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Pectinolytic Enzymes from Actinomycetes for the Degumming of Ramie Bast Fibers

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Actinomycetes isolated from 10 different soil and compost samples were screened for production of pectinolytic enzyme activities when grown on pectin-containing solid and liquid media. Pectinolytic enzymes, detected by using plate diffusion tests with a medium containing ramie (*Boehmeria nivea*) plant material as the sole carbon source, were mainly pectate lyases, but low activities of pectinesterases were also observed. Polygalacturonases and polymethylgalacturonases were not produced. Multiple forms of pectate lyases were detected in the culture supernatants of some of the strains by using the zymogram technique of isoelectric focusing gels. Xylanolytic and cellulolytic activities were always found to be associated with pectinolytic activities. None of the pectinolytic enzymes were produced in a medium with glucose as the sole carbon source. Treatment of ramie bast fibers with crude enzyme preparations from a selection of strains showed a good correlation between the pectate lyase activity applied and the degumming effect, resulting in good separation of the bast fibers.

Actinomycetes play an important role in the degradation of plant residues (21). Biodegradation of pectin, a polysaccharide built mainly of α-(1-4)-linked D-galacturonic acid and its methylester, occurs as the result of a synergistic action of different extracellular enzymes (19, 32). Two different classes of pectinolytic enzymes depolymerize pectin or its nonesterified form either by transeliminative cleavage (lyases) or by hydrolysis (hydrolases). In addition, the ester bonds in pectin are hydrolyzed by a pectinesterase. Pectinolytic enzymes have been found in plants and microorganisms such as moulds, yeasts, and bacteria (33). Only limited information is available on the pectinolytic enzyme system of actinomycetes. Studies on pectinolytic actinomycetes have mainly revealed the presence of pectate lyases (23, 24, 28). In *Streptomyces viridochromogenes*, a pectin lyase has been detected (1). A pectate lyase from the thermophilic actinomycete *Thermomonospora fusca* has been purified and characterized (29).

Pectinolytic enzymes are known to play a key role in the maceration of plant tissues by degrading the pectin located in the middle lamella and in the primary cell wall of higher plants (7, 15). They are involved in the retting and degumming of jute, flax, hemp, and ramie bast fibers (3, 4, 13, 25). Cellulose fibers obtained from ramie are considered the longest, strongest, and silkiest plant fibers. The fibers are obtained by mechanical removal of the bast from the stem of the plant (decortication). Decorticated ramie fibers contain about 20 to 30% incrusting material (gum) consisting mainly of pectin and hemicellulose. This material is removed in a chemical degumming process by treatment of the decorticated fibers with hot alkaline solutions, with or without application of pressure (4, 9). This process produces polluting effluents and can cause damage of the fibers. The incrusting material could also be removed by polysaccharide-degrading microorganisms or their enzymes in a biotechnical degumming process. Combined microbial and chemical processes have been proposed to reduce the consumption of chemicals and energy (10, 20).

In this work, we report the isolation of pectinolytic actinomycetes and the identification of their polysaccharide-degrading enzymes. The particular objective was to study the application of pectinolytic enzymes in the ramie-degumming process.

MATERIALS AND METHODS

Isolation of actinomycetes. Ten soil and compost samples from Brazil (ramie compost), Nepal (forest soil), Thailand (forest soil and sand), and Switzerland (forest soil and grass-and-flower compost) were dried for up to 2 weeks at 37°C. One gram of each sample was added to 10 ml of sterile water and sonicated (50 W) for 15 s (Branson Sonifier B12). The samples were diluted 10- to 10^5-fold with sterile water. An aliquot (0.1 ml) was plated on HMS agar [2 g of $(NH_4)_2SO_4$, 1 g of K_2HPO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 1 g of NaCl, 2 g of $CaCO_3$, 1 mg of $FeSO_4 \cdot 7H_2O$, 1 mg of $MnCl_2 \cdot 4H_2O$, 1 mg of $ZnSO_4 \cdot 7H_2O$, 50 mg of cycloheximide, 0.1 g of yeast extract, 18 g of agar (pH adjusted to 7.3) per liter of deionized water] containing a ramie fiber extract overlay. The overlay consisted of 0.2 ml of an aqueous suspension of a precipitate obtained after extraction of decorticated ramie fibers with a phosphate buffer at 100°C for 2 h after cooling down to room temperature. The plates were incubated at 30°C for 4 days. Colonies were picked and plated on PYG agar (5 g of Bacto Peptone, 1 g of yeast extract, 5 g of glucose, 18 g of agar per liter of deionized water). Gram staining of the isolates was done with a Gram stain kit (Difco). Cultures were stored as suspensions of spores and hyphae in a 25% (vol/vol) aqueous solution of glycerol at −70°C. Isolates BW 80 and BW 86 were obtained from B. Winter at our institute.

Detection of pectinolytic actinomycetes. Colonies from PYG plates were picked and transferred to DMC plates (17) containing 0.5% citrus pectin (Sigma) instead of glucose. After 3 days of incubation at 30°C, plates were stained with 0.05% aqueous ruthenium red (8).

Liquid culture conditions. Cells were precultured in a

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medium with 5 g of glucose, 1 g of yeast extract, 100 mM phosphate buffer (pH 7.0), 0.1 g of $(NH_4)_2SO_4$, 0.3 g of NaCl, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCO_3$, 40 μg of $ZnCl_2$, 200 μg of $FeCl_3 \cdot 6H_2O$, 10 μg of $CuCl_2 \cdot 2H_2O$, 10 μg of $MnCl_2 \cdot 4H_2O$, 10 μg of $Na_2B_4O_7 \cdot 10H_2O$, and 10 μg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ per liter of deionized water in 300-ml flasks at 150 rpm and 30°C. Cells were harvested after 48 h and transferred to medium containing either 1% finely cut ramie fibers, 1% milled, dried ramie leaves, 0.5% citrus pectin (Sigma), or 0.5% sodium polygalacturonate (Sigma) instead of glucose. Carbon sources were autoclaved in deionized water and added to sterile double-strength medium after being cooled.

Enzyme assays. A cup-plate assay (11) was used to detect polysaccharide-degrading enzymes in the culture supernatants. For detection of pectate and pectin lyases, polygalacturonases, and polymethylgalacturonases, a ruthenium red-staining method for detection of pectinolytic enzymes in polyacrylamide gels (22) was adapted. The substrate was either sodium polygalacturonate (Sigma) or a highly esterified pectin with a 93% degree of esterification (Sigma). Pectin lyases were also tested in the absence of calcium. Plates for detection of polygalacturonases and polymethylgalacturonases contained 1 mM EDTA to inhibit pectate lyases. Pectinesterases were detected on citrus pectin (Sigma) by the method of McComb and McCready (18). Cellulase and xylanase activities were measured by the methods of Teather and Wood (30) and Farkaš et al. (12), respectively. Cell-free supernatants (20 μl) were pipetted into cups 5 mm in diameter. Plates were incubated at room temperature for 15 h to detect enzyme activities, which were indicated by halo formation around the cups. A further overnight incubation of the ruthenium red-stained plates at 4°C resulted in better halo contrast and thus improved the sensitivity of the assay.

Lyase activity was also estimated spectrophotometrically by measuring the increase in A_{235} (2). Reaction mixtures contained 300 μl of 0.2 M Bis-Tris propane buffer (pH 9.5) with 2 mM $CaCl_2$ (freshly prepared), 300 μl of 0.2% sodium polygalacturonate (Sigma) or 0.2% highly esterified (degree of esterification, 93%) pectin from citrus (Sigma), and 50 μl of diluted enzyme. One enzyme unit was defined as the enzyme amount which forms 1 μmol of a product per min with a molar extinction coefficient of 4,600 liters mol^{-1} cm^{-1} (33).

Pectinesterase activity was determined at pH 7 and room temperature by measuring the liberation of methanol from citrus pectin (34). One enzyme unit was defined as the enzyme amount which releases 1 μmol of methanol per min.

Polygalacturonase and polymethylgalacturonase activities were determined at room temperature by measuring the release of reducing sugars from 0.1% polygalacturonic acid or citrus pectin in 100 mM sodium acetate buffer (pH 5.3). Reducing sugars were measured with the copper–2,2′-bicinchoninate reagent (26). One enzyme unit was defined as the enzyme amount which releases 1 μmol of galacturonic acid equivalents per min.

Xylanase activity was determined at room temperature by measuring the release of reducing sugars from 0.8% oat spelt xylan (Fluka) in 100 mM TES buffer (Sigma) (pH 7). The suspended substrate was sonicated at 50 W for 15 min (Branson Sonifier B12) (6). Reducing sugars liberated were measured with the copper–2,2′-bicinchoninate reagent (26). One enzyme unit was defined as the enzyme amount which releases 1 μmol of xylose equivalents from oat spelt xylan per min.

Cellulase was determined at room temperature by measuring the release of reducing sugars from 0.5% carboxymethyl cellulose in 100 mM TES buffer (pH 7). Reducing sugars liberated were measured with the copper–2,2′-bicinchoninate reagent (26). One enzyme unit was defined as the enzyme amount which releases 1 μmol of glucose equivalents from carboxymethyl cellulose per min.

Isoelectric focusing. Analytical isoelectric focusing was carried out on 0.5% agarose gels with a broad-range ampholyte ranging from pI 3.5 to pI 10 (Pharmacia). Separations were achieved as recommended by the manufacturer. A protein calibration kit with pI values from 4.65 to 9.6 was purchased from Bio-Rad. Pectate lyases were detected by the replica gel technique as described by Ried and Collmer (22). Pectate-agarose overlays were placed in contact with the isoelectric focusing gel for 12 h at room temperature before they were stained with ruthenium red. Protein bands were also visualized by silver staining as described by Vesterberg and Gramstrup-Christensen (31).

Degumming. Degumming of ramie bast fibers was carried out in 50-ml Erlenmeyer flasks containing 0.5 g of decorticated dried raw fibers and 20 ml of cell-free supernatants from 3-day-old liquid cultures (supplemented with 0.01% sodium azide). Flasks were shaken at room temperature for 15 h at 150 rpm. Controls contained sterile medium instead of culture supernatants. The gum content of the fibers was determined gravimetrically by sequential degumming of the fibers with hot sodium hydroxide solutions with different concentrations (9). In addition, a commercial enzyme preparation for flax retting (Flaxzyme from Novo Nordisk) was tested.

RESULTS

Isolation of actinomycetes. Isolation of strains by using selective conditions for enrichment of actinomycetes (16), such as heat treatment of the soil and compost samples and addition of cycloheximide to prevent fungal growth, resulted in 77 gram-positive, mycelium-forming isolates from 10 different soil and compost samples. Microscopical observations showed that most strains resembled streptomycetes. One strain (no. 52) was tentatively identified as an *Amycolata* sp. (E. Wellington, University of Warwick, Warwick, United Kingdom). Most of them grew at 30°C; three of the strains isolated from ramie compost showed growth at 50°C.

Detection of pectinolytic actinomycetes. Seventy-nine actinomycete isolates were tested for production of pectinolytic enzymes on citrus pectin-containing agarose plates. About 66 strains showed growth. Ruthenium red staining of these plates revealed 39 isolates producing white halos, 13 isolates producing dark pink halos, and 14 isolates producing a combination of white and dark pink halos around the colonies. According to Cotty et al. (8), white halos correspond to pectin-depolymerizing activities and dark pink halos correspond to pectinesterase activities. As judged by the diameters of the halos produced, actinomycetes isolated from ramie compost (Brazil) and from a sand sample (Thailand) showed significantly higher pectinolytic activities than the isolates from the other sources.

Detection of polysaccharide-degrading enzymes. The plate diffusion test (Fig. 1) allowed fast and convenient detection of extracellular enzyme activities in the culture supernatant of the 79 isolates grown with raw ramie fibers as the sole carbon source. Pectinolytic, xylanolytic, and cellulolytic activities were detected after day 2 of cultivation throughout the incubation period of 5 days. Pectinolytic activities among the strains tested were found on pectate- and pectin-containing agarose plates at pH 8.5, indicating the presence of pectate and pectin lyases. All strains showing a pectate lyase activity also showed activity on plates used for detection of pectin lyases, although at a

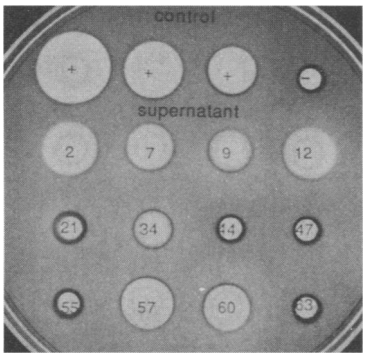

FIG. 1. Plate diffusion test for detection of pectate lyases in supernatants of 3-day-old cultures of strains grown on ramie fibers. The numbers refer to the isolates. Controls consisted of diluted culture supernatant from strain 57 containing 162, 32, and 16 mU of pectate lyase per ml (+) and noninoculated medium (−).

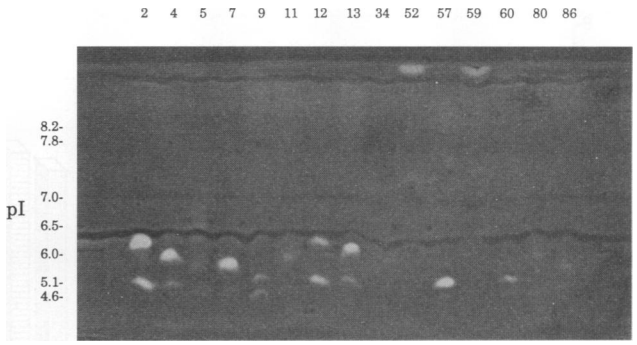

FIG. 3. Zymogram of pectate lyases in culture supernatants of actinomycetes separated by isoelectric focusing. The numbers at the top refer to isolates. The positions of silver-stained pI markers are shown on the left.

reduced level. Further discrimination between these two activities with a spectrophotometric assay showed that the lyase activity was always higher on pectate than on highly esterified pectin. Therefore, pectate lyases rather than pectin lyases are responsible for the observed pectin-depolymerizing activities. Polygalacturonases and polymethylgalacturonases could not be detected under the assay conditions used (pHs 5 to 7). Very low pectinesterase activities (less than 10 mU/ml) were observed in some of the strains. Xylanolytic and cellulolytic activities were found in a larger number of strains than were pectinolytic activities (Fig. 2). Generally, strains with high pectinolyticactivity also showed high xylanolytic and cellulolytic activities. Furthermore, xylanases and cellulases were always found to be associated with pectate lyase activities. The cellulolytic activity in the culture supernatants still increased after 5 days of cultivation, whereas pectinolytic and xylanolytic activities remained constant after day 3 of cultivation. This could indicate that after removal of the incrusting material, the exposed cellulose fibers were utilized as a carbon source. When glucose was used as the sole carbon source, no pectate lyases were detected, although low levels of xylanase and cellulase activities were observed in some of the isolates.

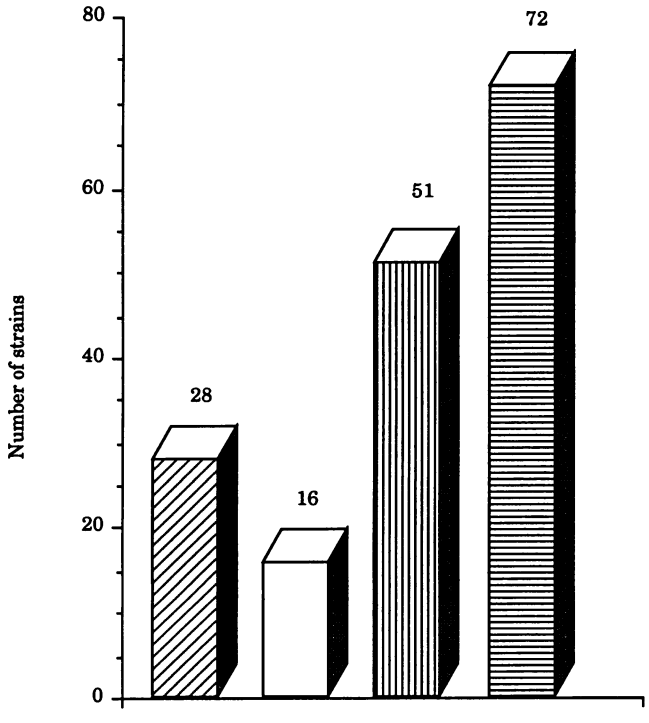

FIG. 2. Distribution of enzyme activities detected with a plate diffusion test in culture supernatants of 79 isolates of actinomycetes grown on ramie fibers. ▨, pectate lyase; □, pectinesterase; ▥, carboxymethyl cellulase; ▤, xylanase.

The zymogram of isoelectric focusing gels revealed the presence of multiple forms of pectate lyases in some of the culture supernatants of actinomycetes grown on ramie fibers (Fig. 3). Most pectinolytic strains produced acidic pectate lyases with pIs ranging from 4.6 to 6.5. Two highly pectinolytic strains, 52 and 59, produced a pectate lyase with a pI higher than 9.6.

Induction of pectate lyases by different pectic substances. Production of pectate lyases could be induced in the cultures by different carbon sources containing pectic substances, such as dried raw ramie fibers or ramie leaves, pectin from citrus, or polygalacturonic acid. The strains produced very different pectate lyase activities, depending on the carbon sources used (Fig. 4). The results show that strains producing high pectate lyase activities when grown on ramie plant material as the sole carbon source did not necessarily produce high pectate lyase activities on other pectin-containing carbon sources. Most strains produced high pectinolytic activities when grown on dried, milled ramie leaves. The strains tested did not show a uniform induction pattern with the different carbon sources.

Degumming of ramie bast fibers. Supernatants from cultures of a selection of strains containing pectinolytic, xylanolytic, and cellulolytic enzymes were used to degum decorticated, dried ramie fibers. A strong correlation between the pectate lyase activities in the supernatants (Fig. 5A) and their degumming effects was found (Fig. 5B). Degumming of bast fibers at pH 7 seemed to be influenced more by the presence of pectate lyases than by that of xylanases or cellulases. Degumming could not be improved by increasing the pH in the culture supernatant from pH 7 to pH 9, towards the pH optimum of the pectate lyases from actinomycetes reported previously (23, 29, 33).

Decorticated, dried, raw ramie fibers contained approximately 24% incrusting materials (gum). After treatment with sterile medium as a control for 15 h, the fibers still contained 22% gum. By treatment with cell-free culture supernatants of

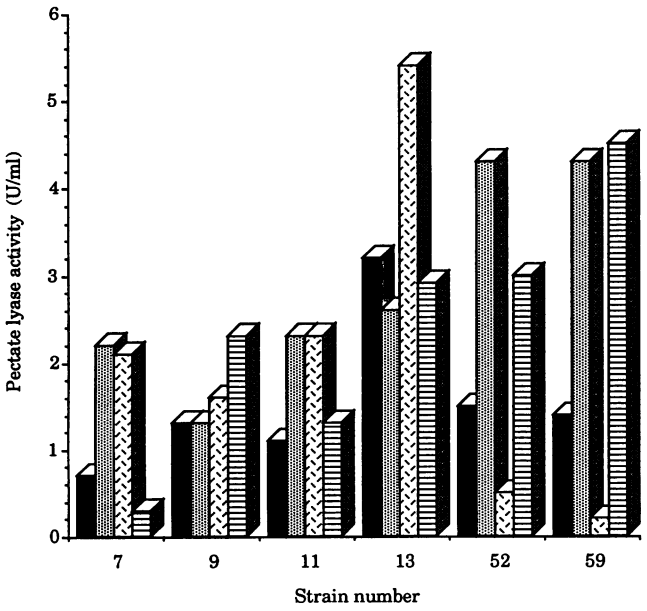

FIG. 4. Production of pectate lyases in cultures of strains containing different carbon sources. Maximum activities observed during 10 days of cultivation are shown. Carbon sources: ■, raw ramie fibers; ▨, dried ramie leaves; ▩, citrus pectin; ▤, polygalacturonic acid (sodium salt).

pectinolytic strains, the gum content of the fibers was reduced to 18%. The same degumming effect could be obtained by using a commercial enzyme preparation for flax retting (Flaxzyme from Novo Nordisk). Prolongation of the degumming time to up to 3 days or application of a supernatant concentrated fivefold by ultrafiltration (7.5 U/ml of pectate lyase) resulted in fibers still containing 15% gum. Degumming could be improved when nondried ramie fibers directly obtained from the green stem were used. The gum content of fresh fibers treated with pectinolytic cell-free culture supernatants was reduced to 15% after 15 h. Pectate lyase activities applied to the fibers remained active throughout the incubation time. It can be concluded that only a fraction of the incrusting material was removed by treatment of the ramie bast fibers with cell-free culture supernatants from the best strains. However, degumming with the supernatants showing high pectinolytic activities did result in good fiber separation.

DISCUSSION

Pectate lyases were the dominant pectinolytic enzymes in 79 isolates of actinomycetes grown in liquid cultures with ramie fibers as the sole carbon source. Most of the pectinolytic isolates also produced very low levels of pectinesterases. Pectate lyases have been reported previously in actinomycetes (23, 24, 28, 29), while other pectinolytic enzymes have not been described.

A larger number of pectinolytic strains was detected on solid medium by ruthenium red staining than in liquid cultures by the plate diffusion test. This could be explained by the different medium compositions and different cultivation conditions used but also by the different sensitivities of the assays. Recently, pectinase productivities and regulatory phenomena were reported to be different in the two types of cultivation (solid-state and submerged-state fermentation) for *Aspergillus niger* (27).

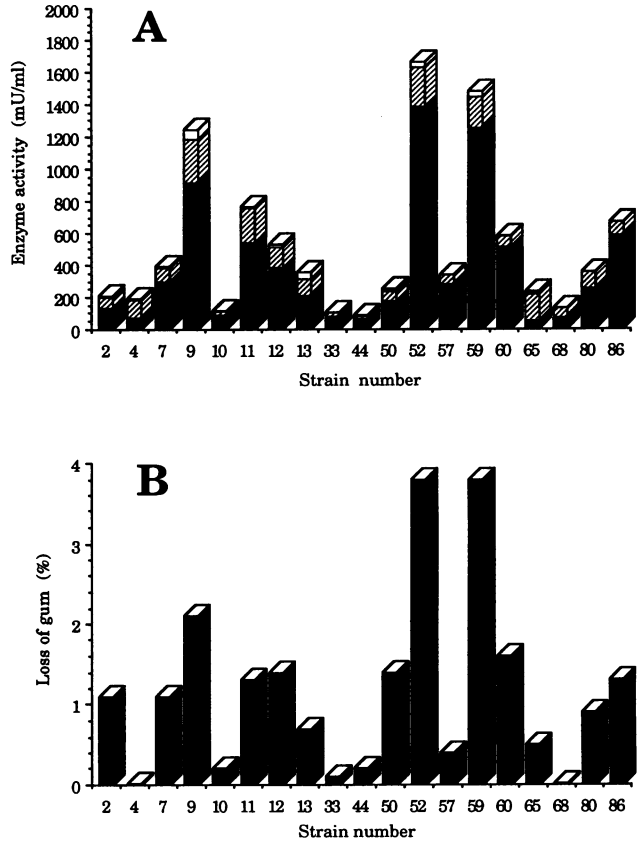

FIG. 5. (A) Activities of pectate lyases, xylanases, and carboxymethyl cellulases in cultures of strains grown on ramie fibers. The numbers refer to the isolates. ■, pectate lyase; ▨, xylanase; □, carboxymethyl cellulase. (B) Percent loss of gum by raw ramie fibers after treatment with cell-free culture supernatants of the strains. The water-soluble fraction of the gum (2%) has been subtracted.

In contrast to the cellulolytic and hemicellulolytic enzymes, multiple forms of pectate lyases have hardly been observed in actinomycetes. One can assume that these pectinolytic isozymes contribute to the high potential of actinomycetes to degrade a wide spectrum of polysaccharides from plants under changing environmental conditions. In liquid cultures, pectate lyase production was very strongly carbon source and strain dependent. Although the strains were originally selected on ramie fiber extract overlays, some strains produced the highest pectate lyase activities on commercial pectins. The composition of the pectin substances seems to play a major role in the induction of total pectate lyases. Different pectin concentrations, as well as soluble sugars left in the carbon sources used, do not explain the different total pectate lyase activities observed in various isolates of actinomycetes.

Paul et al. (20) found that ramie fibers still contained 15% incrusting material after degumming with mixed bacterial cultures. With cell-free culture supernatants from our actinomycete strains, only a slightly higher remaining gum content was obtained, although the time required for degumming was much shorter (15 h instead of 7 days). Since a good correlation was found between the pectate lyase activities of various actinomycetes applied to the fibers and the resulting degumming effect, it is likely from this point of view that the pectate lyases observed were similar in substrate preference and mode

of action. Only pectinolytic enzymes of the endo type, not of the exo type, have shown good macerating activities (14, 24) and are therefore important for the degumming of ramie fibers. A close relationship between the pectate lyase activity applied and the degree of fiber separation was not found when bast fibers from flax were treated with pectinolytic enzymes from *Erwinia* spp. (14). A concerted action of a pectate lyase and a pectin lyase with the aid of xylanase was proposed. Cao et al. (5) also reported that high pectinolytic activity in the medium of an alkalophilic bacillus did not necessarily show high degumming efficiency on ramie bast fibers. A synergistic action of different polysaccharide-degrading enzymes is therefore more likely to be responsible for the degumming and defibration of bast fibers than a single enzyme activity alone. However, no significant contribution of xylanases also present in the culture supernatants to ramie degumming was observed with our isolates. Higher esterified pectins may also require the presence of further enzyme activities, such as pectinesterases, to achieve good degradation. Therefore, the pectinesterases, although detected at very low levels, are probably important in actinomycetes, where pectinolytic activity is due mainly to pectate-depolymerizing enzymes.

For the manufacture of textiles from ramie fibers, a gum content of less than 6% is desired (4, 9). However, application of ramie fibers for other purposes (e.g., biodegradable fabrics, composites) may require fibers with a higher gum content. Since the residual gum content of the fibers after treatment with the culture supernatants of the strains tested was still 15 to 18%, combined biological and chemical treatment of the raw ramie fibers is likely to be necessary for sufficient degumming. Such a combined process could further reduce the amounts of chemicals and energy used in the conventional chemical degumming process. Because of the milder degumming conditions during biological treatment, the quality of the fibers could also be improved.

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