COMPARISON OF GROWTH AND PECTOLYTIC ENZYME PRODUCTION BY *BACILLUS POLYMYXA*

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ABSTRACT

NAGEL, CHARLES W. (University of California, Davis) AND REESE H. VAUGHN. Comparison of growth and pectolytic enzyme production by *Bacillus polymyxa*. J. Bacteriol. **83**:1-5. 1962.—Studies were made of pectolytic enzyme production by *Bacillus polymyxa* during growth. It was found that elaboration of enzyme occurred during logarithmic growth and ceased when the stationary phase was reached. The specific activity of the extracellular enzyme remained relatively constant until lysis occurred. The increased specific activity of the intracellular pectolytic enzyme may be explained if one assumes that the rate of secretion of the intracellular enzyme is dependent upon the concentration of the extracellular enzyme. The concentration of the intracellular pectolytic enzyme dropped markedly at the end of the logarithmic growth phase; the enzyme was released into the medium during the stationary growth phase and subsequent lysis of the cells. It was shown that the intra- and extracellular enzymes were similar or identical in that both were calcium-dependent pectic acid transeliminases.

During the course of previous studies on the pectolytic enzymes produced by *Bacillus polymyxa* (Nagel and Vaughn, 1961*a*, *b*), it became apparent that information on the synthesis and secretion of such enzymes was lacking. However, the relationship between cell multiplication and synthesis and secretion of other enzymes has been studied in some detail. For example, Rogers (1954) reported that production of hyaluronidase and coagulase by *Staphylococcus aureus* was correlated with growth. In another study, Mandels (1956) showed that synthesis and secretion of invertase by *Myrothecium verrucaria* was also dependent upon growth. Similarly, Dworschack and Wickerham (1958) and Wickerham (1958) demonstrated that secretion of invertase by *Saccharomyces uwarum* NRRL Y-972 closely paralleled growth. The review by Phaff (1959) also may be consulted.

The present investigation was made to determine the relationship between cell multiplication and synthesis and secretion of the pectic acid-decomposing fraction of the pectolytic enzyme complex produced by *B. polymyxa*. At least three enzymes are present in the pectolytic complex produced by *B. polymyxa*. These include two extracellular enzymes, pectin esterase and an eliminase, as well as an intracellular digalacturonase. All have been described in part by Nagel and Vaughn (1961*a*, *b*). This paper deals with the pectic acid transeliminase.

MATERIALS AND METHODS

To 200 ml of basal mineral medium (Nagel and Vaughn, 1961*a*) containing 2% (w/v) pectin as the carbon source in a 1-liter Erlenmeyer flask was added 10 ml of a 72-hr culture of *B. polymyxa* grown in the same medium. The flask was shaken on a rotary shaker in an incubator at 30 C; 10-ml samples were removed after different time intervals and centrifuged in the cold (4 C). The supernatant solution was frozen; the cells were resuspended in 10 ml of 0.067 M phosphate buffer (pH 7.0), recentrifuged, and again suspended in 10 ml of buffer. After determination of the optical density, the washed cells were frozen. Prior to determination of gross pectolytic activity and protein concentration, the cells were disrupted by sonic oscillation with a Raytheon 10 kc magnetostrictive sonic oscillator. The cell supension was placed in the cup, flushed with hydrogen for 3 min, and oscillated for 5.5 min. Cell debris was then removed by centrifugation (Servall SS1) at 8,000 × *g* for 10 min in the cold. The supernatant liquid and the original culture solution were dialyzed at 4 C against distilled water.

The protein concentrations in the dialyzed

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cell-free extracts and supernatant culture solutions were determined by the method of Lowry et al. (1951). The optical density method described in a previous publication (Nagel and Vaughn, 1961*a*) was used to determine cell mass. Because of its greater sensitivity under the conditions of these experiments, the viscosimetric technique was used to determine pectic acid degradation. The reaction mixture consisted of pectic acid (Sunkist Growers, Ontario, California) at a final concentration at 0.5% (w/v), 0.1 M HCl-tris(hydroxymethyl)aminomethane (tris) buffer, 0.001 M $CaCl_2$, and the properly diluted enzyme solution (total volume, 10 ml). The substrate and enzyme solutions were allowed to come to equilibrium with the water bath (30 C) before they were mixed. Then 8 ml of the reaction mixture were transferred to an Ostwald-Cannon-Fenske capillary viscosimeter. The pH of the reaction mixture was 7.0. Although this was considerably below the optimum (Nagel and Vaughn, 1961*a*), the sensitivity of this technique was such that there was no difficulty in measuring activity.

The initial flow time was determined on a reaction mixture without enzyme. The reaction time when a viscosity determination was made was arbitrarily selected as the time elapsed between addition of the enzyme and the start of the reading plus one-half of the flow time. The enzyme activity was defined as one over the time in minutes necessary to obtain 50% reduction in relative viscosity. Therefore, one viscosimetric unit would be the amount of enzyme activity necessary to give a 50% reduction in relative viscosity in 1 min. A linear relationship is obtained (Fig. 1) when viscosimetric activity is plotted against enzyme activity, as determined by the hypoiodite technique (Nagel and Vaughn, 1961*a*).

After it was determined that the extracellular enzyme produced by *B. polymyxa* probably was a pectic acid transeliminase (Nagel and Vaughn, 1961*b*), enzyme activity was determined by measuring the optical density (OD) change at 232 mμ vs. time with a Beckman DU spectrophotometer.

To determine whether the intracellular enzyme also was a calcium-dependent pectic acid transeliminase, 100 ml of the basal mineral medium containing 2% (w/v) pectin as the substrate were inoculated with 10 ml of a 68-hr culture of *B.*

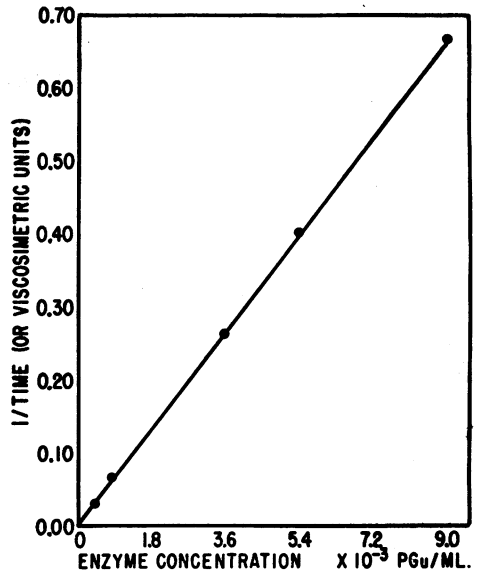

FIG. 1. *Relationship between polygalacturonase activity and reduction of viscosity.*

polymyxa grown in the same medium. The flask was shaken on a rotary shaker in an incubator at 30 C for 40 hr. The cells were separated by centrifugation at 10,000 × *g* for 5 min in the cold. Cells equivalent to 30 ml of the original culture were suspended in 0.067 M phosphate buffer (pH 7.0). The cells were recentrifuged and then resuspended in 30 ml of 0.067 M phosphate buffer at pH 7.0. The cells were then disrupted by sonic oscillation for 5.5 min. Cell debris was removed by centrifugation at 10,000 × *g* for 20 min in the cold. The supernatant solution was dialyzed against distilled water, also in the cold. The dialyzed crude enzyme solution was then assayed for pectic acid transeliminase activity and calcium requirement by measuring the OD change at 232 mμ vs. time. The reaction mixture contained 0.1% (w/v) acid-soluble pectic acid prepared by the procedure of McCready and McComb (1953), 0.001 M $CaCl_2$, 0.05 M tris buffer, and 2.0% (v/v) enzyme at a final pH of 8.5. In addition, the versene mixture contained 0.001 M ethylenediaminetetraacetic acid.

In another experiment to determine the fate of the intracellular enzyme, cells grown as just described were centrifuged at 10,000 × *g* for 5 min, resuspended in 0.067 M phosphate buffer at pH 7.0, and recentrifuged. The cells were then resuspended in filtered basal mineral medium

without pectin and incubated on a rotary shaker at 30 C. Samples (10 ml) were taken at 0, 1, 2, and 3⅔ hr and the OD measured immediately. Then the cells were removed from the sample by centrifugation at 10,000 × *g* for 5 min, and the supernatant was dialyzed against distilled water for 64 hr in the cold. The cells were sonically disrupted as before. Then both the supernatant medium and the sonic-treated, dialyzed cell samples were tested for transeliminase activity and protein content. The reaction mixture was the same as that used for the previous experiment, except that the final enzyme concentration was 2.5% (v/v).

RESULTS

The changes in cell population and extracellular and intracellular pectolytic enzyme activities which occur with time are presented in Fig. 2. As the cells enter the stationary phase of growth, the intracellular enzyme activity drops abruptly. Thus, one must conclude that pectolytic enzyme synthesis ceases at the same time as growth. The extracellular activity reaches a maximum at the same time as the intracellular enzyme fraction and remains approximately constant thereafter.

If enzyme synthesis closely parallels growth, one would expect the ratio of intracellular enzyme to cells (or protein) to remain constant during the active growth phase. Instead, the specific activity of the intracellular enzyme (Fig. 3), whether calculated in terms of cell mass or protein, approximately doubled during the active growth phase.

The ratio of extracellular enzyme to cells also approximately doubled during the active growth phase. The specific activity (ratio of viscosimetric units to mg protein) remained essentially constant during active growth and, as expected, decreased during lysis as a result of nonselective release of cell protein (Fig. 4). These data support the view that the enzyme is selectively secreted during the active growth phase.

However, this view raised the question concerning the identity of the intracellular enzyme. It had been assumed that the intracellular enzyme was similar to or identical with the extracellular pectic acid transeliminase. The latter enzyme requires calcium ions and catalyzes the production of α, β-unsaturated uronides from pectic acid. It is shown by the data in Fig. 5 that the

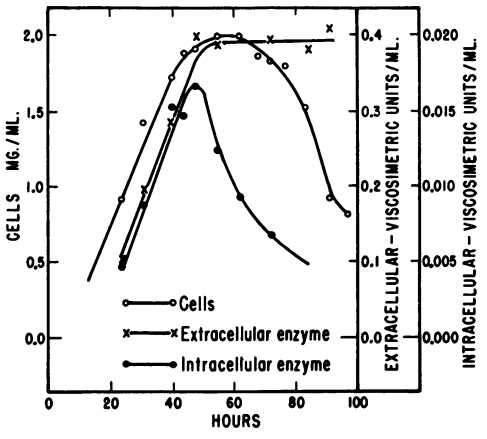

FIG. 2. *Production of intracellular and extracellular pectolytic enzymes during the growth of Bacillus polymyxa.*

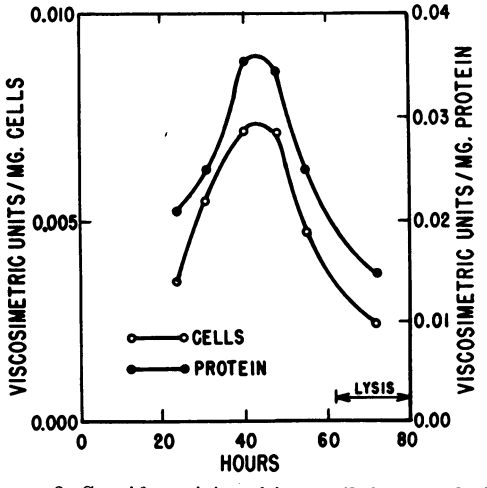

FIG. 3. *Specific activity of intracellular pectolytic enzyme during the growth cycle of Bacillus polymyxa.*

intracellular enzyme also is a calcium-dependent pectic acid transeliminase. Therefore, it would appear that the assumption that the two are similar or identical is warranted.

Two possible explanations exist for the abrupt drop in intracellular enzyme at the end of the active growth phase. There can be little doubt that in either case enzyme synthesis has ceased. The first explanation would be that the enzyme is released into the medium, and since no new enzyme is produced the concentration in the cell decreases. The second explanation would be that the enzyme is broken down within the cell and used for synthesis of other cell proteins. Cells

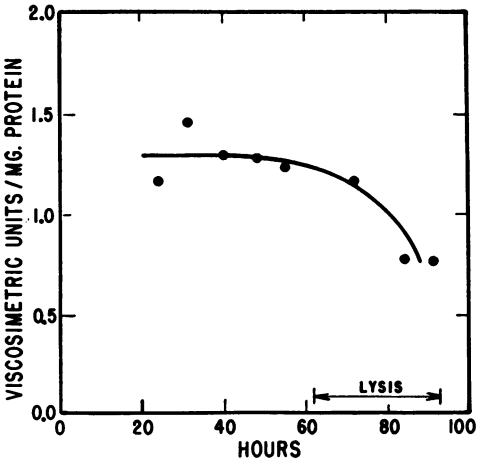

FIG. 4. *Specific activity of extracellular pectolytic enzyme during the growth cycle of Bacillus polymyxa.*

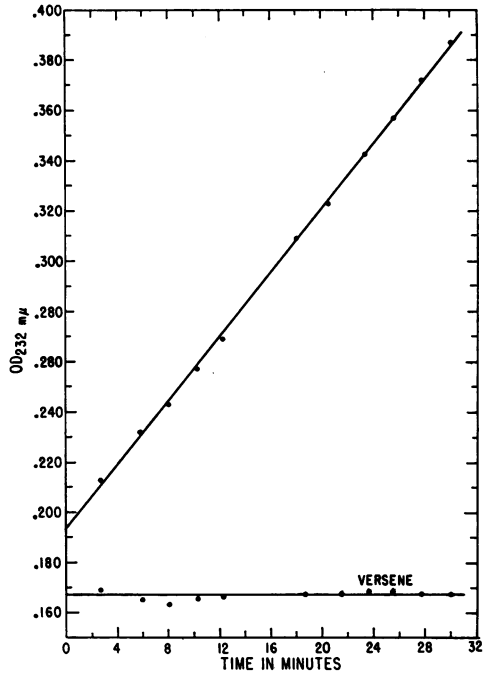

FIG. 5. *Calcium ion dependence of the intracellular enzyme.*

previously washed in 0.067 M phosphate buffer and resuspended in filtered basal mineral medium without pectin were used to determine the fate of the intracellular enzyme. As shown in Table 1 the intracellular enzyme is released into the medium, so that there is an increase in extracellular transeliminase activity with a concomitant decrease of such activity within the cells. Therefore, it is reasonable to conclude that the intracellular enzyme is selectively released (secreted) into the medium, even though it was not possible to completely control lysis; there may also have been a very small amount of contamination of the cells with extracellular enzyme under the conditions of this experiment.

TABLE 1. *Release (secretion) of enzyme from washed cells*

Cells				
Time	Enzyme	Protein	Activity*	Specific activity†
hr	*mg/ml*	*mg/ml*		
0	1.25	0.138	0.01900	0.1380
1	1.00	0.119	0.00904	0.0760
2	0.93	0.110	0.00624	0.0567
3⅔	0.95	0.066	0.00476	0.0723

Supernatant liquid			
Time	Protein	Activity*	Specific activity†
hr	*mg/ml*		
0	0.0060	0.00596	0.993
1	0.0110	0.02010	1.830
2	0.0130	0.02370	1.820
3⅔	0.0225	0.02770	1.230

* Expressed as OD units divided by the number of minutes times the number of milliliters.

† Expressed as OD units divided by the number of minutes times the milligrams of protein.

DISCUSSION

The results obtained in this investigation of pectic acid degradation by *B. polymyxa* are similar to those reported by Rogers (1954) for hyaluronidase production by *S. aureus*. In both cases, the enzymes are synthesized during the exponential growth phase. Enzyme synthesis ceases at the end of the logarithmic growth stage, and the intracellular enzyme concentration drops abruptly. The ratios of both the intracellular and extracellular pectolytic enzymes to cells approximately doubled during logarithmic growth. The specific activity of the intracellular enzyme also increased during exponential growth, whereas the specific activity of the extracellular enzyme remained relatively constant. These results could be interpreted as indicating that

the rate of synthesis of the pectolytic enzyme complex is greater than the rate of synthesis of total protein. However, the results can be explained in another way: if the rate of secretion of the intracellular enzyme is dependent upon the concentration of the enzyme in the medium, the concentration of intracellular enzyme will increase as the concentration of extracellular enzyme increases. Then, when enzyme synthesis stops, the intracellular enzyme is released from the cell into the medium during the stationary growth phase and subsequent lysis, so that the ratio of extracellular to intracellular enzyme increases (Fig. 2).

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