 37°C for 10 min. Crude enzyme solution

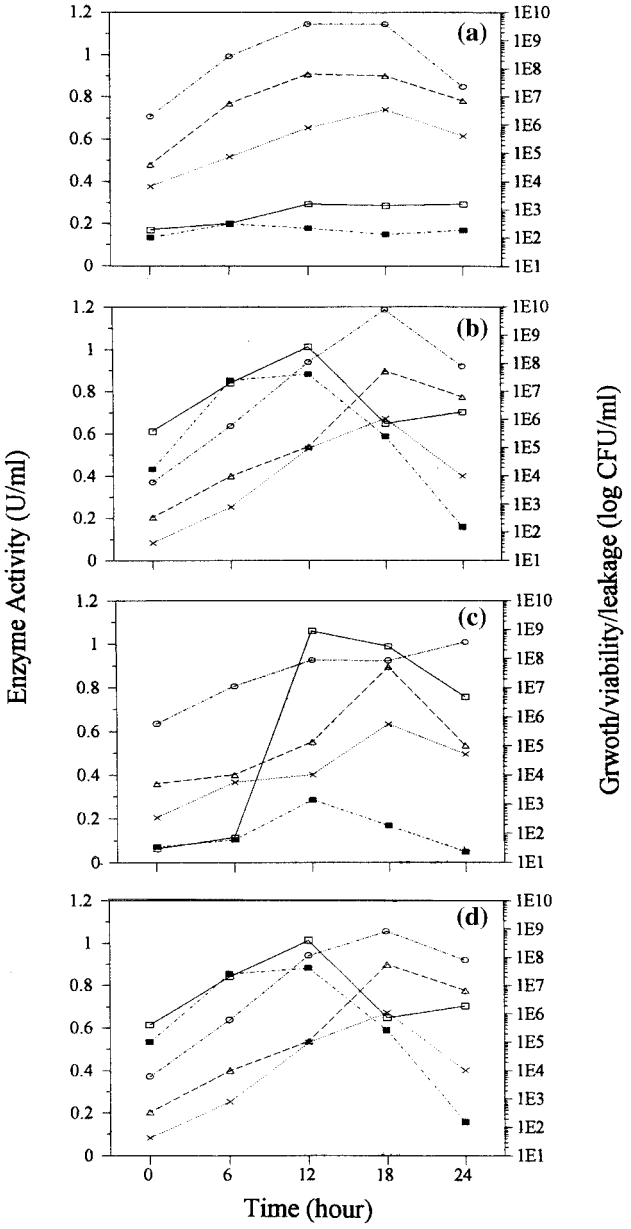

Figure 1 Pectate lyase production by free and immobilized cells and the viability of *E. chrysanthemi* ECS (a), SR120 (b), Ech6/2 S$^+$ (c), and SR237 (d) strains grown in high-calcium *X. campestris* medium containing sodium polypectate as carbon source, at 30°C, 180 rpm over 24 h. (——□——) Immobilized cells' enzyme activity. (– – △– –) Immobilized cells' viability. (···×···) Leakage. (-·-■-·-) Free cells' enzyme activity. (-···O···-) Free cells' growth.

(0.5 ml) was added to the substrate–buffer mixture, and incubated for 30 min. The reaction was then stopped by adding 0.5 ml of 1.5 M $HClO_4$. Assay blanks were prepared by adding 0.5 ml of 1.5 M $HClO_4$ to substrate–buffer mixture before adding enzyme solution. The reaction mixture was centrifuged at 9000 rpm for 2 min. Then 0.5 ml of the supernatant was mixed with 0.5 ml of 1 M NaOH and absorbancy was read at 440 nm. One unit of the enzyme is defined as the amount that catalyzed the hydrolysis of 1 μg of azocasein per minute at 37°C. An extinction coefficient of 35 for azocasein solution (1% w/v in 0.1 NaOH) was used to calculate enzyme activity in units per milliliter. A conversion factor of 29.411 was used to convert nett A_{440} to units per milliliter. Nett A_{440} was obtained by subtracting the absorbancy at 440 nm of the blank tube from that of the assay tube [3].

Results

The results for enzyme production by free and immobilized cells of the different strains of the phytopathogenic *E. chrysanthemi* as well as the viability of these microbial cells in the two experimental systems used in this study are summarized in Figures 1 and 2 for pectate lyase and protease, respectively.

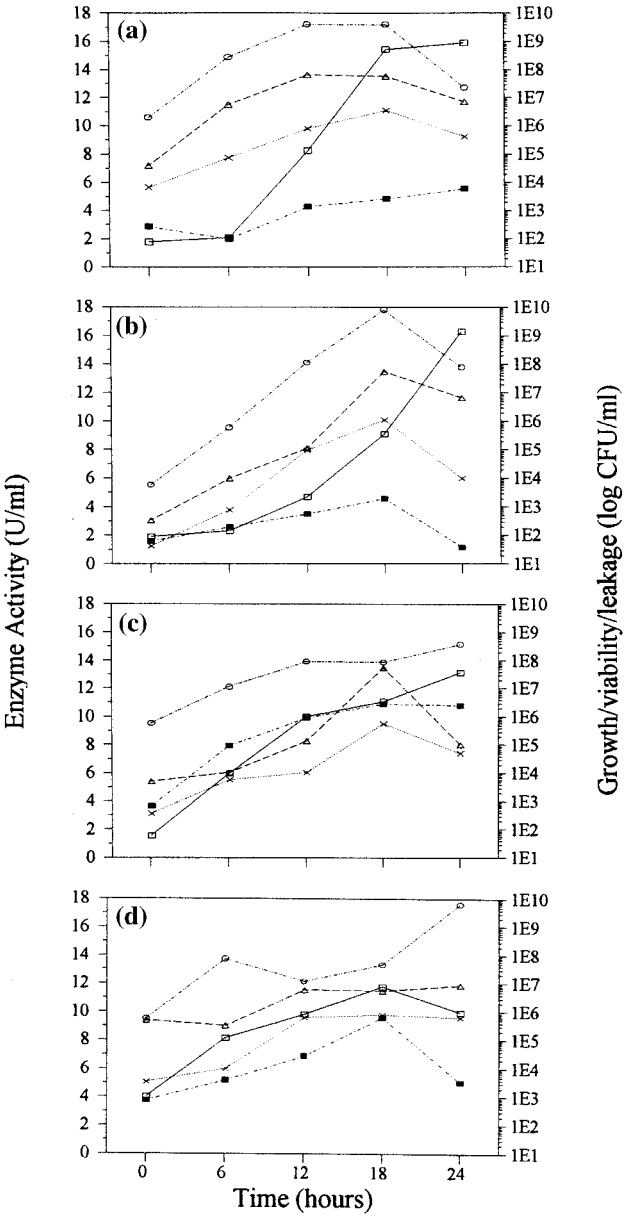

Figure 2 Protease production by free and immobilized cells and the viability of *E. chrysanthemi* ECS (a), SR120 (b), Ech6/2 S$^+$ (c), and SR237 (d) strains grown in high-calcium *X. campestris* medium containing sodium polypectate as carbon source, at 30°C, 180 rpm over 24 h. (——□——) Immobilized cells' enzyme activity. (– – △– –) Immobilized cells' viability. (···×···) Leakage. (-·-■-·-) Free cells' enzyme activity. (-···O···-) Free cells' growth.

Discussion

All *E. chrysanthemi* strains used in this study survived immobilization in k-carrageenan beads (Figures 1 and 2). However, the microbial cells grew better when free in liquid suspension than inside the carrageenan beads. This could be explained by such factors as oxygen limitation and nutrient availability [19,21]. Both immobilized and free cells displayed a typical growth curve with the stationary phase being reached after 12 h of incubation.

Some *E. chrysanthemi* cells in the immobilized experimental system escaped from the carrageenan beads into the bulk medium. Leakage might be due to the relatively low concentration of the carrageenan used (4%). Such leakage might be minimized by increasing the concentration of carrageenan and/or using additional coating techniques. Leaked cells also displayed a typical growth pattern indicating that they were viable. Similar findings have been reported by Muyima and Cloete [15] on the survival of leaked *Acinetobacter* cells out of alginate beads.

Overall, the total numbers of culturable cells in the free cell experimental systems were always higher than the respective combined numbers of cells recovered from the carrageenan beads plus those that leaked out of the beads into the medium, that is, the total numbers of culturable cells in the immobilized systems. For pectate lyase production, immobilized *E. chrysanthemi* cells showed higher enzyme activity than free cells in liquid medium. With the exception of the free ECS cells, pectate lyase activity for both the free and immobilized cells increased with time starting from time 0 and reached a peak after 12 h of incubation (Figure 1). This maximum enzyme activity at around 12 h corresponded with the onset of the stationary phase. This observation confirmed the report by other investigators that *E. chrysanthemi* start producing more enzyme when they have stopped growing actively [10,11,13]. During the first 6 h of incubation, immobilized and free cells showed similar pectate lyase activity. The low secretion of the enzyme observed at this initial stage could also be explained by the fact that the cells were actively growing. According to several investigators, when an *Erwinia* strain attacks a plant, the microbial cells first spend much of their energy in multiplying until they reach a satisfactory cell density before they stop growing and start producing the enzyme [10,11,13]. Comparing the performance of the four strains, *E. chrysanthemi* Ech6/2 S^+ showed the highest pectate lyase activity.

For protease production, both free and immobilized cells of *E. chrysanthemi* strains displayed similar enzyme activity from time 0 to 6 h of incubation. The peak for protease production by *E. chrysanthemi* varied with the strain and the environmental growth conditions (i.e., free or immobilized). Immobilized cells of strains SR120, Ech6/2 S^+, and ECS did not reach a peak for protease production during the 24-h study period, while strain SR237 reached its peak after 18 h of incubation (Figure 2). For free cells, strains SR120, SR237, and Ech6/2 S^+ reached their maximum enzyme production at 18 h, while strain ECS did not reach a peak to protease production during the study period. Immobilized cells constantly produced the enzyme for a much longer period before reaching the peak than their free cell counterparts. This behaviour could be of economic value when considering large-scale production of protease. Immobilized cells produced more protease than their free cell counterparts.

The findings in this study showed that immobilized *E. chrysanthemi* cells produced more pectate lyase and protease than free cells in suspension. In general, the peak of pectate lyase production was reached earlier, that is, after 12 h of incubation, than the peak for protease production. Since the situation of this phytopathogenic bacterium inside the tissue of the infected plant is thought to be quite similar to the situation of the cells in the carrageenan beads, this could therefore indicate that in the infected plant tissue, *E. chrysanthemi* would behave in the same manner. The indications that *E. chrysanthemi* would produce more enzyme in the plant tissue than in free culture could bring new insights to the study of the pathogenicity of this bacterium.

Although the culturable cell density in the free cell experimental system was higher than in the immobilized cell system, higher enzyme activity was recorded in the immobilized systems. This indicated that high enzyme activity in this case was a result of the immobilization rather than the density of cells in the system. The results for both pectate lyase and protease production by the *E. chrysanthemi* strains used were in agreement with the report by Hartmeier [9], which states that immobilized microbial cells show higher enzyme activity than microbial cells growing free in liquid suspension. In general, the enzyme activity varied with the strain and the conditions under which the cells were grown, that is, free or immobilized.

The results indicate that *E. chrysanthemi* cells survived entrapment in k-carrageenan beads. Except for free ECS cells, pectate lyase activity for both free and immobilized cells increased with time and reached its peak after 12 h of incubation, which corresponded with the onset of the stationary growth phase. The peak for protease activity varied with the strain as well as the growth conditions of the cells. In general, immobilized cells did not reach a peak for protease production within the 24-h study period, while free cells reached their peak after 18 h of incubation. Overall, immobilized *E. chrysanthemi* cells displayed higher pectate lyase and protease activity than free cells. Entrapment of *E. chrysanthemi* cells in k-carrageenan could provide a system to mimic the conditions of this pathogen in the infected plant tissue. This could prompt a thorough study of the factors governing biosynthesis of virulence factors for a better understanding of the pathogenicity of this bacterium.

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