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Polygalacturonate Lyase Production by *Bacillus subtilis* and *Flavobacterium pectinovorum*

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Nutritional factors relating to the production of polygalacturonate lyases by strains of *Bacillus subtilis* and *Flavobacterium pectinovorum* were examined. Studies were carried out in shake flask cultures. In the case of *B. subtilis* the enzyme was produced constitutively, whereas in the case of *F. pectinovorum* it was only produced in quantity in the presence of pectic substances. Glucose was the most suitable carbon source for production of the polygalacturonate lyase of *B. subtilis;* of the nitrogen sources examined, the highest activities per milliliter of supernatant and per milligram of cells were obtained with glutamine and ammonium sulfate, respectively. The pattern of enzyme production and growth was similar although enzyme production ceased at pH 5.3. Sodium polypectate was the best inducer of polygalacturonate lyase with *F. pectinovorum*. Highest activity per milliliter of cell-free supernatant was obtained with skim milk powder as nitrogen source, although ammonium sulfate gave highest enzyme production per unit of biomass. Growth of *F. pectinovorum* occurred between pH 5.7 and 7.2. Enzyme production occurred during active growth and was independent of the pH of the medium.

Many species of wood are naturally resistant to treatment with preservatives, even when applied under pressure. During storage of Sitka spruce (*Picea sitchensis*) in water, bacterial degradation of the tori and bordered pit membranes takes place and this results in a marked increase in the permeability of the wood (1, 2). A number of bacterial species have been isolated from the sap of Sitka spruce poles that had been stored under water; two of these isolates were shown to possess high pectinolytic enzyme activity (4). This observation was significant because the pit membranes in the sapwood contain much pectic material. When these isolates were inoculated into sapwood blocks under laboratory conditions an increase in the permeability of the blocks occurred (3).

The bacterial isolates have been identified as strains of *Bacillus subtilis* and *Flavobacterium pectinovorum*. Both isolates elaborate polygalacturonate lyase extracellularly and grow well and produce this enzyme in sapwood blocks in laboratory experiments. Polygalacturonate lyase activity has also been detected in sap, expressed from water-stored Sitka spruce (18). Polygalacturonate lyase was the only pectic enzyme produced by the bacterial isolates and was likewise the only pectinase detectable in expressed sap of water-stored Sitka spruce. The importance of pectinases in the process is indicated by the observation that a commercial pectinase was also capable of increasing the permeability of sapwood blocks (3).

This investigation is a comparative investigation of some factors affecting production of polygalacturonate lyase by the isolates in batch culture.

MATERIALS AND METHODS

The basal mineral medium consisted of, in grams per liter: K_2HPO_4, 5.0 g; KH_2PO_4, 1.0 g; KCl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $MnSo_4 \cdot 4H_2O$, 0.001 g; $FeSO_4 \cdot 7H_2O$, 0.0005 g. Carbon sources, 0.5% (wt/vol), were added to this medium. The nitrogen sources consisted of complex nitrogen, (peptone, casein, etc.), 1.0% (wt/vol), amino acids 0.5% (wt/vol) or inorganic N, 0.2% (wt/vol). In studies on the effects of carbon and nitrogen sources on enzyme production, the initial pH (pHi) of the medium was 7.0 to 7.2. Media were dispensed in 50-ml volumes in 250-ml Erlenmeyer flasks, and autoclaved at 121 C for 15 min. A standard inoculum was prepared as follows: actively growing cells were centrifuged from the basal mineral medium containing bacteriological peptone (0.5%, wt/vol), washed, and suspended in sterile saline to give an optical density (OD) reading of 10.0 in an EEL spectra colorimeter at 600 nm. All media were inoculated with 1.0 ml of this standard suspension and shaken at 150 rpm in a New Brunswick orbital incubator (model G25) set at 27 C.

Enzyme activity was measured in the cell-free supernatant (CFS). In studying the effect of pH on growth and enzyme production, biomass and enzyme activity estimations were carried out throughout the growth cycle of the organism. In other investigations, enzyme activity was assayed when enzyme production had ceased in the stationary phase of growth. Variations in activity between duplicate culture flasks were negligible, and results were reproducible on repetition of experiments.

Acid-soluble pectic acid (ASPA) was prepared by the method of McCready and Seegmiller (11). Polygalacturonate lyase activity was measured with a solution containing 0.2% (wt/vol) ASPA, 0.001 M $CaCl_2$, and 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.0. The substrate was prepared immediately before use. Appropriately diluted, cell-free supernatant (0.1-ml samples) was added to 2.0 ml of substrate in all assays. The rate of change in absorbance at 235 nm was measured in a 1-cm cell, using a Pye Unicam SP 500 spectrophotometer equipped with a constant temperature unit. An enzyme unit is defined as the lyase activity releasing 1 μmol of product per min at 30 C. A molar absorbtivity of 4,600 M^{-1}/cm was used for this calculation (16). Activities are expressed in units per milliliter of CFS. However, to establish that differences in enzyme activities were not merely due to differences in bacterial growth, activity was also determined as a function of biomass (i.e., in units per milligram of biomass). This value provides a better measure of enzyme induction.

Biomass was measured in OD units at 600 nm in an EEL spectrophotometer. OD units were converted to milligrams (dry weight) of cells by reference to a standard curve. Results of biomass determinations are expressed as milligrams per milliliter of CFS. When an insoluble carbon source was used in the growth medium, biomass was not measured.

RESULTS

Enzyme production by B. subtilis. The effect of carbon source on polygalacturonate lyase production by *B. subtilis* is summarized in Table 1. A 0.2% (wt/vol) amount of $(NH_4)_2SO_4$ was added as nitrogen source to the basal medium. Although considerable enzyme activity was produced on many carbohydrates, glucose was clearly the best carbon substrate. Saccharides containing two or more glucose units were the poorest inducers.

Glucose was incorporated as carbohydrate in media used to investigate the effect of nitrogen source on enzyme production. The results are presented in Table 2. Although highest enzyme activity per milliliter of CFS was measured in media containing glutamine, highest induction as related to biomass was detected with $(NH_4)_2SO_4$ as nitrogen source. However, significant levels of enzyme activity were measured with all nitrogen sources.

The effect of pH on growth and polygalacturonate lyase production by the *B. subtilis* isolate is illustrated in Fig. 1. $(NH_4)_2SO_4$ and glucose were incorporated into the basal salts medium. Although bacterial growth occurred between pH 4.7 to 7.5, the growth rate decreased with increase in pH. Because growth was accompanied by acid production, growth stopped when the pH dropped to about 4.5.

TABLE 1. *Effect of carbon source on polygalacturonate lyase production by B. subtilis*[a]

Carbon source (0.5%, wt/vol)	Biomass (mg/ml)	Polygalacturonate lyase act	
		Units/ml of CFS	Units/mg of biomass
Glucose	0.9	8.4	9.3
Galactose	0.6	2.7	4.6
Lactose	0.24	0.01	0.04
Maltose	1.4	1.8	1.3
Cellobiose	0.9	0.6	0.6
Melibiose	1.4	8.4	6.0
Raffinose	0.9	2.1	2.3
Starch	1.4	2.1	1.5
Amylose		0.6	
Xylan		6.0	
Pectin	1.5	6.0	4.0
Na-polypectate (Sigma, London)	1.6	6.6	4.1

[a] The basal mineral medium contained ammonium sulfate (0.2%, wt/vol) as nitrogen source.

TABLE 2. *Effect of nitrogen source on polygalacturonate lyase production by B. subtilis*[a]

Nitrogen source	Biomass (mg/ml)	Polygalacturonate lyase act	
		Units/ml of CFS	Units/mg of biomass
Peptone	2.5	4.3	1.7
Casein	3.0	6.6	2.2
Casamino Acids	2.0	7.7	3.9
Yeast extract	2.4	9.2	3.8
Skim milk	2.4	3.8	1.6
Alanine	1.8	8.7	4.8
Glutamine	1.8	12.0	6.7
Methionine	0.1	0.6	6.0
Tryptophane	0.1	0.7	7.0
Leucine	0.2	0.6	3.0
Asparagine	1.5	6.0	4.0
$NaNO_3$	0.9	1.6	1.7
$(NH_4)_2SO_4$	0.9	8.0	8.9
NH_4NO_3	0.8	5.1	6.4

[a] The basal mineral medium contained glucose (0.5%, wt/vol) as carbon source.

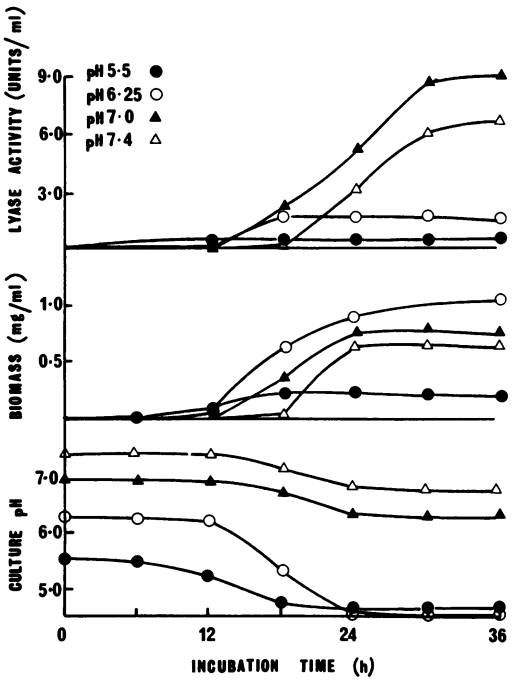

FIG. 1. *Effect of pH on growth and polygalacturonate lyase production by B. subtilis. The initial pH values of the culture media were 5.5, 6.25, 7.0, and 7.4.*

Generally, the pattern for enzyme production was similar to the growth pattern. However, production stopped when the pH of the medium dropped to 5.3, although growth was not impaired.

Enzyme production by F. pectinovorum. In determining the effect of carbon source on polygalacturonate lyase production by *F. pectinovorum*, $(NH_4)_2SO_4$ was added as nitrogen source to the basal medium. The results are presented in Table 3. The enzyme was only induced by pectic substances. On other carbohydrates only a very low level of activity was observed.

Sodium polypectate was added as inducer to media used in investigating the effect of nitrogen source on enzyme elaboration by this organism (Table 4). While highest activity per milliliter of CFS was obtained when skim milk was used as a nitrogen source, activity reflected the high biomass production. The carbohydrate content of skim milk made little or no contribution to the level of activity produced (Table 4). $(NH_4)_2SO_4$ caused highest enzyme production per unit of biomass. Apart from skim milk, enzyme production on complex nitrogen sources was completely inhibited or low (Table 4).

The influence of pH on growth and enzyme production is illustrated in Fig. 2. Sodium polypectate and $(NH_4)_2SO_4$ were added to the basal medium in this experiment. Bacterial growth, which in this case was accompanied by a slight rise in pH, occurred between pH 5.7 and 7.2. Within this range the growth rate increased with increase in pH. Enzyme production occurred during active growth by the organism. Although enzyme production was affected by the growth rate, it appeared to be otherwise independent of the pH of the medium.

TABLE 3. *Effect of carbon source on polygalacturonate lyase production by F. pectinovorum*[a]

Carbon source (0.5%, wt/vol)	Biomass (mg/ml)	Polygalacturonate lyase act	
		Units/ml of CFS	Units/mg of biomass
Glucose	1.9	0.0	0.0
Galactose	0.8	0.06	0.07
Lactose	0.05	0.003	0.06
Maltose	1.7	0.4	0.2
Cellobiose	1.8	0.3	0.2
Gentiobiose	2.0	0.8	0.4
Starch	1.9	0.1	0.05
Amylose		0.3	
Xylan		1.3	
Pectin	0.6	8.2	1.4
Na-polypectate	0.9	36.0	40.0

[a] The basal mineral medium contained ammonium sulphate (0.2%, wt/vol) as nitrogen source.

TABLE 4. *Effect of nitrogen source on polygalacturonate lyase production by F. pectinovorum*[a]

Nitrogen source	Biomass (mg/ml)	Polygalacturonate lyase act	
		Units/ml of CFS	Units/mg of biomass
Peptone	2.6	0.0	0.0
Casein	2.6	0.0	0.0
Casamino Acids	2.1	10.0	4.3
Yeast extract	2.6	8.0	3.1
Skim milk	2.5	40.0	16.0
Asparagine	1.3	25.0	19.4
$NaNO_3$	0.4	6.0	15.0
$(NH_4)_2SO_4$	0.9	35.0	39.0
NH_4NO_3	1.2	26.0	21.6

[a] The basal mineral medium contained sodium polypectate (Sigma, London) (0.5%, wt/vol) as carbon source.

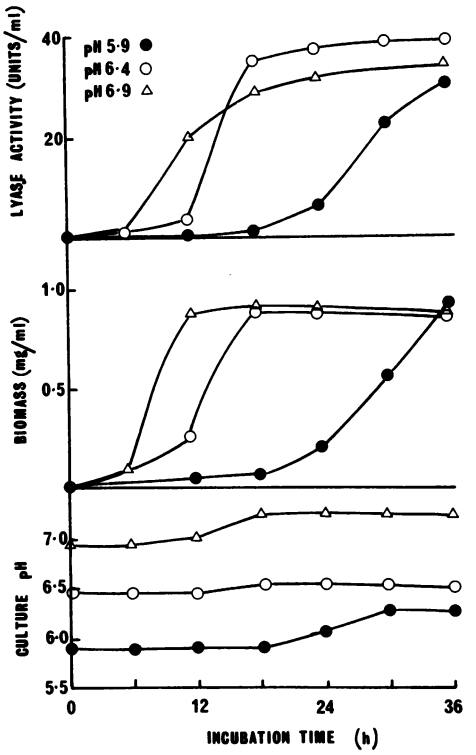

FIG. 2. *Influence of pH on growth and polygalacturonate lyase production by F. pectinovorum. The initial pH values of the culture media were 5.9, 6.4, and 6.9.*

DISCUSSION

Although both organisms perform a similar function in increasing wood permeability, the above study reveals that the polygalacturonate lyase is constitutively produced by the *B. subtilis* isolate but is only induced in *F. pectinovorum* when the medium contains pectic substances. Apart from *F. pectinovorum*, inducible polygalacturonate lyase has been observed with *Erwinia* spp. (21), *Pseudomonas fluorescens* (5, 7, 20), *Pseudomonas marginalis* (13), xanthomonads (17), *Xanthomonas campestris* (14), and *Clostridium multifermentans* (9). On the other hand, although constitutive polygalacturonate lyase synthesis was observed with *Aeromonas liquefaciens* (8) and with *Erwinia* spp. (12), in contrast to *B. subtilis*, activity was repressed by glucose. Repression of constitutive enzyme production by glucose is a very common phenomenon (D. D. Brown and J. Monod, Fed. Proc., **20**:222, 1961; 10). In a number of rumen bacteria (19) and in a strain of *P. marginalis* (21), constitutive polygalacturonate lyase production was not repressed by glucose. With both *B. subtilis* and *F. pectinovorum*, different degrees of repression were observed when nitrogen sources other than $(NH_4)_2SO_4$ were added to the medium. In contrast to this, Hancock et al. (6) observed that polygalacturonate lyase elaboration by *Hypomyces solani* was reduced when individual amino acids or inorganic nitrogen sources were substituted for casein hydrolysates in the growth medium.

As in the case of *B. polymyxa*, polygalacturonate lyases of *F. pectinovorum* and *B. subtilis* are produced mainly in the log phase of growth. The lyases of these two isolates are classical extracellular enzymes, according to the criteria established by Pollock (15), in that they are produced during active growth, are found in the cell-free supernatant, and are secreted before extensive cell lysis has occurred.

The pH range for growth of *B. subtilis* was wider than that of *F. pectinovorum*. Enzyme production occurred in *F. pectinovorum* over its complete growth range, but not at lower pH values in the case of *B. subtilis*. Optimal elaboration of polygalacturonate lyase by *H. solani* (6) occurred within the pH range for production of this enzyme by the two isolates.

During water storage, the pH of the sapwood generally varied from 7.0 to 5.0 (2). It has been illustrated that both organisms grow and elaborate the enzyme within this pH range. The growth rate of the *B. subtilis* isolate was higher at lower pH values, whereas *F. pectinovorum* had an increased growth rate at higher pH values.

This study is part of a research project undertaken to investigate the possibility of utilizing artificial water storage tanks, seeded with selected bacteria, to increase permeability of softwoods to treatment with preservative. The high pectinolytic activity and resultant ability of the isolates to increase sapwood permeability renders them suitable for use in commercial storage plants for increasing wood penetrability of preservatives.

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LITERATURE CITED

1. Dunleavy, J. A., and A. J. McQuire, 1970. The effect of water storage on the cell structure of Sitka spruce (*Picea sitchensis*) with special reference to its permeability and preservation. J. Inst. Wood Sci. **6**:20-28.
2. Dunleavy, J. A., and W. M. Fogarty, 1971. The preservation of spruce poles using a biological pretreatment. Proc. Brit. Wood Pres. Ann. Conv. 1-28.

3. Dunleavy, J. A., and W. M. Fogarty. 1972. Studies on the permeability increase of refractory spruce wood during water storage, p. 320-335. *In* A. H. Walters and E. H. Heuck-van der Plas (ed.), Biodeterioration of materials, vol. 2. Applied Science Publishers Ltd., London.
4. Fogarty, W. M., and O. P. Ward. 1972. Enzyme production by bacteria isolated from water-stored Sitka spruce (*Picea sitchensis*). J. Appl. Bacteriol. **35**:685-689.
5. Fuchs, A. 1965. The *trans*-eliminative breakdown of Na-polygalacturonate by *Pseudomonas fluorescens*. Antonie Van Leeuwenhoek J. Microbiol. Serol. **31**:323-340.
6. Hancock, J. G., C. Eldridge, and M. Alexander. 1970. Characteristics of pectate lyase formation by *Hypomyces solani*. Can. J. Microbiol. **16**:69-74.
7. Heuther, J. P., and G. A. McIntyre. 1969. Pectic enzyme production by two strains of *Pseudomonas fluorescens* associated with the pinkeye disease of potato tubers. Amer. Potato J. **46**:414-423.
8. Hsu, E. J., and R. H. Vaughn. 1969. Production and catobolite repression of the constitutive polygalacturonic acid *trans*-eliminase of *Aeromonas liquefaciens*. J. Bacteriol. **98**:172-181.
9. Macmillian, J. E., and R. E. Vaughn. 1964. Purification and properties of a polygalacturonic acid *trans*-eliminase from *Clostridium multifermentans*. Biochemistry **3**:564-572.
10. Macquillan, A. H., S. Winderman, and H. O. Halvorson. 1960. The control of enzyme synthesis by glucose and the repressor hypothesis. Biochem. Biophys. Res. Commun. **3**:77-80.
11. McCready, R. M., and C. G. Seegmiller. 1954. Action of pectic enzymes on oligogalacturonic acids and some of their derivatives. Arch. Biochem. Biophys. **50**:440-450.
12. Moran, F., and M. P. Starr. 1969. Metabolic regulation of polygalacturonic acid *trans*-eliminase in *Erwinia*. Eur. J. Biochem. **11**:1-5.
13. Nasuno, S., and M. P. Starr. 1966. Pectic enzymes of *Pseudomonas marginalis*. Phytopathology **56**:1414-1415.
14. Nasuno, S., and M. P. Starr. 1967. Polygalacturonic acid *trans*-eliminase of *Xanthomonas campestris*. Biochem. J. **104**:178-195.
15. Pollock, M. R. 1963. Exoenzymes, p. 121-178. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 4. Academic Press Inc., New York.
16. Spectrophotometry nomenclature. 1965. Analyt. Chem. **37**:1814.
17. Starr, M. P., and S. Nasuno. 1967. Pectolytic activity of phytophathogenic *Xanthomonads*. J. Gen. Microbiol. **46**:425-443.
18. Ward, O. P., and W. M. Fogarty. 1973. Bacterial growth and enzyme production in Sitka spruce (*Picea sitchensis*) sapwood during water storage. J. Inst. Wood Sci. **6**:8-12.
19. Wojciechowicz, M. 1971. Partial characterisation of pectinolytic enzymes of *Bacteroides ruminicola* isolated from the rumen of a sheep. Acta Microbiol. Polonica Ser. A. **111 (XX)**:45-50.
20. Zucker, M., and L. Hankin. 1970. Regulation of pectate lyase synthesis and phytopathogenicity of *Pseudomonas fluorescens*. Can. J. Microbiol. **17**:1313-1318.
21. Zucker, M., L. Hankin, and D. Sands. 1972. Factors governing pectate lyase synthesis in soft rot and non-soft rot bacteria. Physiol. Plant Pathol. **2**:59-67.