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MICROBIAL DECOMPOSITION OF PECTIC SUBSTANCES

III. Evidence for the role of Protozoa in the retting of plant straws

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ABSTRACT

Evidence has been adduced to show that retting liquors of plant straws of *Catotropis* and *Hibiscus* carry a large load of protozoa. Crude acetone powder extract of the associated protozoal population has been shown to possess pectic enzymes characterized as pectin methylesterase, pectin polygalacturonase and pectin trans-eliminase.

INTRODUCTION

The ability of many species of bacteria, yeasts and fungi to attack pectic substances and the possible role of these microorganisms in the retting of plant tissue have been ably brought out in a review[1]. That actionomycetes too share this power with other types of marine organisms has also been revealed recently[2-6]. Interestingly, about the same time, it was reported that there exist in the rumen of animals protozoa capable of attacking pectin[7-10]. However, this group of organisms has not so far been associated with the retting process notwithstanding the fact that these 'animalcules' have for long been known to decompose many other complex plant residues as cellulose and starch. It is the purpose of this paper to show that certain protozoa not only possess pectic enzymes but are apparently concerned in the retting of certain plant straws which yield good fibre.

Bilimoria[4], during his early studies, observed that rets of *Calotropis* (probably the species *procera*[11]) and *Hibiscus cannabinus* teem with protozoa at certain stages of retting. His further observation that rets made in copper containers, in contradistinction to those made in glass or porcelain wares[12], were free of protozoal fauna was interesting and promoted the query whether or not the fauna was associated in the process. Subsequent observations made by the authors have substantiated the above findings and have thrown some light on the possible role of the fauna in the retting process.

Materials, Methods and Results

Ecosystem. Straws of *Calotropis* sp. weighted under tap water kept in glassware constituted the ecological system. In this system the retting was invariably completed within 4-5 days at room temperature (25-27°C) in contrast to those made in copper containers which took as many as 7-8 days to yield comparatively poor fibre. Furthermore, the retting liquors from the copper-ware were free of the fauna which in the glass-ware appeared conspicuously in the form of a scum on or about the fourth day. This material was comprised invariably of free swimming ciliates (see photomicrograph) and their harvest for experimentation was possible before the appearance in the rets of filamentous fungi. In other words, the scum matter comprising predominantly of the protozoal fauna from the 4-6 days old retting liquors constituted the system used in the experiments.

In practice this retting liquor along with the scum was strained through absorbent cotton by applying suction. This removed the plant debris and other heavier suspending matter. The crude protozal suspension in the filtrate was treated according to the recommendation of Wright[7] with this difference that galactose was not added in the system. Also, centrifugation at 1000 g for 10 minutes was preferred to the settling procedure (in a separatory funnel). At every step microscopic checks were made to ensure that the material predominantly was still comprised of the fauna observed initially. It may however be mentioned here that the procedure affects adversely the free mobility of the protozoal cells, though not their population or activity.

Preparation of acetone powder. The protozoal suspension as made above was precipitated in the cold as a powder according to the procedure adopted previously[3] for obtaining streptomyectal preparation. An extract of this powder, as and when required, was made by homogenising this powder in water in a tissue homogenizer and by centrifuging off the insoluble matter at 10,000 g for 20 minutes. The supernatant usually contained an equivalent of 5 mg of the acetone powder/ml with a protein concentration of 63.6% as measured by the Biuret method[13].

Tests for Pectolytic Activity

1. *Pectin methylesterase.* (*PE*). The extract was tested for its pectin methylesterase activity by the method of Lineweaver and Ballou[14] using a pure pectin

sample (S. B. Penick & Co. Ill.), as substrate. Its PE activity was relatively low and the reaction stopped after 4 hours (Figure I). That the acids formed in the reaction was the result of PE activity was ascertained by testing of the demethylated pectin in the reaction mixture with calcium ions for gel formation. The results indicated the presence of an active PE in the protozoal preparation.

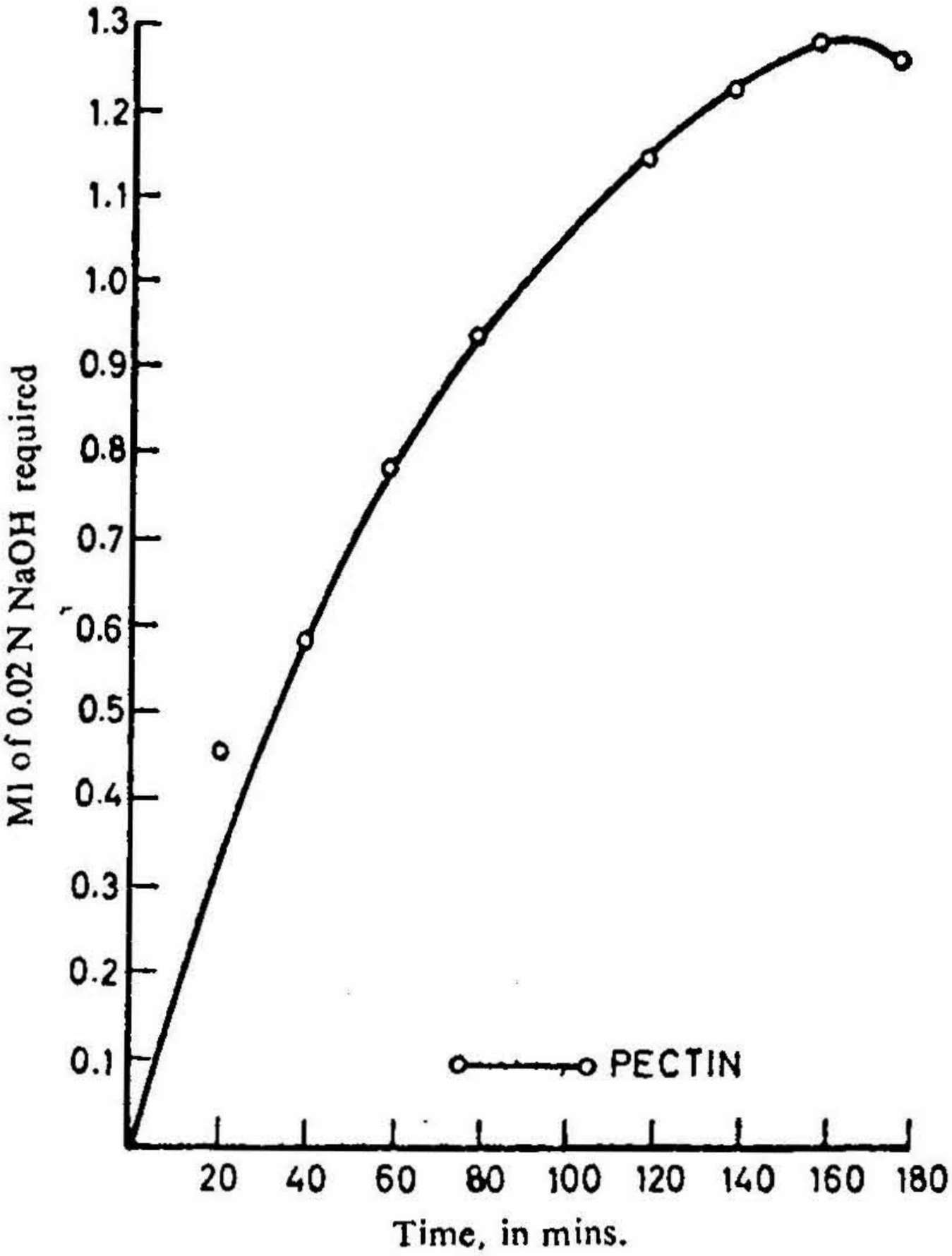

FIG. I

Pectin methylesterase activity of the protozoal extract

The effect of pH on PE activity was then studied using a series of $NaHCO_3$ buffers of pH values 6.0, 6.3, 6.6, 6.9, 7.2, 7.5 and 7.6. The activity was observed to be increasing with the rise in pH as may be evidenced from Figure II. No attempt was made to study this activity at pH 8 and above as pectin is known to get spontaneously demethylated at those ranges.

2. *Pectin polygalacturonase (PG).* This was carried out by Kertesz's method[15] using both pectin and polygalacturonic acid as subtrates. It is evident from the graph (Figure III) that both the subtrates were attacked by the preparation though pectin was degraded at a relatively slower rate than polygalacturonic acid. The optimal activity of PG, when studied in citrate buffers ranging

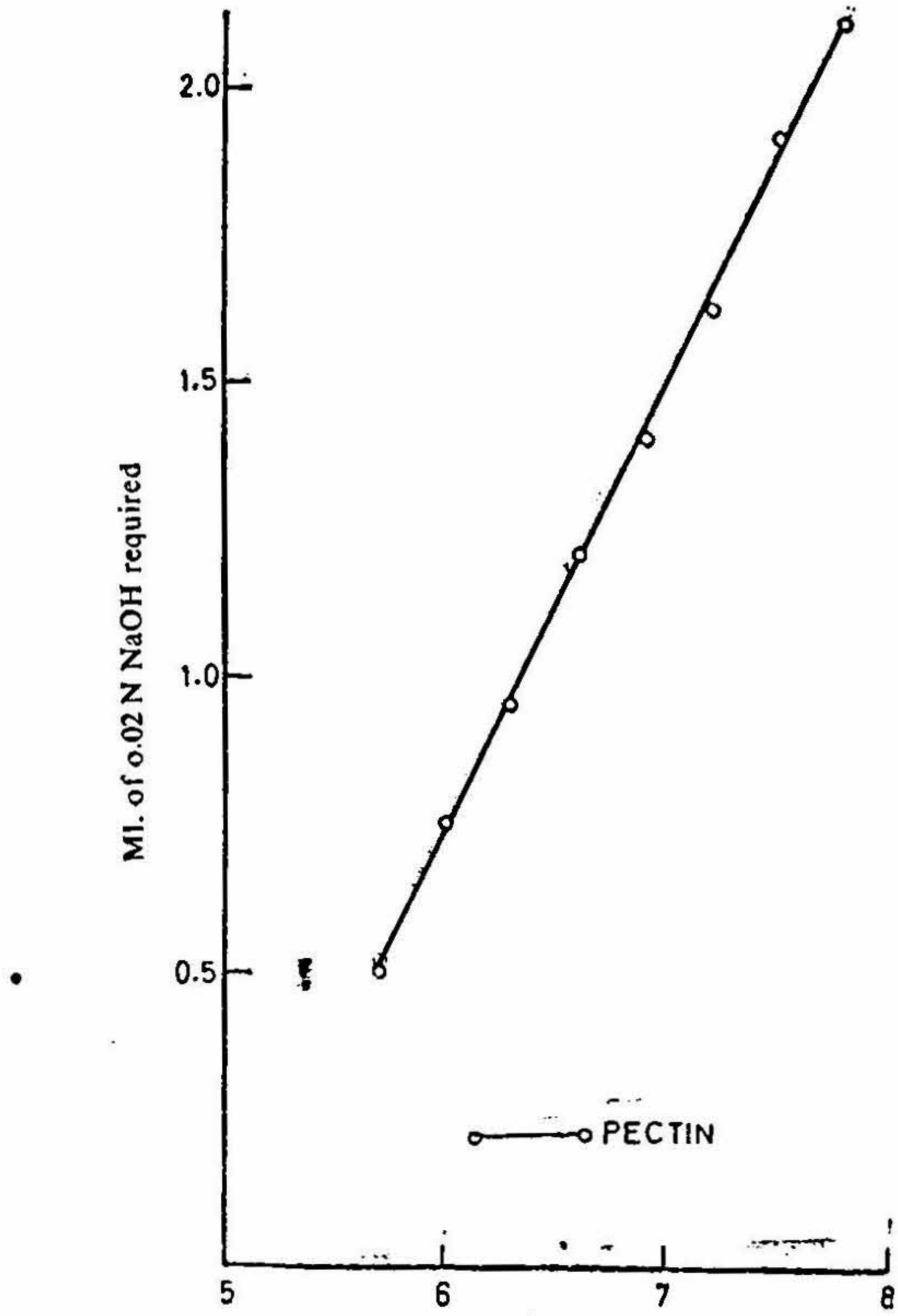

FIG. II

Effect of pH on pectin methylesterase activity of the protozoal extract

from 5.0 to 6.2 and phosphate buffers pH 6.5 to 7.5, was observed (Figure IV) to lie between pH 6.6 and 6.9 at room temperature (25 to 27°C).

3. *Pectin trans-eliminase (PTE).* To detect the presence of this enzyme, the extract was tested by a method similar to that of Nagel and Vaughn[16] and employed by the authors[5] previously. The active presence of this enzyme was indicated by a peak given at 235 mμ in the reaction mixture by the dianer formed due to the action of this enzyme on pectin as well as on polygalacturonic acid at room temperature (Figure V).

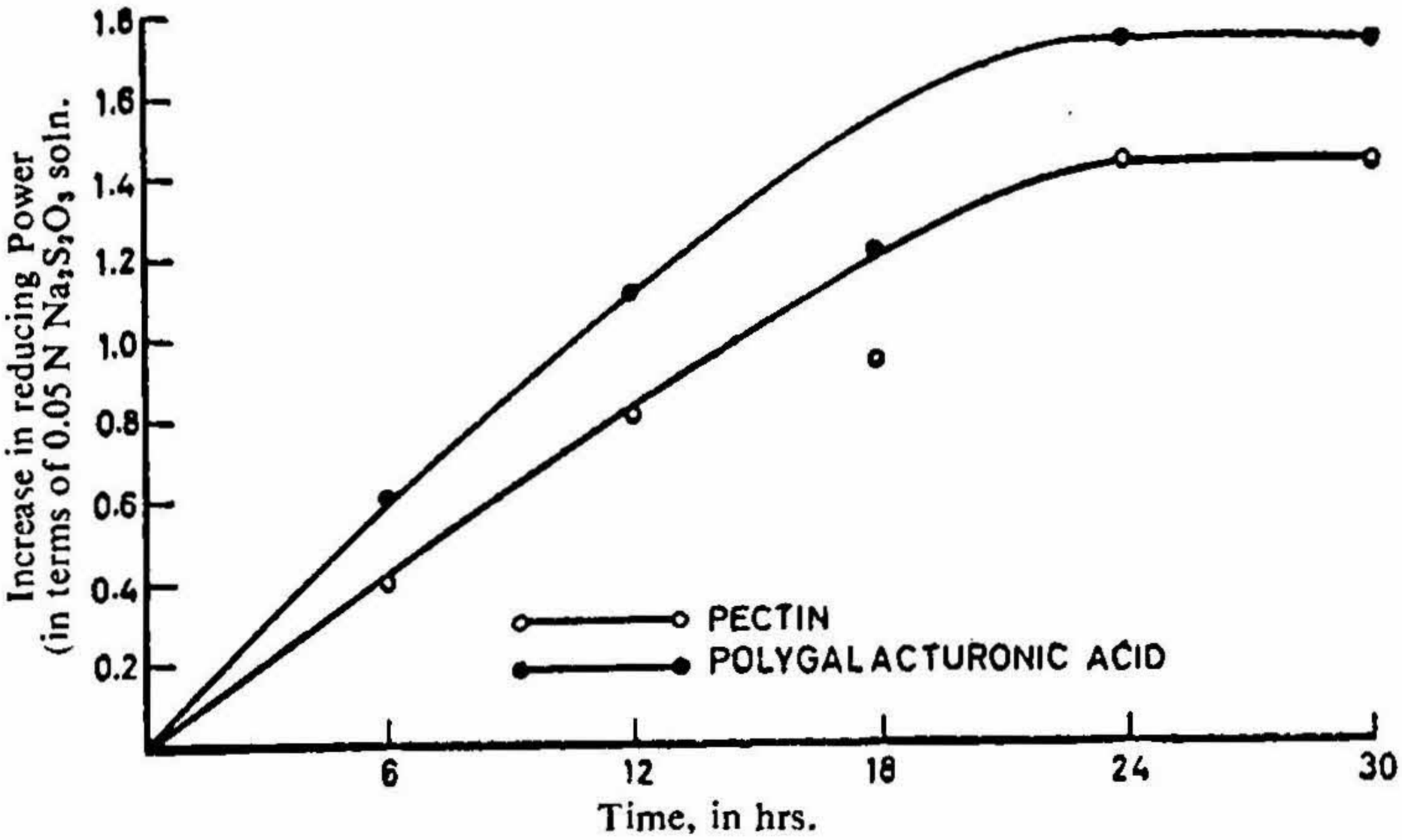

FIG. III

Polygalacturonase activity of the protozoal extract

Chromatography of pectin solutions. The products of decomposition of pectin and polygalacturonic acid solutions were examined by employing paper chromatographic technique (descending). Whatman No. 1 paper, ethyl acetate-acetic acid—water (1 : 1 : 1) solvent system[17], and anilene diphenyl-amine phosphoric acid as the reagent, were used for detecting sugar spots[18]. The chromatograms were run for 12 hours. The reaction mixture containing pectin gave a strong spot similar to galacturonic acid and a fainter spot with $R_f = 0.16$ while that containing polygalacturonic acid gave a series of four spots, trailing behind a much larger spot of galacturonic acid with R_f values 0.16, 0.088, 0.054 and 0.032 respectively. Their R_m values [$\log (1/R_f - 1)$] were suggestive respectively of the mono-, di-, tri- and tetra-galacturonic acids of the oligouronide series[19], though their exact chemical nature remains to be established.

DISCUSSION

The results of this study are in agreement with the earlier studies and tend to demonstrate the presence of PG, PE and PTE in the protozoal preparation. This would mean that the protozoa associated with retting of these plants have the capacity to decompose pectic substance to galacturonic acid and other lower oligouronides. The exact chromatographic behaviour of lower oligouronides with the authentic samples, however, remains to be established. But, the observation that the decomposition of pectin gave only galacturonic acid and that of polygalacturonic acid gave a series of oligouronides suggests the specificity of the polygalacturonase and confirms a similar observation made by Hathway and Seakins in connection with commercial pectic enzymes[20].

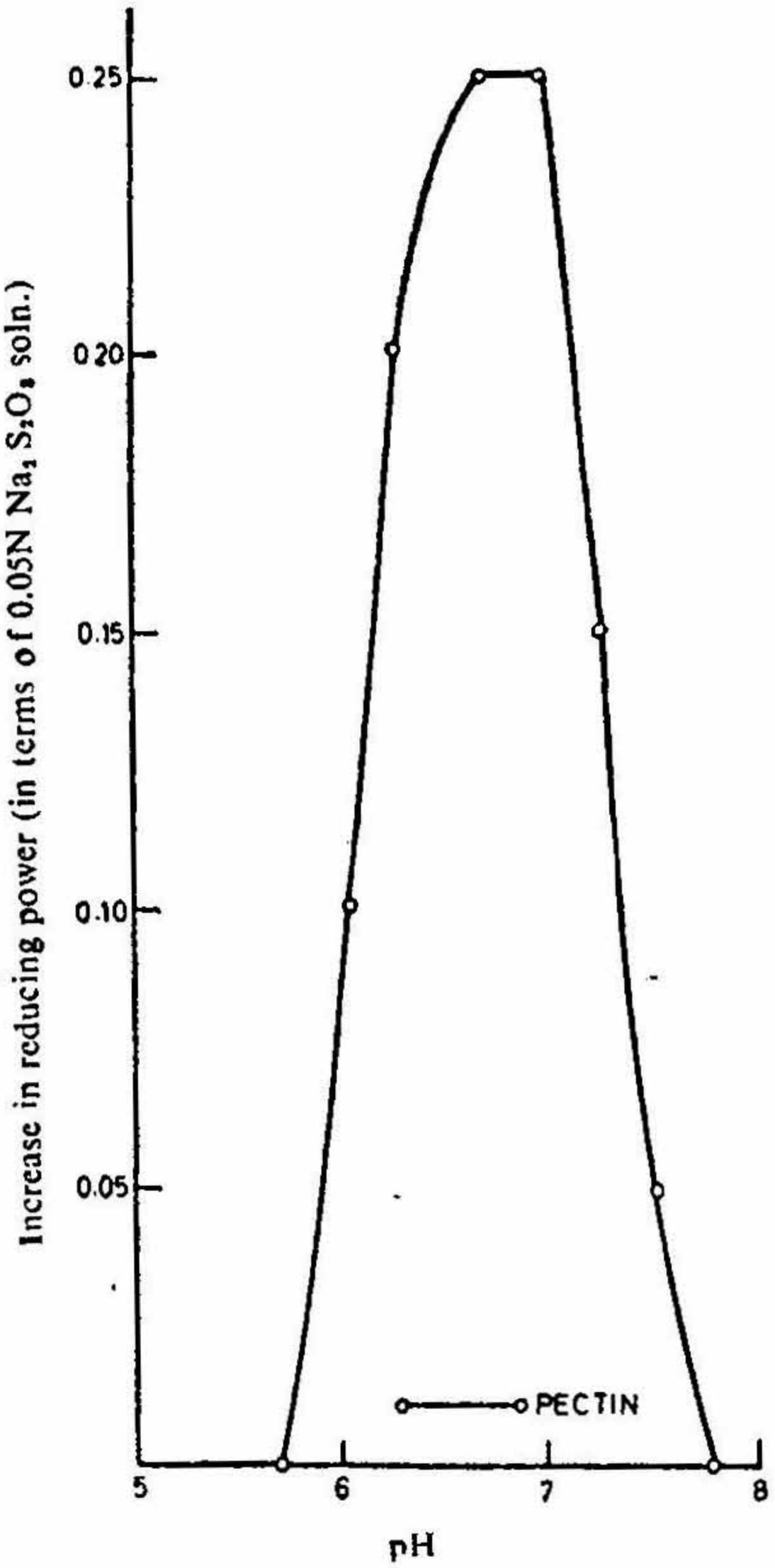

FIG. IV

Effect of pH on pectin polygalacturonase activity of the protozoal extract

The most disturbing factor is the complicity caused by PE which can deesterify pectin constantly, thus changing the substrate with time. (The faint digalacturonic acid-like spot detected in solutions containing pectin explains the situation). Therefore, unless and until pure enzyme systems are worked out, the difficulty of complex enzyme system operating at a time on the same given subtrate will always arise.

Another complicating circumstance is the presence of a few bacteria. Though the protozoal preparation was thoroughly washed with a view to remove all possible external bacteria, the chances are that some bacteria might

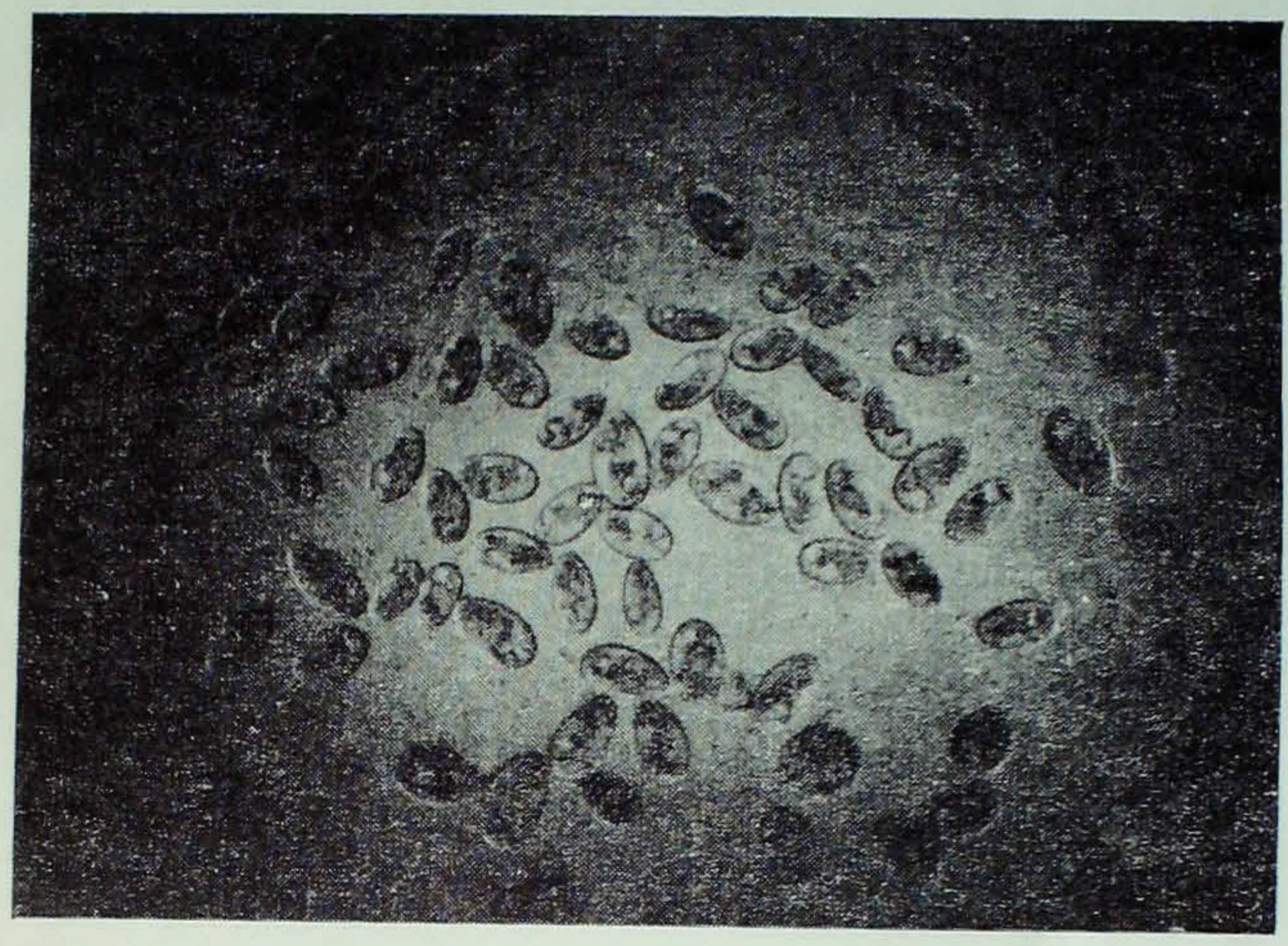

PLATE I

The dominant protozoal sp. encountered in *Calotropis* rets (X 1100)

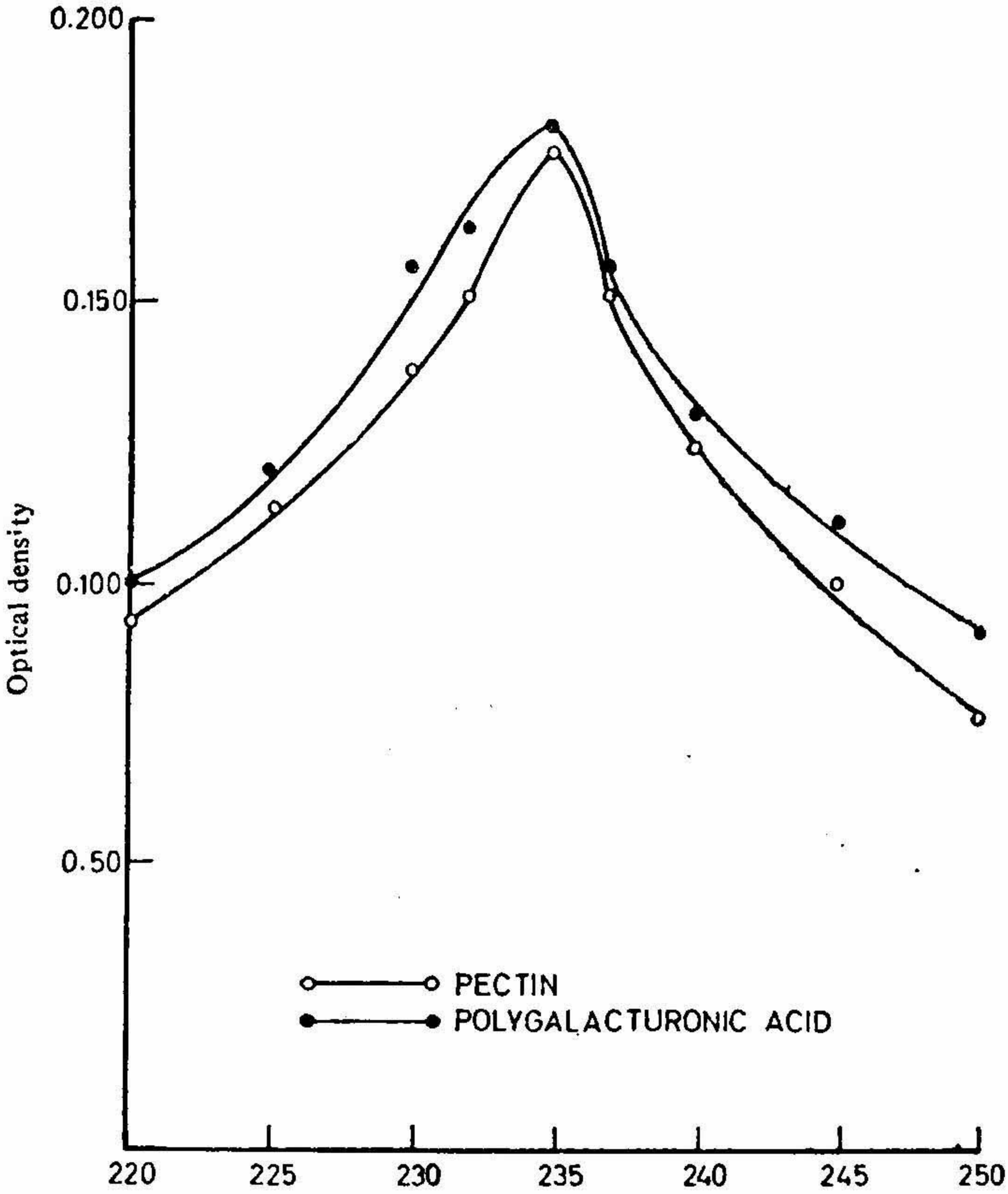

FIG. V

Petin trans-eliminase activity of the protozoal extract

have remained within the ciliates. However, it is not likely that they would affect the enzyme make-up of the extracts to a significant degree.

The role played by protozoa in the process of retting is quite difficult to assess although it has been possible to show that they possess pectolytic enzymes. It is not unlikely that they bring about many changes in the process of retting by their hitherto undiscovered properties. These can, however, be ascertained only after pure cultures of protozoa have been made and examined for their pectolytic and other activities. In the meantime it may be stated that the preliminary efforts made in this direction indicate that PG and PE can be shown quantitatively in nearly pure cultures, relatively free of bacteria

(unpublished data). Further efforts in this direction are underway and may bring some interesting and useful results in the future.

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