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Effect of Cellulase Pretreatments on Red Algae Agar Extractability

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SUMMARY

Pretreatment of Gracilaria lemanaeformis *with cellulases before extraction enhanced agar gel strength and yields when the algae was not ground. The algae macrostructure remained unchanged, facilitating subsequent filtration operations. Microphotographs of the algae showed that cellulases produced microcracks in the external wall of the algae thallus, and collapsed the internal cells walls. These structural changes apparently increased agar yield and gel strength by facilitating diffusion of the gelling polysaccharides.*

INTRODUCTION

Agar is a gelling hydrocolloid of commercial value, present in the cell walls of *Rhodophycae*. It consists of several polymers based on the repeating unit agarobiose, a disaccharide composed of 3-linked β-D-galactopyranose and 4-linked α-L-galactopyranose (Chapman and Chapman, 1980). The D-galactose units are partially substituted with either *o*-methyl ether, pyruvic acid ketal or sulfate groups (Yaphe, 1984). Studies have shown that agar is a mixture of polysaccharides between three extreme structures, namely neutral agarose, pyruvated agarose and galactan sulfate (Duckworth and Yaphe, 1971).

Though many countries produce large quantities of algae, few reports have been published on microstructural modifications of the algae and the extractability of agar. In general, commercial processes use pretreatments (presoaking, grinding, alkali and/or acid pretreatments and others) that modify the microstructure of the algae and chemical properties of the agar, and thereby enhance agar yields (Okazaki, 1971).

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However, it is not clear what function these pretreatments have in increasing the yield and/or the quality of the agar. It is important that the pretreatment must be sufficiently mild to avoid depolymerization of the agarophytes, and to preserve the algae macrostructure for subsequent filtration operations. If not, large amounts of filter aid must be used, increasing costs and operational difficulties. The preprocess should also help in removing sulfate groups from the agaropectin type molecules, as these polysaccharides are soluble in cold water and could be lost in the initial washing steps (Yaphe, 1984).

Experimental evidence suggests that native agar is present in close association with other cell wall polysaccharides such as cellulose (Matsushashi, 1977). Painter (1983) reported that cellulose type II (regenerated cellulose) was identified by X-ray diffraction in the cell wall of red algae. In addition, algae photomicrographs indicate that higher concentrations of agar occur in the external cell layers (San Martin and Aguilera, 1986). Thus, experiments were performed to test whether pretreatment with cellulase enzymes could disrupt cell walls and also free the agar from them, enhancing extraction yields due to higher diffusional transport rates. This pretreatment should be mild enough to preserve the ease of separation between extract and residue.

MATERIALS AND METHODS

Materials

Air dried *Gracilaria lemanaeformis* was supplied by Chile's largest exporter and agar manufacturer (ALGAMAR, Chile). Its average moisture was 12·5% (wet basis). Commercial cellulase, from *Thrichoderma reesei* (Celluclast), was obtained from Novo Industries (Copenhagen, Denmark).

Cellulase assays

Overall enzyme activity was measured by the filter paper activity test (Mandels *et al.*, 1976); β-glucosidase activity was determined as reported by San Martin *et al.* (1986). Cellulase preparations were supplemented with cellobiase (Novozym 188, Novo Industries), to obtain a final β-glucosidase to filter paper activity ratio of 1:1. This activity ratio reduces cellulase inhibition due to accumulation of cellobiose (Chahal *et al.*, 1981). Both filter paper and β-glucosidase activities

are reported in terms of International Units (1 IU = 1 micromol of glucose produced per min-ml). Cellulase to substrate ratio is expressed as filter paper International Units per gram of air dried algae (FP IU g^{-1}).

Agar properties

Extraction yields were expressed as the weight of agar at 18% moisture, divided by the weight of the natural algae (12·5% H_2O). Moisture was determined using AOAC standard method No. 31.005 (AOAC 1970).

Gel strength was determined using a Kiya Sefakusho Gelometer (Japan); gels were prepared by dissolving 1·5 wt% agar (4 wt% H_2O) by boiling in water for 15 min. After allowing the gel to cool, its surface was covered with a polyethylene film of 0·2 mm thickness and conditioned in a water bath at 20°C for 2 h. The gel strength was measured in a PVC container of $9 \times 12{\cdot}5$ cm^2 section and 3·5 cm depth, and was defined as the weight that produced the rupture of the gel with a 1 cm^2 plunger after 20 s.

Experimental procedure

Preliminary experiments determined the effect of enzyme loading and reaction time on the hydrolysis of cellulose. For this purpose, natural algae were hydrolyzed at 6% concentration in 250 ml flasks. The pH was adjusted to 5·0 with acetate buffer and the temperature was kept constant at 45°C in a controlled temperature bath. Enzyme loadings were 2·5, 10, 20 FP IU g^{-1}. Reducing sugars and glucose were measured at 1, 2 and 4 h, using 3,5-dinitrosalicylic acid (Miller, 1959), and glucose-oxidase-peroxidase tests (Merckotest-3393, Merck, West Germany), respectively. A control flask, without cellulase, was run to measure solubilization of free sugars.

Following this preliminary screening, the algae were extracted using the procedure shown in Fig. 1. NaOH pretreatment is recommended for *Gracilaria* species to enhance agar extraction yields and gelling strength by removing sulfate groups (Tagawa and Kojima, 1971). Cellulase pretreatments were performed after the alkali treatment, at 3 FP IU g^{-1} for 1 h, at pH 5 and 45°C. This enzyme load resulted in higher yields, as determined in preliminary experiments. The effect of grinding the algae prior to extraction was also investigated. All experiments were performed in duplicate.

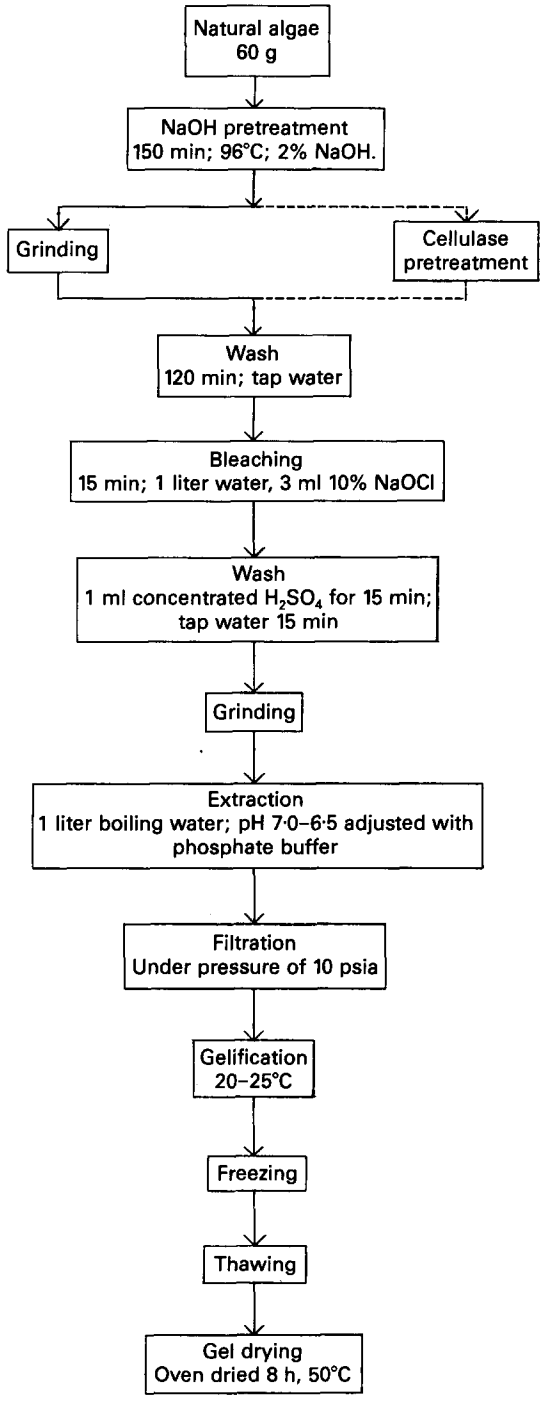

Fig. 1. Basic and modified agar extraction processes.

Microscopy

Scanning electron microscopy (SEM) was performed in JEOL, JMS-25 SII Microscope at 25 Kv. Algae sections were previously fixed in FAA (formaldehyde, acetic acid, alcohol) for a week, dehydrated in acetone, and finally critical-point dried. These samples were mounted in stubs with graphite and covered with a 100 Å gold-palladium layer.

Light microscopy (LM) was performed in a Nikon HFX microscope (Japan), and pictures were taken with a Nikon FX-35A camera. Samples were fixed as for SEM, dehydrated in butanol for three days and embedded with Paraplast (Sherwood Medical Ind., St. Louis, Missouri) in an oven at 60°C for a week. Sections between 10 and 15 μm were cut with a Jung Microtome (West Germany). Fast-green dye was used to identify cellulosic tissues and safrenine was used for the lignified tissues.

RESULTS AND DISCUSSION

The production of reducing sugars and glucose from algae pretreated with cellulase is presented in Table 1. The data show that the hydrolysis occurred primarily during the first hour of operation. This high initial hydrolysis rate was probably due to the absence of lignin in the cell walls of the algae, a fact that was corroborated by the absence of safrenine dyed tissue in LM preparations. In highly lignified materials, the hydroly-

TABLE 1
Enzymatic Hydrolysis of Natural Algae: Reducing Sugars (RS) and Glucose Production in $g\,l^{-1}$

Cellulase loading FP IU g^{-1}		*Time (h)*		
		1	*2*	*4*
No enzyme	RS ($g\,l^{-1}$)	0·1	0·2	0·2
No enzyme	Glucose ($g\,l^{-1}$)	0·1	0·1	0·1
2·5	RS ($g\,l^{-1}$)	1·1	1·1	1·2
2·5	Glucose ($g\,l^{-1}$)	0·2	0·2	0·2
10	RS ($g\,l^{-1}$)	4·3	4·6	5·4
10	Glucose ($g\,l^{-1}$)	0·3	0·4	0·7
20	RS ($g\,l^{-1}$)	9·6	10·0	10·8
20	Glucose ($g\,l^{-1}$)	0·6	1·0	1·3

sis period normally ranges from 24 to 48 h. The data also show that the hydrolysis rate was directly proportional to the enzyme loading. However, for industrial applications it is desirable to keep the enzyme dosage low to reduce enzyme production costs and filtration problems due to major macrostructural modifications.

Table 2 gives agar yields and gel strength for algae exposed to three different pretreatments. The use of cellulase after the alkali treatment resulted in a gel strength and agar yield comparable to samples that were ground before extraction. The enzymatic pretreatment had the advantage of rendering the product easier to filter, avoiding the need for additional time to clean the equipment during the industrial process. In terms of cost, however, the situation must be evaluated to compare filtration costs with cellulase pretreatment costs before giving a definitive answer.

Figure 2 shows cross-section light micrographs of algae with different treatments. In Figure 2A (natural algae), high concentrations of intercellular material (agar/cellulose) are observed in the external cell layers. Figure 2B shows the removal of intracellular material in the algae extracted following the traditional process (includes NaOH treatment). Figure 2C shows there is a high disruption of cell layers in algae treated with NaOH and cellulases. Finally, Fig. 2D shows the algae extracted after treatment with NaOH and cellulases. It is clear from these LM photographs that cellulases produce disruption of cell walls within the algae thallus, and thereby facilitate agar diffusion out of the cells during the extraction step.

Figure 3 presents side views of algae thalli obtained using SEM. Figure 3A depicts the natural algae. Figure 3B shows the removal of the surface material from external cells, after treatment with NaOH. Figure 3C exhibits algae treated with NaOH and cellulases. The enzymatic attack produced microcracks in the surface, probably due to cellulose breakdown, that apparently also facilitate outward diffusion of agar during extraction.

TABLE 2
Agar Yields and Gel Strength for *G. lemanaeformis* Processed with Different Pretreatments

Treatment	*Agar yield [% w/w]*	*Gel strength [g per cm²]*
NaOH/Grinding	17	1100
NaOH/Cellulase (5 FP IU g^{-1})	15	800
NaOH/No grinding	11	550

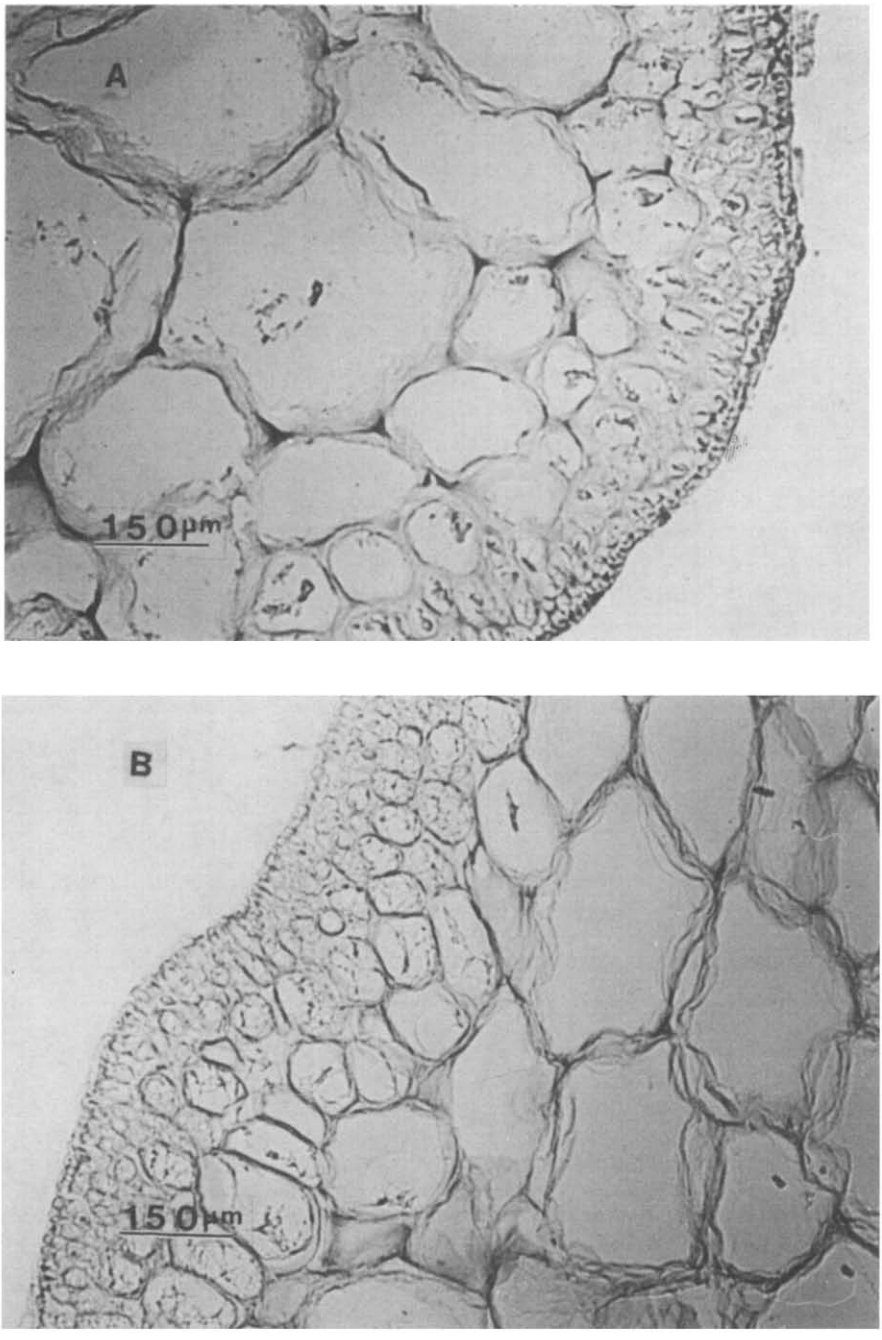

Fig. 2. Light micrographs from cross-sections of (A) natural algae, (B) extracted (NaOH pretreated) algae, (C) NaOH + cellulase pretreated algae, and (D) extracted (NaOH + cellulase pretreated) algae.

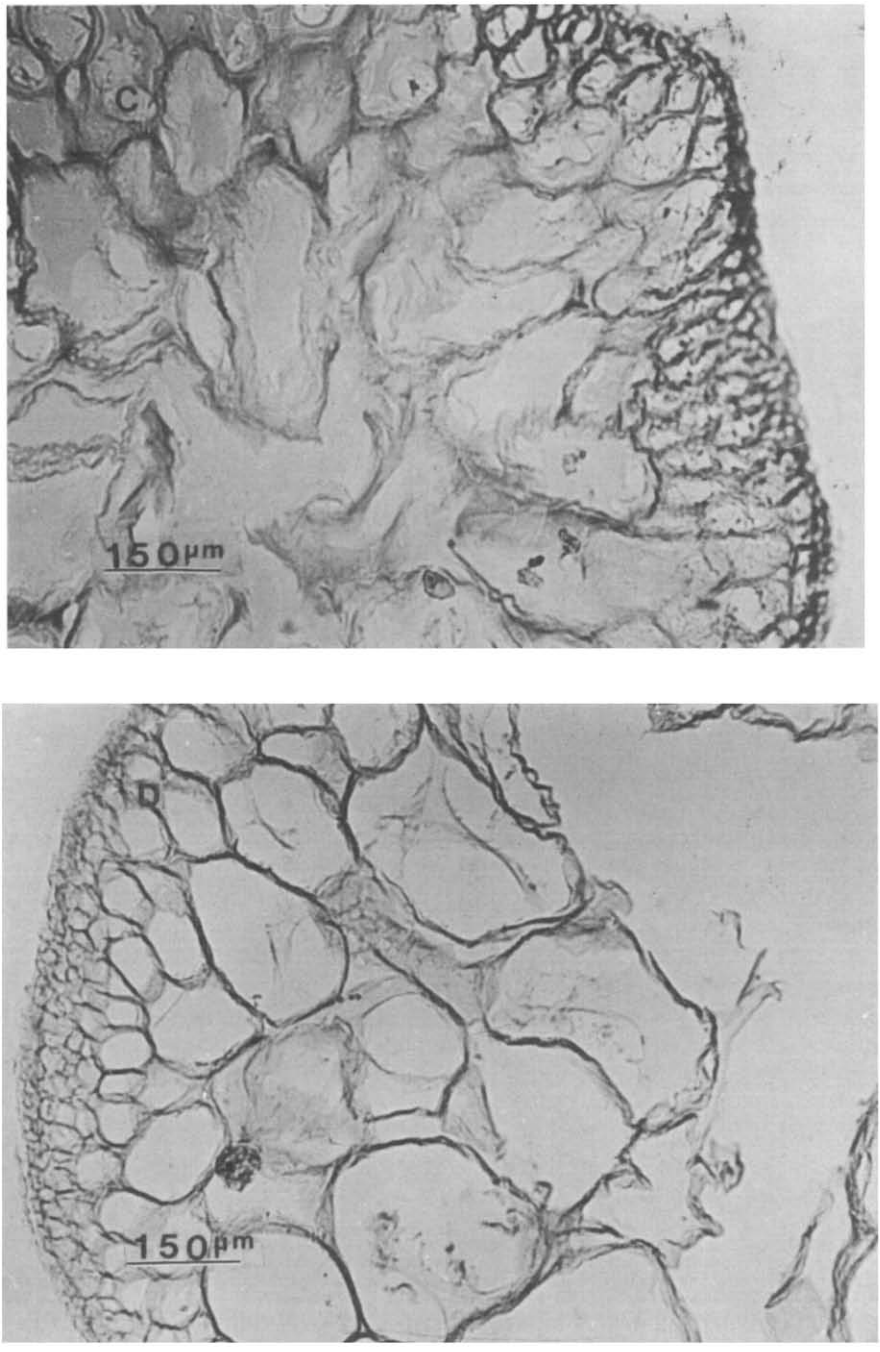

Fig. 2. — *contd.*

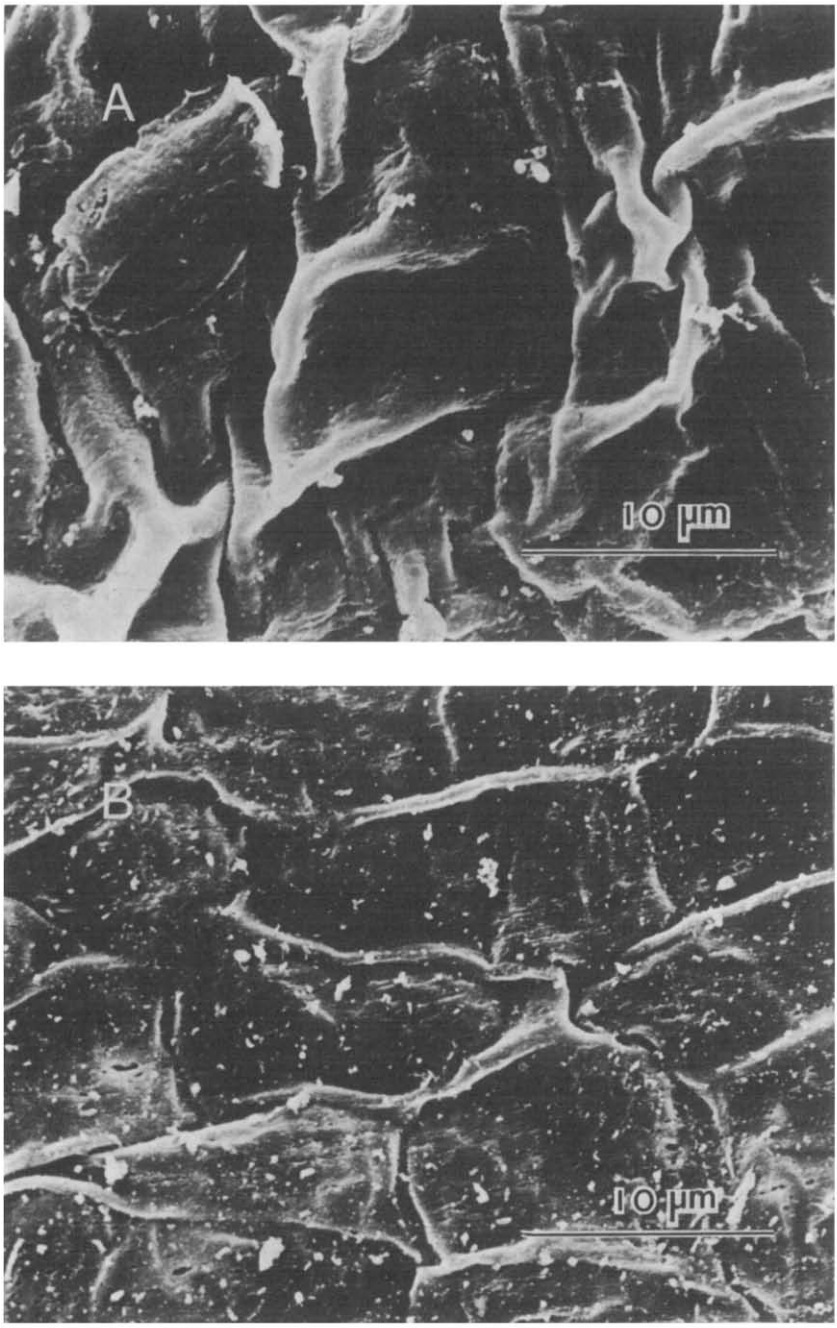

Fig. 3. SEM micrographs from thallus surfaces of (A) natural algae, (B) NaOH pretreated algae, and (C) NaOH and cellulase pretreated algae.

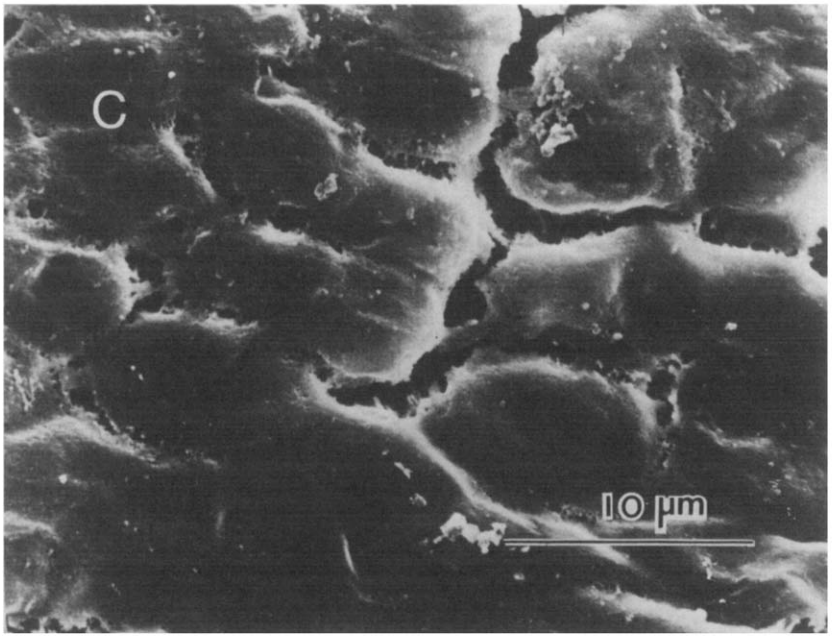

Fig. 3 — *contd.*

CONCLUSIONS

Microscope and yield results indicated that the use of cellulase pretreatments in *Gracilaria* red algae enhanced agar extractability. Cellulase was very effective against algae cellulose, as most hydrolysis occurred during the first hour of reaction. The pretreatment resulted in significantly improved agar yield and gel strength compared with untreated samples, but did not give better results than ground algae commonly used for the extraction process. Even though the grinding step improved yield and gel strength it also resulted in a significant increase in the cost of filtration, so economic considerations must decide whether cellulase pretreatment is advantageous relative to the traditional grinding process for agar extraction.

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