APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1989, p. 1612–1616
0099-2240/89/061612-05$02.00/0

Vol. 55, No. 6

Pectin Lyase Production by a *Penicillium italicum* Strain

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Received 29 November 1988/Accepted 18 March 1989

Growth and concomitant production of an extracellular pectin lyase (PL) [poly(methoxylgalactosiduronate) endolyase; EC 4.2.2.10] were investigated in a group of 16 fungi grown in liquid medium containing pectin as a supplementary carbon source. Culture filtrates of both *Penicillium italicum* (CECT 2294) and *P. expansum* (CECT 2275) showed the highest PL activity and contained polygalacturonase but not pectinesterase activity. The effect of the inoculum size, the carbon source (sucrose and glucose syrup), and the presence of pectin on the production of PL by *P. italicum* was studied. The presence of 2.6 mM glycerophosphate in the culture medium enhanced the appearance of PL but was not inhibitory for the in vitro activity. However, glycerol inhibited the enzyme nearly 50% at such a concentration.

Many plant-pathogenic bacteria and fungi have long been known to produce pectolytic enzymes useful for invading host tissues (1, 4). Moreover, pectolytic enzymes are essential in the decay of dead plant material by nonpathogenic microorganisms and thus assist in recycling carbon compounds in the biosphere.

As far as the food industry is concerned, these enzymes from fungal sources play a decisive role in fruit juice technology by degrading pectins and clarifying juices, steps which are essential for processing juices which must be concentrated. The available commercial preparations generally contain a mixture of these enzymes: pectinesterase (PE) and polygalacturonase (PG) in addition to pectin lyase (PL). However, PL is the only enzyme known to be able to hydrolyze, without the prior action of other enzymes, highly esterified pectins such as fruit pectin.

Therefore, the use of isolated PL in the fruit juice industry seems advantageous for two reasons. First, no methanol can be formed in the course of the enzyme action; hence, the volatile ester content, responsible for the specific aroma of the various fruits, is not damaged. Second, the use of PG- and PE-containing enzyme complexes decreases fruit juice stability because of the coagulant processes caused by the interaction of the de-esterified pectin derivatives with the endogenous Ca^{2+} content of juices.

Fruit and vegetable liquefaction was also made feasible by the commercial availability of suitable enzymes (23). This technological process is based on the synergetic action of cellulases, PEs, PGs, and/or PLs.

Penicillium italicum has been described as one of the microorganisms responsible for the decay of citrus fruit (8). Infection by this fungus, which gives rise to restricted colonies with blue-green spores, reduces the fruit to a soft pulpy condition.

In this study growth in liquid medium and the concomitant release of PL from a group of 16 fungi were studied. *P. italicum* (CECT 2294) and *P. expansum* (CECT 2275) seemed to be the best PL producers among the studied fungi. The presence of other extracellular pectolytic activities in these two strains is also reported. To enhance PL production by *P. italicum*, we investigated the effect of pectin as an inducer, the inoculum size, the carbon source, and the presence of glycerophosphate.

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MATERIALS AND METHODS

Microorganisms. All the strains used (see Table 1) were obtained from Colección Española de Cultivos Tipo (CECT), Valencia, Spain, and maintained in potato dextrose agar (bioMérieux) medium.

Growth conditions. Cultures were grown at 28°C in shaken (160 rpm) Erlenmeyer flasks (250 ml) containing 50 ml of modified Czapek Dox medium (Oxoid Ltd., London, England; containing, in grams per liter, the following: $NaNO_3$, 2; KCl, 0.5; magnesium glycerophosphate, 0.5; $FeSO_4$, 0.01; sucrose, 30; and K_2SO_4, 0.35) supplemented with 0.5% (wt/vol) citrus pectin (grade I; Sigma Chemical Co., St. Louis, Mo.) with a degree of esterification of 70%. The medium was sterilized for 15 min at 1.3 atm (1 atm = 101.29 kPa) and inoculated with a suspension of spores (5×10^4/ml in 20% [vol/vol] glycerol) grown for 4 days in potato dextrose agar.

Biomass determination. Cultures were filtered on glass fiber filters (GF/C; Whatman, Inc., Clifton, N.J.) under reduced pressure and dried at 85°C to a constant weight.

Enzyme assays. Culture filtrates were concentrated by ultrafiltration (Diaflo YM-5000 membrane) before enzyme assays. PL activity was determined spectrophotometrically (Shimadzu UV-260 spectrophotometer) by monitoring the increase in the A_{235} as described by Albersheim and Killias (2). The reaction mixture (see Table 1) contained the following: 1.25 ml of 0.15 M citrate-phosphate buffer (pH 5.5), 0.25 ml of concentrated culture filtrate, and 1.0 ml of 1% (wt/vol) citrus pectin (grade I; Sigma) (degree of esterification, 70%). The reaction mixture of the standard assay used elsewhere for *P. italicum* PL contained the following: 0.35 ml of 0.5 M citrate-phosphate buffer (pH 6.0), 0.4 ml of dialyzed culture filtrate, and 0.5 ml of 1.25% (wt/vol) citrus pectin. Preincubations were carried out at 40°C for 15 min. The reaction was started by adding pectin. Control tubes contained the enzyme previously inactivated by incubation for 10 min at 100°C. One unit of PL activity was the amount of enzyme which produced an increase of one unit of A_{235} per minute.

PG (endo-, EC 3.2.1.15, plus exo-, EC 3.2.1.67 and EC 3.1.2.82) activity was assayed by measuring the reducing groups released in the reaction mixture by the Nelson-Somogyi copper reduction method described by Spiro (31)

TABLE 1. Production of PL by several fungi after 63 h of growth[a]

Strain	Final pH of cultures	Dry wt (mg/ml)	PL (U/mg of protein)
Aspergillus carbonarius (CECT 2086)	2.6	3.3	0.9
A. niger (CECT 2088)	3.9	7.6	0.9
Penicillium expansum (CECT 2275)	3.5	2.1	4.7
P. italicum (CECT 2294)	3.7	3.1	12.0

[a] Liquid Czapek Dox (modified) medium containing 0.5% (wt/vol) citrus pectin was inoculated with 5×10^4 spores per ml in all cases. After 63 h of growth, aliquots were sampled and the pH, biomass, and PL activity were determined as described in Materials and Methods. During this period of time no PL activity was detected in culture filtrates of *Saccharomycopsis fibuligera* (CECT 1238), *A. niger* (CECT 2775, 2090, and 2091), *A. oryzae* (CECT 2094 and 2095), *R. arrhizus* (CECT 2339 and 2340), *R. nigricans* (CECT 2344), *R. rhizopodiformis* (CECT 2763), *R. stolonifer* (CECT 2672), and *R. thailandensis* (CECT 2774).

with Somogyi copper reagent (29) and Nelson arsenomolybdate reagent (21). The reaction mixture contained the following: 50 μl of 0.2 M acetate buffer (pH 4.5), 50 μl of concentrated culture filtrate, and 50 μl of 1% (wt/vol) polygalacturonic acid from orange (Sigma). Preincubations were carried out at 37°C for 15 min. To avoid interference from other contaminant enzymes, we omitted the substrate in the control tubes. The reaction was started by adding the substrate, and incubation was carried out at 37°C for 10 min. One unit of galacturonase activity was the amount of enzyme which released 1 μeq of galacturonic acid per min.

PE (EC 3.1.1.11) activity was assayed by measuring the pH decrease during the reaction at 25°C. The reaction mixture (pH 7.0) contained 1 ml of concentrated culture filtrate, 3 ml of distilled water, and 5 ml of 0.5% (wt/vol) citrus pectin. One unit of PE activity was the amount of enzyme which released one microequivalent of carboxyl groups per minute.

Data in all assays represent the averages of at least two separate experiments, each one consisting of samples from duplicate flasks whose PL activities were assayed twice.

Fractional precipitation of PL. After culture filtrates were adjusted to pH 8.0, solid ammonium sulfate was added at room temperature. The 30 to 95% saturated ammonium sulfate pellet was dissolved in an appropriate volume of distilled water, dialyzed overnight at 4°C against 200 volumes of 50 mM Tris hydrochloride buffer (pH 8.0), and stored at 4°C.

Protein assay. Protein was determined by the method of Bradford (6) with bovine serum albumin as the standard.

Analysis of pectic substances. The galacturonic acid content was estimated as anhydrogalacturonic acid by the carbazole colorimetric method (10) as modified by Dietz and Rouse (9).

RESULTS

Preliminary screening. The production of PL by 16 fungi after 63 h of growth is summarized in Table 1. *P. italicum* (CECT 2294) culture filtrates showed the highest specific activity (12 U/mg of protein). Mycelial dry weight increased fairly rapidly until day 6, after which there was only a slight increase in growth. The specific activity of PL decreased after 63 h despite increasing biomass (data not shown). Although *P. expansum*, the second-best producer among the fungi studied, showed maximum growth at day 4, the highest PL specific activity (4.7 U/mg of protein) was reached after 63 h of growth.

TABLE 2. Production of PG by two *Penicillium* strains at different times of growth[a]

Strain	PG (U/mg of protein) at:			
	63 h	111 h	159 h	255 h
P. italicum	41.4	26.2	11.9	0.0
P. expansum	447.7	16.0	130.8	28.2

[a] PG activity was assayed as described in Materials and Methods. No PE activity was detected in the filtrates.

Although no PL activity was found in culture filtrates of most of the fungi studied after 63 h of growth, low activities (≤0.8 U/mg of protein) were observed in *Aspergillus niger* (CECT 2090 and CECT 2275) and *A. orizae* (CECT 2094) at 111 and 159 h of growth, respectively. Biomass increased in all the *Aspergillus* strains studied during a 255-h culture period. *Rhizopus arrhizus* (CECT 2340) and *R. nigricans* (CECT 2344) culture filtrates showed PL activities of 1.1 and 2.8 U/mg of protein at 111 and 159 h of growth, respectively.

The presence of PG and PE activities was investigated in culture filtrates of both *Penicillium* strains. Both of them contained PG but not PE activity (Table 2).

PL production by *P. italicum*. The effect of pectin on the production of PL was investigated (Fig. 1). Maximum PL activity (which coincided with a major decrease in the pectin content and with an intensive growth phase) appeared after 48 h of growth in filtrates of cultures grown with 0.5% (wt/vol) pectin (31.6 mU/ml, corresponding to 15.8 U/mg of protein) and 1% (wt/vol) pectin (67 mU/ml, corresponding to 35.6 U/mg of protein). The residual pectin present in our culture filtrates did not interfere in the enzyme assay, since similar results were obtained (data not shown) when PL was salt precipitated from filtrates and then assayed by the

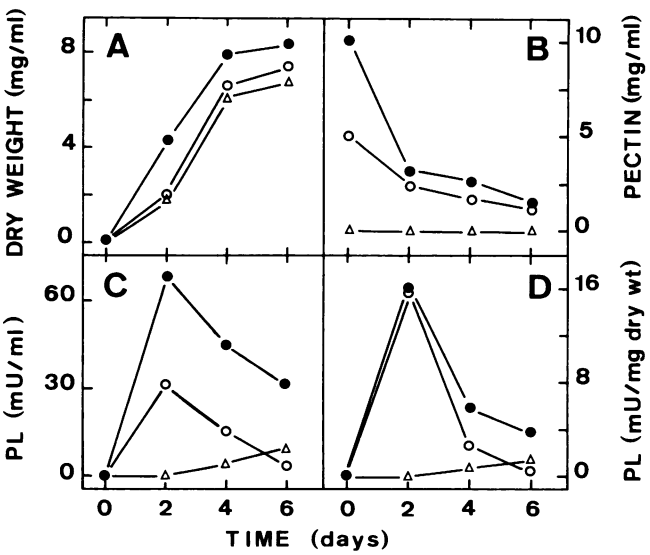

FIG. 1. Effect of pectin on the growth of *P. italicum* (A), the disappearance of pectin (B), PL activity (C), and the relationship between growth and PL production (D). Flasks containing 30 ml of Czapek Dox (modified) medium (pH 3.5) supplemented with different pectin concentrations were inoculated with 10^4 spores per ml. The pectin concentrations (percent [weight/volume]) were as follows: 0 (△), 0.5 (○), and 1 (●).

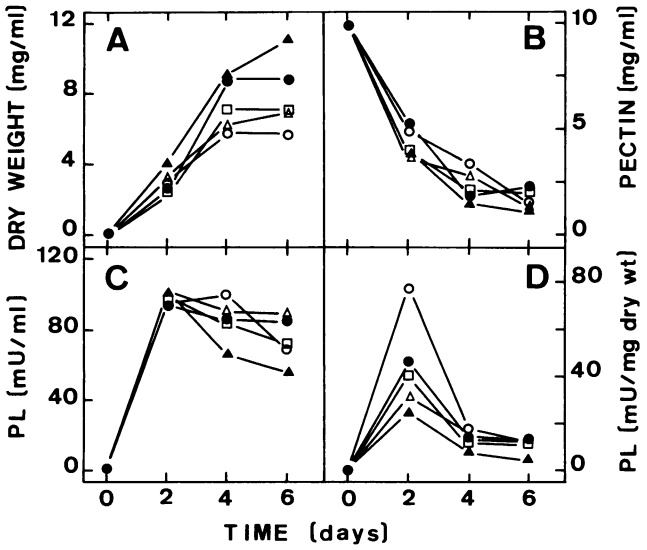

FIG. 2. Effect of the inoculum size on the growth of *P. italicum* (A), the disappearance of pectin (B), PL activity (C), and the relationship between growth and PL production (D). Flasks containing 30 ml of Czapek Dox (modified) medium (pH 3.5) supplemented with 1% (wt/vol) pectin were inoculated with the following numbers of spores per milliliter: 2.5×10^3 (○), 5×10^3 (△), 10^4 (□), 2×10^4 (●), and 5×10^4 (▲).

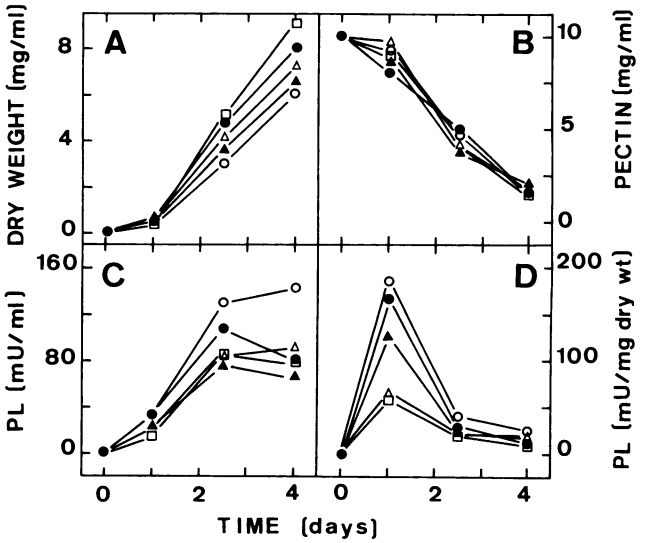

FIG. 3. Effect of the carbon source and glycerophosphate on the growth of *P. italicum* (A), the disappearance of pectin (B), PL activity (C), and the relationship between growth and PL production (D). Flasks containing 30 ml of the indicated media (pH 3.5) supplemented with 1% (wt/vol) pectin were inoculated with 10^4 spores per ml. The Czapek media used were a commercial Czapek Dox (modified) medium (□) and different laboratory-produced media containing the following modifications: none (△), 3% (wt/vol) glucose syrup as the carbon source (○), no glycerophosphate (▲), and 3% (wt/vol) glucose syrup as the carbon source and no glycerophosphate (●). Glucose syrup contained (percent [weight/volume]) the following: glucose, 93.06; fructose, 1.67; maltose, 4.11; trehalose, 0.63; and ribulose, 0.5.

standard procedure. In all the experiments the final pH of cultures grown for 6 days in the presence of pectin was less than 4.0.

The time course data of growth and PL production (Fig. 1D) revealed a nonlinear relationship between the two parameters. Although similar levels of PL activity per unit of growth (about 16 mU/mg of dry weight) were measured in filtrates of cells grown with 0.5 and 1% (wt/vol) pectin, the respective PL specific activities (15.8 and 35.6 U/mg of protein) were quite different.

The effect of the inoculum size on different parameters related to growth and PL production by *P. italicum* is shown in Fig. 2. As expected, the final biomass of the cultures depended on the initial number of the germinated spores used as the inoculum (Fig. 2A). As far as the substrate was concerned, pectin disappearance was similar in all cases, maximum rates of consumption being observed during the first 2 days of growth (Fig. 2B) and coinciding with both the beginning of the exponential growth phase and the maximum rates of PL release to the medium (Fig. 2C). After 4 days of growth, when the stationary phase was reached, pectin utilization by the cells ceased and the PL activity of the filtrates diminished. Maximum PL activity per unit of growth, which was independent of the inoculum size used, was measured after 2 days of growth and indicated a nonlinear relationship between PL production per unit of growth and time (Fig. 2D). The specific PL activity of our preparations varied from 27.1 to 35.6 U/mg of protein, smaller inocula (up to 10^4 spores per ml) always giving higher values.

Because of the observed decrease in PL activity 2 days after inoculation, the effects of the carbon source and the presence of glycerophosphate on *P. italicum* growth and PL production were only investigated during the first 4 days (Fig. 3). Despite the pectin utilization being similar in all cases (Fig. 3B), maximum biomasses were obtained in cultures grown in commercial Czapek Dox (modified) medium, although our modified medium, containing glucose syrup as the carbon source and no glycerophosphate, was also effective (Fig. 3A). Filtrates of 24-h-old cultures of *P. italicum* grown with glucose as the carbon source showed as much as twice the PL activity as did those grown with sucrose as the carbon source, despite the fact that similar biomasses were measured in all cases. After 60 h of growth, the presence of glycerophosphate in glucose-containing medium seemed crucial for extending PL production (Fig. 3C). The specific activity of PL varied in a similar manner in all cases, maximum values being measured at 60 h (from 24.5 U/mg of protein in modified Czapek medium containing glycerophosphate and glucose syrup to 17 U/mg of protein in the commercially available medium). The same slopes were observed (Fig. 3D) when the relationship between PL production per unit of growth and time was studied in *P. italicum* grown in different media.

To elucidate the effect of the presence of glycerophosphate in the culture medium on PL production, we studied the in vitro effect of glycerol and glycerophosphate on the ammonium sulfate-fractionated *P. italicum* enzyme. The inhibition caused by glycerophosphate at 2.6 mM was negligible, whereas the same concentration of glycerol caused nearly 50% inhibition of the *P. italicum* enzyme (Table 3).

DISCUSSION

From the results obtained after the preliminary screening, *P. italicum* seemed to be the best producer of PL activity among the tested microorganisms grown in liquid media supplemented with pectin. This fungus, which has been reported to be a PL producer when grown on solid media (8),

TABLE 3. Inhibition of *P. italicum* PL activity by glycerol and glycerophosphate[a]

Inhibitor and concn (mM)	% Inhibition
Glycerophosphate	
2.5	3
6.5	6
Glycerol	
0.5	15
2.5	46
6.5	54

[a] An ammonium sulfate (30 to 95% saturation)-fractionated preparation of PL (9.1 U/mg of protein) was incubated with the inhibitor for 15 min and assayed afterwards by the standard procedure. Similar results were obtained when the enzyme preparation was incubated with the inhibitor for 210 min.

is responsible for the decay of citrus fruit. *P. expansum*, previously described (30) as a PL producer when grown in a pectin-polypectate medium as the only carbon source, also appeared to be a good PL producer. In our studies, the presence of another pectolytic enzyme involved in the splitting of pectin, PG, has been detected in both *Penicillium* species. Since PG attacks the main uronide chains of demethylated pectin at the 1,4-α-glycosydic links, such a depolymerizing system would have to involve PE. Although the latter enzyme has been demonstrated in several fluid cultures of *Penicillium* strains (7, 22), it was not detected in our studies. Several workers involved in the study of the PG-PE system have reported problems in detecting PE activity (13, 16) which, incidentally, was also absent in *P. paxilli* (32).

The biosynthesis of many of the pectolytic enzymes studied to date requires the presence of their substrates in the medium. The effect of pectin on the production of PL, despite this induction not being a universal feature, has been reported in both fungi (5, 22, 26, 28) and bacteria (3, 11, 12). In filamentous fungi, PL is considered to be an extracellular enzyme (24), the release of which can be enhanced by the presence of highly esterified pectins in the medium. In our studies, PL from *P. italicum* seemed to be inducible when cells were grown in medium containing different pectin (degree of esterification, 70%) concentrations, and negligible activity was detected in its absence. Similar results were reported for *P. paxilli* grown in modified Czapek medium containing 1% (wt/vol) sucrose as the sole carbon source (33). The low activity measured in 6-day-old culture filtrates could have been due to the induction of PL by some products arising from cell lysis rather than to the constitutive nature of the enzyme. Nevertheless, in autolyzed cultures of *Botrytis cinerea* (18) PL was a constitutive enzyme, although the presence of pectin in the medium did enhance its production. No pectate lyase activity was detected in our filtrates of *P. italicum* when sodium polypectate (Sigma) was used as a substrate, thus confirming the results reported by Bush and Codner (8).

Maximum PL activities obtained with *P. italicum* grown in different Czapek media, containing 0.5 or 1% (wt/vol) pectin as the supplementary carbon source, differed in all the experiments from those found in *A. niger* (35), in which PL formation proved to be non-growth related, as no enzyme production could be detected in the phase of intensive growth (exponential growth phase).

The time course data of growth and PL production were also different from those reported for *P. expansum* (30), in which PL production per unit of growth on a pectin-polypectate medium continued to increase linearly for 9 days.

It is generally agreed that the initial pH of the medium as well as the pH development in the growing culture plays an important role in both enzyme composition and the yield of the enzyme fractions. In *P. paxilli* grown in Czapek medium containing 1% (wt/vol) pectin as the sole carbon source, PL production was detected as long as the pH did not fall below 5.0 (33). In our case, the final pH of cultures grown in the presence of pectin was always less than 4.0. Low pH values, like those found in *P. italicum*, are favorable for the production of pectinases for industrial purposes (17).

As far as the inoculum size is concerned, it is interesting to note that no significant differences in PL specific activities were observed when the inocula used were even fivefold more concentrated, although inocula with lower spore contents always gave slightly higher values.

Maximum biomass was obtained in *P. italicum* cultures grown in commercial Czapek Dox (modified) medium, as was observed in growth studies with cultures of *Fusarium oxysporum* and *F. moniliforme* (20). In *P. italicum* the effect of glycerophosphate on biomass production depended on the nature of the carbon source used for growth. Maximum PL activity (143 mU/ml) was measured with glucose and 1% (wt/vol) pectin as a supplementary carbon source after 4 days of growth. However, in *P. citrinum* and *P. paxilli* maximum production was observed 7 days after inoculation (22, 33). The PL activity of *P. italicum* was about 70-fold higher than that reported for *P. citrinum* grown in a basal medium containing 1% (wt/vol) pectin as the sole carbon source (22). However, in *Pseudomonas fluorescens* (27) a high level of PL activity was reported in cells grown in media containing sucrose or glycerol as the sole carbon source. In this case, maximum enzyme activity was measured after 32 h (exponential phase) or 52 h (stationary phase) of growth in media containing sucrose or glycerol, respectively.

Although it is difficult to compare the rates of PL production from various organisms reported in the literature because of the different enzyme assays and substrates used, *P. italicum* appears to be more effective than other *Penicillium* strains (22). Also, *P. italicum* produced more PL than did *Erwinia chrysanthemi* or *E. carotovora* treated with mitomycin C and UV irradiation, two inductive processes used to increase PL production (34). Recently, a combination of nalidixic acid and plasmid pPE24 containing the *Escherichia coli lexA*$^+$ gene was also described (19) as an effective tool for increasing the production of PL by *E. carotovora* subsp. *carotovora* 71.

Since glycerol has been reported to be an inhibitor of PL from several fungi (25), the in vitro effect of glycerol and glycerophosphate on the ammonium sulfate-fractionated *P. italicum* enzyme was investigated to determine the possible effect of the presence in the culture medium of glycerophosphate on PL production. Glycerol was much more effective than glycerophosphate in inhibiting PL activity at concentrations similar to those used in the original culture medium (2.6 mM). Although 6.5 mM glycerol inhibited 54% of the *P. italicum* enzyme, concentrations about 10-fold higher were required to produce a similar degree of inhibition of the enzyme in *B. cinerea*, *Monilinia laxa*, *M. fructigena*, and *Alternaria alternata*. *Drechslera halodes* PL, reported to be a glycerol-sensitive enzyme, was unaffected at such concentrations (25). The positive effect of glycerophosphate on PL production in cultures of *P. italicum* could probably be explained by assuming the spontaneous or enzyme-catalyzed conversion of glycerophosphate into glycerol, which would act as a powerful inhibitor of the enzyme present in the culture medium and so would promote the release of

more PL from the cells to counterbalance its inhibitory effect. In the last few years, the importance of glycerol and other polyols in inhibiting pectolysis has been stressed as a mechanism for providing a natural protective function in plant pathology, as initially suggested by Lewis and Smith (14, 15).

ACKNOWLEDGMENTS

We thank J. Anasagasti for technical assistance.

A.A. was the recipient of a scholarship (PFPI) from the Spanish Ministry of Education and Science. This work was partially supported by grants from Diputación Foral de Vizcaya (47-I/85) and Universidad del País Vasco/Euskal Herriko Unibertsitatea (310-0122/88).

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