

TECHNOLOGICAL APPLICATION OF AN EXTRACELLULAR CELL LYTIC ENZYME IN XANTHAN GUM CLARIFICATION

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Submitted: October 05, 2003; Returned to authors for corrections: September 12, 2004; Approved: March 28, 2005

ABSTRACT

An extracellular cell lytic enzyme from *Pseudomonas* sp. was active on heat killed cells of *Xanthomonas campestris*. The lytic activity caused enzymatic digestion of *X.campestris* xanthan gum. Digestion was effective for highly viscous native xanthan 2.0% (w/v) and 2.5% (w/v) commercial Sigma xanthan. Scanning electron microscopy and SDS-PAGE observations confirmed the cell lytic action on *X.campestris* cells.

Key words: Xanthan, *Xanthomonas campestris*, cell lytic enzyme

INTRODUCTION

Xanthan gum secreted from *Xanthomonas campestris* is viscous or jelly in aqueous state. The high viscosity of xanthan broth is a major problem during removal of bacterial cell biomass from the fermented broth. Centrifugation and heat treatment are either ineffective at high viscosity or lead to degradation of xanthan during isolation of cell free gum (4,11). The enzymatic lysis of bacterial cells in microbial broth is preferred for removal of cells, as structure of the gum is preserved and effective at high viscosity. The present report describes technological application of cell lytic activity against *X. campestris* in xanthan gum isolation.

MATERIALS AND METHODS

Microorganisms

Microbial strains of *Xanthomonas campestris* and *Pseudomonas* spp. were local CFTRI isolates, maintained at 30°C for 48 h on sucrose agar medium containing (g/L): sucrose 20, Na₂HPO₄ 3, KH₂PO₄ 2, MgSO₄.7H₂O 1, tryptone 5, yeast extract 1 and 2% (w/v) agar pH 7.0.

Cell lytic enzyme production

The growth medium for *Pseudomonas* spp. had the following composition (g/L): sucrose 30, Na₂HPO₄ 5, KH₂PO₄ 3, K₂SO₄ 1,

NaCl 1, MgSO₄.7H₂O 0.2, CaCl₂.2H₂O 0.02, FeSO₄.2H₂O 0.002, tryptone 11, yeast extract 9, pH 7.0. The organism was grown at pH 7.0 in 500 mL Erlenmeyer flasks containing 100 mL sterilized growth medium, inoculated with 5% (v/v) overnight culture obtained at 28±2°C on a 250 rpm rotary shaker for 18 h. The inoculated medium was incubated at 37°C for 24 h, on a 250 rpm rotary shaker. Cell free supernatant was obtained by centrifugation at 8000 rpm for 20 min and used as crude enzyme.

Production of Xanthan gum

Xanthomonas campestris was cultivated in 500 mL Erlenmeyer flasks containing 100 ml sterilized medium composed of (g/L): sucrose 45, peptone 4, Na₂HPO₄ 5, KH₂PO₄ 3, MgSO₄.7H₂O 1, pH 7.0, and inoculated with 5% v/v overnight culture and incubated at 28±2°C on a 250 rpm rotary shaker for 72h. The xanthan broth was pasteurized at 70°C for 30 min. and precipitated with 2.5% (v/v) isopropanol for xanthan isolation. The precipitate was dried in an oven at 60°C. The precipitate was dissolved in distilled water to various concentrations, referred to as hydrated Xanthan solution (CFTRI) in experiments.

Alkaline protease assay and cell lytic activity

Caseinolytic activity was determined at pH9/45°C in 10 mM Tris-HCl buffer (9). One unit of protease activity was defined as

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the amount of enzyme that released 1 µg tyrosine per min in the assay conditions.

Cell lytic activity on *X. campestris* cell biomass in hydrated xanthan solution was observed by decline of absorbance at 540 nm. The proteolytic activity associated with cell lytic activity was assayed by pre-incubation of crude enzyme with 5 mM PMSF for 2 h at 27°C. The 1% (w/v) hydrated xanthan solution diluted 1:1 (v/v) with distilled water was mixed with 10% (v/v) with/without crude enzyme pre-incubated PMSF and incubated at 50°C/pH8.0 for 3 h. Xanthan solution incubated without the enzyme was used as control.

Lipase activity

Lipolytic activity was determined at pH 7.0/37°C in 0.1M phosphate buffer (5). One unit of lipase activity was defined as the amount of enzyme that released µmol fatty acid per min in the assay conditions.

β1-3 Glucanase activity

β1-3 Glucanase was determined at pH 7.0/40°C in 0.1M citrate-phosphate buffer (2). A unit of activity was defined as the amount of enzyme that released 1 µg of glucose in 20 min in the assay conditions.

Filter paper activity

Filter paper activity was determined at pH 4.8/50°C in 0.1M citrate phosphate buffer (6). One unit of activity was defined as the amount of enzyme that released 1 µg of glucose in 60 min.

Protein determination

Protein was assayed using coomassie blue with BSA as the standard (12).

Optimization of cell lytic activity

The different dilutions of pasteurized xanthan broth were mixed with 10% (v/v) crude enzyme and incubated at pH 6.0/50°C for 6h in stationary condition, to determine the minimum dilution required in highly viscous xanthan gum for effective cell lytic activity. The decline in absorbance was recorded at 540 nm (1). Hydrated xanthan (0.25% w/v, CFTRI) 2 mL was mixed with 1 mL crude enzyme at pH 6.0 and incubated at various temperatures to optimize cell lytic activity. Lytic activity was optimized at 50°C and carried out at final pH of the enzyme-xanthan mixture i.e. pH 6.0.

Application of cell lytic activity in xanthan broth and crude hydrated xanthan solution

Xanthan broth and distilled water in 1:1 (v/v) ratio were mixed with 5% (v/v) crude enzyme at pH 6.5 and incubated on 100rpm shaker incubator for 18 h at 50°C. Commercial xanthan (Sigma, 2% and 2.5% w/v) with biomass was hydrated and mixed with 10% (v/v) crude enzyme at pH 5.5 ± 0.5 for incubation on

100 rpm shaker incubator for 4.5 h at 50°C. Xanthan was diluted with distilled water to a minimum of 1:3 (v/v) before measurement of absorbance, as high viscosity of xanthan broth interfered in the filling of the cuvette. The decline in absorbance was recorded at 540 nm. These experiments were performed at the final pH of the enzyme-xanthan mixture and pH adjustment was not performed.

Xanthan hydrated solution (2% w/v, CFTRI, pH8.0) mixed with 10% (v/v) crude enzyme was incubated at 50°C for 13.5 h at stationary condition. The viscosity of xanthan solution was recorded by Brookfield viscometer, spindle 1 (100, 50, 20 and 10 rpm) during the process of incubation. Xanthan solution was diluted with distilled water by 1:1 (v/v) before absorbance reading, as high viscosity of xanthan solution interfered in the filling of the cuvette for absorbance reading. Cell lytic activity was measured as decline in absorbance at 540 nm, due to enzymatic cell digestion of *Xanthomonas campestris* present in crude xanthan hydrated solution.

Detection of cell lysis by Scanning Electron Microscopy (SEM) and SDS-PAGE

The viscous fermented xanthan broth was diluted 1:5 (v/v) with distilled water and centrifuged at 10,000 rpm to pellet *X. campestris* cells. Harvested cells were purified by exhaustive dialysis with de-ionized water and lyophilized for storage at 4°C. Mixture of 2 ml (0.6 mg/mL) lyophilized cell suspension and lyophilized enzyme concentrate (190U caseinolytic) was incubated for time intervals from 30-90 min at 50°C. Treated cells were washed with 10 mM Tris-HCl buffer, pH8.0 and fixed in 2% (v/v) glutaraldehyde. Cell suspension was washed with 10 mM Tris-HCl buffer, pH8.0 and dehydrated by an acetone series. Cells were mounted on Al planchets coated with gold-palladium in a Polaron sputter coater and observed in SEM (13).

SDS-soluble membrane proteins were extracted and visualized on SDS-PAGE (10).

Cell lysis was observed on SDS-PAGE as lysis of total SDS-soluble membrane proteins. The enzymatically treated and control cells were washed with 10 mM Tris-HCl buffer pH 8.0, lyophilized, mixed with 2% (w/v) SDS in gel loading buffer and heated at 100°C for 5 min.

RESULTS AND DISCUSSION

Cell lytic enzyme and Xanthan gum production

Extracellular cell lytic enzyme from *Pseudomonas* sp. lysed cells of *X. campestris* in fermented xanthan broth or hydrated xanthan. The crude lytic enzyme was composed of protease (3200 U/mg), lipase (161 U/mg), β-1-3-glucanase (120 U/mg), carboxymethyl cellulase activity (72 U/mg). The concentration of xanthan in 72 h fermented broth was 1.5%-2.1% (w/v) and cell biomass was 0.2-0.35% (w/v). The viscosity of the fermented broth observed at spindle 1, 20 rpm was 2220- 2540 cps.

Enzymatic clarification of xanthan

Maximum decline of absorbance of 76.7% and minimum 37.5% was observed in 1:6 and 1:1 dilutions of xanthan broth at 3h, respectively. The isolated lytic enzyme was active in fermented broth consisting of spent medium and cells (Fig. 1).

Cell lytic activity in xanthan broth diluted 1:1(v/v) recorded decline of 68.3% absorbance at 18 h (Fig. 2). Clarification was effective in highly viscous 2% and 2.5% (w/v) commercial sigma xanthan as 75% and 71.5% decline in absorbance were observed respectively (Fig. 3). The present report shows that optimization of pH conditions was not a pre-requisite for enzymatic clarification of xanthan. Murofushi *et al.* (7) reported clarification of xanthan by an alkaline protease and lysozyme at pH 7.5-8.5, which is optimum for enzymatic action. Ernest *et al.* (3) reported

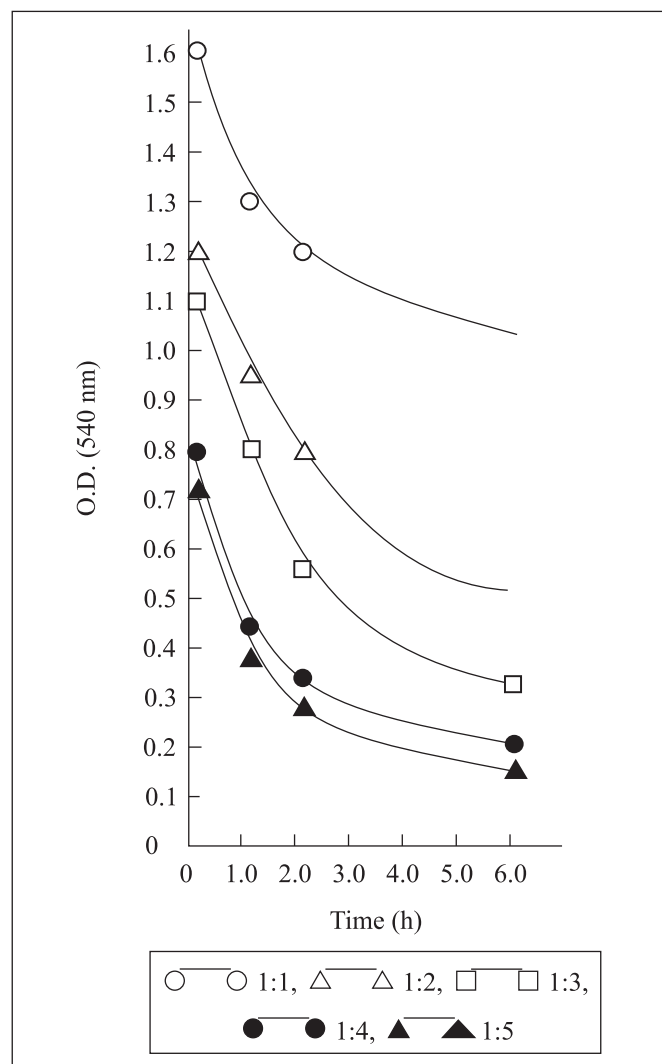


Figure 1. Clarification of pasteurized xanthan broth at different dilutions by *Pseudomonas sp.* cell lytic enzyme.

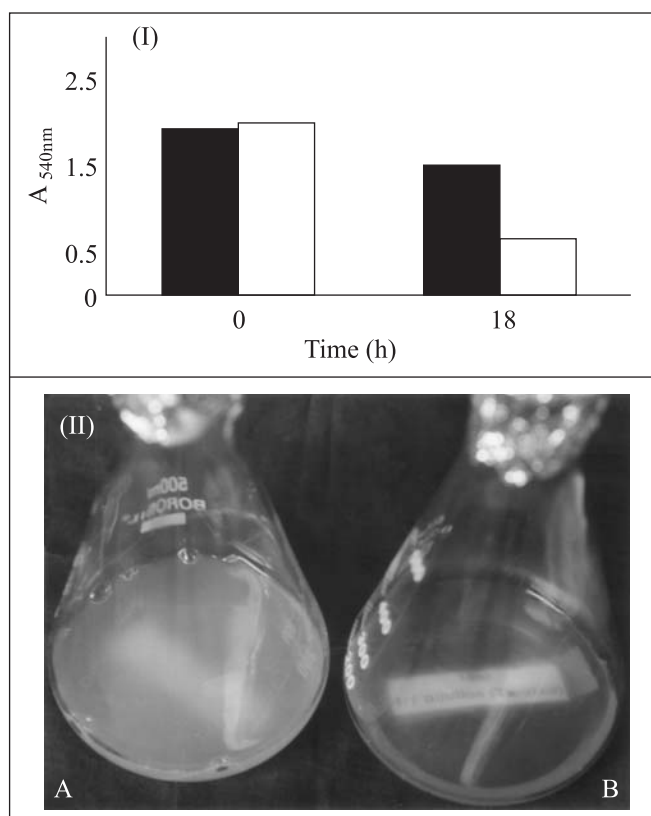


Figure 2 (I). Enzymatic clarification of xanthan broth.

■ Control; Xanthan broth and distilled water in 1:1(v/v) ratio were mixed without crude enzyme at pH 6.5 and incubated on 100 rpm shaker incubator for 18 h at 50°C. □ Test; Xanthan broth and distilled water in 1:1 (v/v) ratio was mixed with 5% v/v crude enzyme at pH 6.5 and incubated on 100 rpm shaker incubator for 18 h at 50°C. (II) (A) Control, Xanthan broth: water (1:1 v/v) composed of spent medium with cells, incubated for 18 h/50°C. (B) Test, Xanthan broth: water (1:1 v/v) incubated with crude cell lytic enzyme for 18 h/50°C.

enzymatic clarification of xanthan by proteases from *Pellicularia sasaki* or *P. filamentosa* in the range of pH 2-10 or 3.4-8 for optimum enzymatic action. An US Patent (8) described a method in which sodium hydroxide was added to the xanthan gum at low concentrations to adjust pH above 11 to effect clarification. These reports required pH adjustment as a pre-requisite for enzymatic clarification of xanthan, however the enzyme we isolated did not require pH optimization. Hence the scale up operation for the isolated cell lytic enzyme in xanthan clarification would be simplified.

Culture filtrate used as a source of cell lytic activity had no effect on the viscosity of xanthan broth. Xanthan solution (2% w/v, CFTRI) on clarification at the optimal condition (pH 8.0/ 50°C) of the cell lytic enzyme recorded 64.5% decline in

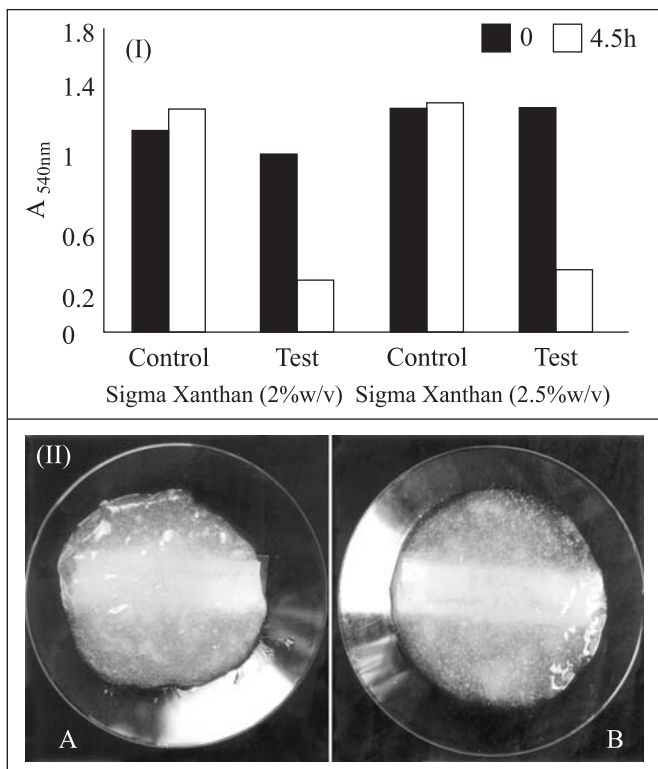


Figure 3. (I) Enzymatic clarification of Sigma Xanthan.

■ Control; Commercial xanthan (Sigma, 2% and 2.5% w/v) with biomass was hydrated and incubated at 50°C/ pH 5.5 ± 0.5 on 100 rpm shaker incubator for 4.5 h. □ Test; Commercial xanthan (Sigma, 2% and 2.5% w/v) with biomass was hydrated and incubated with 10% (v/v) crude enzyme at 50°C/ pH 5.5 ± 0.5 on 100rpm shaker incubator for 4.5 h. (II) Sigma xanthan (2.5% v/v) clarified with crude cell lytic enzyme. (A) Control; Sigma xanthan (2.5% w/v) with biomass was hydrated and incubated at 50°C/ pH 5.5 ± 0.5 on 100 rpm shaker incubator for 4.5 h. (B) Test; Sigma xanthan (2.5% w/v) with biomass was hydrated and incubated with 10% (v/v) crude enzyme at 50°C/ pH 5.5 ± 0.5 on 100 rpm shaker incubator for 4.5 h.

absorbance (Fig. 4). Viscosity of broth at 0h was 3040 cps and after enzymatic clarification 2960 cps, which indicated absence of xanthan gum degradation (Table 1). Xanthan (2% w/v) is gel like in appearance and its clarification at high viscosity has a potential technological application.

Role of alkaline protease in enzymatic clarification

Culture filtrate was composed of alkaline protease, lipase, β1-3 glucanase and carboxy methyl cellulase. The PMSF inhibited 86% protease activity in the crude enzyme and its subsequent cell lytic activity recorded 18% decline in absorbance at 540 nm in hydrated xanthan solution. Lytic activity

in the crude enzyme recorded 68% decline of absorbance. Hence alkaline protease present in the crude lytic enzyme had a major role in cell lysis. The other enzymes might have contributed to cell lysis in crude xanthan solutions (Fig. 5).

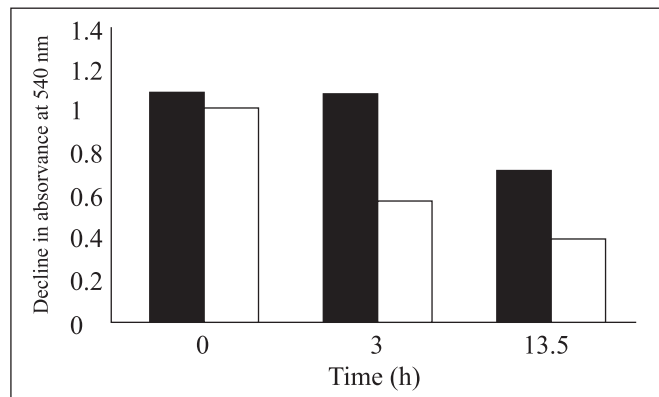


Figure 4. Enzymatic clarification of the reconstituted xanthan (CFTRI) 2% w/v at optimal enzymatic conditions. ■ Control; Xanthan hydrated solution (2% w/v, CFTRI, pH8.0) was incubated at 50°C for 13.5 h at stationary condition. □ Test; Xanthan hydrated solution (2% w/v, CFTRI, pH8.0) was incubated with 10% (v/v) crude enzyme at 50°C for 13.5 h at stationary condition.

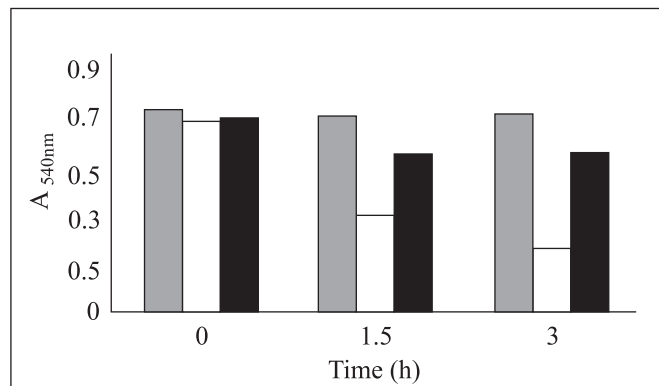


Figure 5. Cell lytic activity with inhibited proteolytic activity of alkaline protease. ■ Control; 1% (w/v) hydrated xanthan solution diluted 1:1 (v/v) with distilled water was mixed incubated at 50°C/pH8.0 for 3 h. □ Test; 1% (w/v) hydrated xanthan solution diluted 1:1 (v/v) with distilled water was incubated with 10% (v/v) crude enzyme at 50°C/pH8.0 for 3 h. ■ Proteolytic Inhibition; 1% (w/v) hydrated xanthan solution diluted 1:1 (v/v) with distilled water was incubated with 10% (v/v) crude enzyme (pre-incubated with 5mM PMSF for 2 h at 27°C) at 50°C/pH8.0 for 3 h.

Table 1. Viscosity measurement by Brook Field viscometer at Spindle1(100, 50, 20,10 rpm) at 50°C, during enzymic clarification 2%(w/v) xanthan (CFTRI), pH 8.0

Spindle No.1 (rpm)	0h		13.5h	
	Control	Test	Control	Test
100	570	512	492	516
50	844	816	792	822
20	1508	1520	1420	1500
10	2320	2360	2112	2200

SEM and SDS-PAGE of enzymatic cell lysis

Enzymatic digestion of *X. campestris* cells initiated from loss of cell shape to aggregated debris as recorded in scanning electron microscopy. Enzymatic action on the cell was generalized action on the outer cell wall, as any localized action was absent. Cell debris were observed after 90 min and decline of 69% in absorbance was recorded (Fig. 6). The cell debris contributed to absorbance and as a result 100% decline in absorbance or total removal of cells during clarification was difficult to achieve.

The enzymatic action on cells was confirmed by the lysis of total SDS soluble membrane proteins. The decline in absorbance

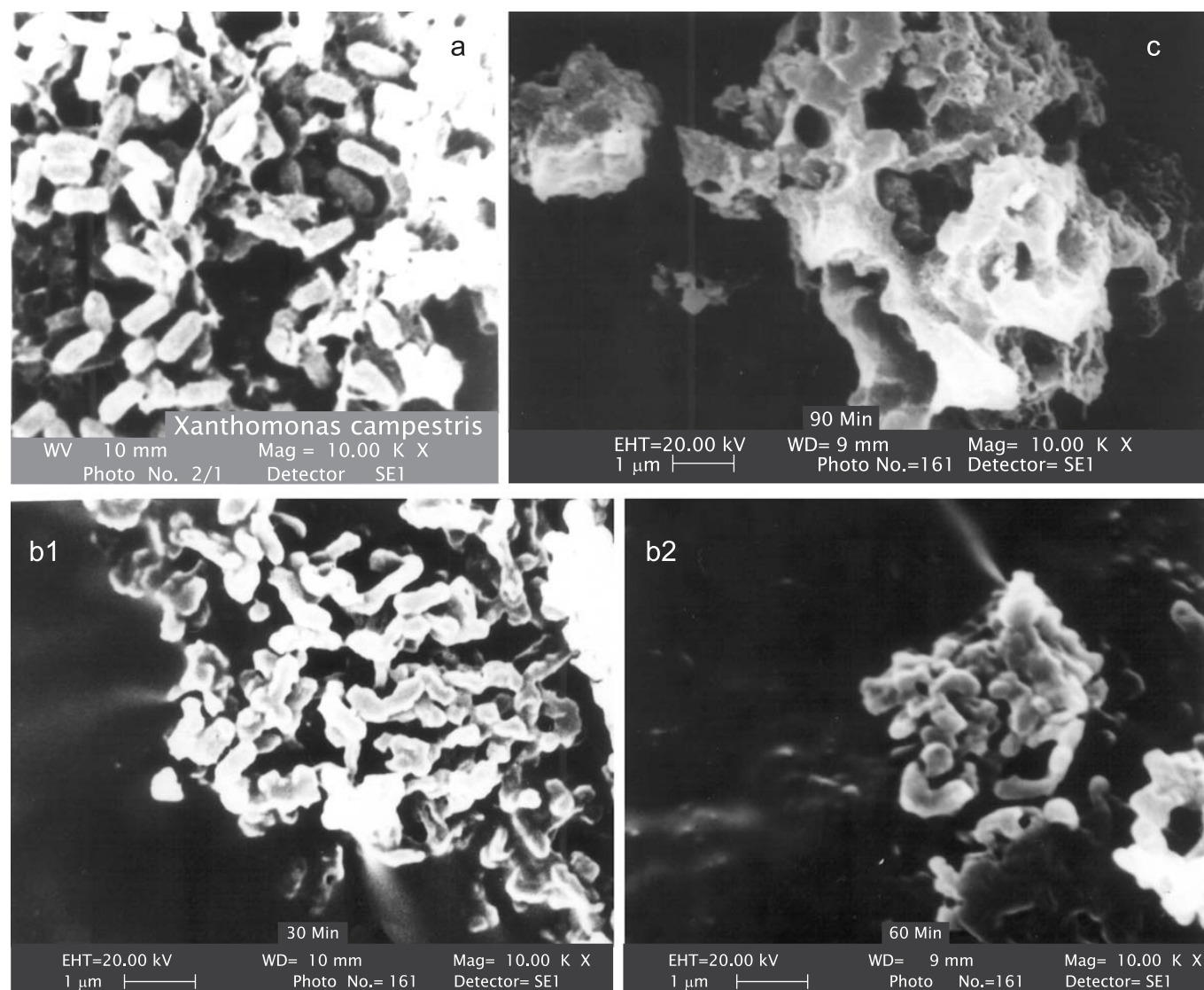


Figure 6. Scanning electron micrograph of *Xanthomonas campestris* treated with crude cell lytic enzyme for 30, 60 and 90 min. a, *Xanthomonas campestris* cells; b1 and b2, intermediate stages of cell lysis at 30 and 60 min; c, cell debris at 90 min.

recorded earlier and lysis of SDS-soluble membrane proteins could be correlated as SDS-soluble membrane proteins have a role in maintenance of cell integrity (Fig. 7).

We conclude that an extracellular enzyme isolated from *Pseudomonas* sp. was active on *Xanthomonas campestris* and could be used for *X. campestris* cell removal from xanthan broth in the recovery step of gum production.

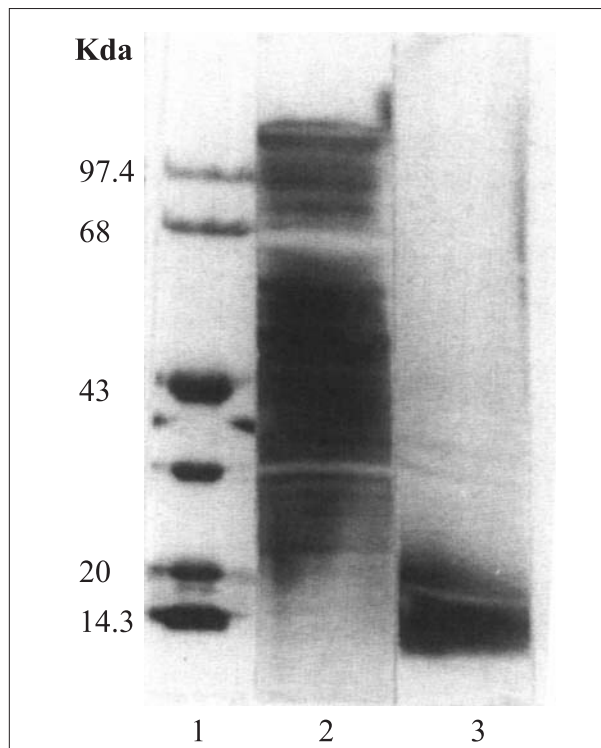


Figure 7. Enzymic action on SDS-soluble membrane proteins of *Xanthomonas campestris*. Lane1: Molecular mass standard corresponds to phosphorylase (97.4 KDa); Bovine serum albumin (68 KDa); Ovalbumin (43KDa); Carbonic anhydrase (29KDa); Soyabean Trypsin inhibitor (20 KDa); Lysozyme (14.3KDa); Lane 2; control; Lane 3; Test.

ACKNOWLEDGEMENTS

Shastry, S. thanks the Council of Scientific and Industrial Research, New Delhi, for the research fellowship.

RESUMO

Aplicação tecnológica de uma enzima celulolítica para clarificação de goma xantana

Uma enzima extracelular celulolítica produzida por *Pseudomonas* sp. foi ativa sobre células de *Xanthomonas campestris* mortas pelo calor. A atividade lítica causou a digestão enzimática de goma xantana de *X. campestris*. A digestão foi eficiente tanto para xantana nativa altamente viscosa (2,0% w/v) como para xantana comercial Sigma (2,5% w/v). Observações por microscopia eletrônica de varredura demonstraram a ação celulolítica sobre células de *X. campestris*.

Palavras-chave: xantana, *Xanthomonas campestris*, enzima celulolítica

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