MICROBIAL DECOMPOSITION OF PECTIC SUBSTANCES

I. Role of Soil Streptomycetes in the Process

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Abstract

One hundred and fifty streptomycete cultures, representing several species, were screened for their pectolytic activity. Of these as many as one hundred and forty-five were observed to be pectolytic, *i.e.*, possessing the ability to bring about either demethylation, glycosidic hydrolysis or both types of breakdown of the pectin molecule. That demethylation was enzymatic was confirmed by testing for pectin methylesterase activity by Kertesz's method in the case of a few species. The favourable effect of calcium carbonate in the test medium on the growth and rate of decomposition of pectin as determined by the calcium pectate method was also shown. The pectin glycosidase elaborated by a strain of *S. viridochromogenes* was obtained as an acetone powder. This enzyme was shown to be active at pH 7.6 but not at pH 4.5.

Introduction

A survey of the available literature on the microbial decomposition of pectic substances indicated that the information collected so far can at best be regarded as meagre and fragmentary (Kertesz, 1951; Betrabet, 1957; Wieringa, 1955). This was indeed surprising when it is considered that a better knowledge of the number and types of micro-organisms capable of decomposing pectin is of considerable economic importance in that it would not only help to explain the process of retting of economic plants as jute, flax, hemp, *kenaf*, etc., but also the loss of economic plants through diseases and the spoilage of fruits and vegetables during storage. Hence a systematic work in the field of microbial decomposition of pectic substances with special reference to the role actinomycetes and yeasts was undertaken in this laboratory.

It is necessary to mention in this connection that the earlier reports (Betrabet, 1957; Betrabet and Bhat, 1958) on the retting of two Malvaceous plants, *viz.*, *Malachra capitata* Linn. and *Hibiscus cannabinus* Linn. brought to light not only the dominant part certain pseudomonads play in the decomposition of pectin but indicated to the possibility of existence in nature of a wide variety of pectolytic micro-organisms. Supporting evidence for this came from Wieringa (1958). Furthermore, the quantitative studies designed at determining the strength of pectolytic micro-organisms in soil have led Wieringa (1947) and investigators here (1957) to the conclusion that every gram of soil may contain as many as one million viable cells. A critical examination of the soil microflora

also revealed that while moulds and bacteria belonging to several genera have been recognised to possess the ability to decompose pectin, similar ability has not been attributed to actinomycetes, although this class of micro-organisms have been encountered in abundance in soil and other environs associated with pectin decomposition. Even the more recent and excellent reviews on pectic substances and pectic enzymes (Demain and Phaff, 1957; Cochrane, 1958; Deuel and Stutz, 1958), surprisingly, provided only scanty information on actinomycetes decomposing pectin, though Norman (1939) reported on the ability of actinomycetes involved in the degradation of plant materials to decompose polyuronides. Wieringa, it would appear (1947), succeeded in isolating pectolytic actinomycetes by directly plating the diluted soil samples on solid media containing pectin, but liquid enrichments carried out in this laboratory proved unfruitful for the isolation of actinomycetes in as much as fluorescent pseudomonads and yeast-like micro-organisms, among other bacteria almost always overwhelmed the growth of the former rendering their isolation difficult. Presence of pectolytic pseudomonads in pectin enrichments has also been reported by Wieringa (1958) and Graham (1958). At any rate, it was difficult for us to believe that actinomycetes, so important in soil transformations and believed by Waksman as of considerable importance for pectolytic enzymes (1953), could prove to be of no consequence from the point of view of laboratory studies on pectin decomposition. Therefore, as a first step, to establish whether or not this class of micro-organisms are at all capable of attacking pectin, a screening of several species of actinomycetes isolated by routine methods on media devoid of pectin was carried out with the results reported below. An account of the nature of enzymes involved also follows. It may be mentioned here that the nomenclature employed for the pectic enzymes is similar to that recommended by Kertesz (1951).

Materials, Methods and Results

Actinomycete cultures:—Most of the actinomycete cultures screened for pectolytic activity were obtained through the courtesy of Dr. (Miss) Yvonne M. Freitas. They were isolated on the routine culture media recommended for their isolation and were maintained for several years on media which did not contain any pectic substances. All the cultures reported in this paper belong to the genus *Streptomyces*. Isolate CF 68 identified as a strain of *Streptomyces viridochromogenes* was selected for a detailed study of the nature of the enzymes produced as this particular culture proved to be highly efficient in pectolysis.

Medium employed for testing pectolytic activity:—For testing their pectolytic activity the streptomycetes were grown in a liquid medium of the following composition in g. or ml. per 100 ml:

$(NH_4)_2SO_4$, 0.05 g; $Na_2HPO_4.12H_2O$, 0.08 g; KH_2PO_4, 0.02 g; NaCl, 0.005 g; $MgSO_4.7H_2O$, 0.02 g; $CaCl_2$, (0.1 M) 1.0 ml; $FeCl_3$, 0.001 g; micronutrient solution 0.1 ml*; yeast extract (Difco) 0.1 g; liver extract (aqueous) 1.0 ml; bromocresol purple 1.0 ml; pectin (purified) 0.5 g; distilled water to make the volume.

The pH of the medium was adjusted to 6.8—7.0 before sterilization. (The medium was tubed in 5 ml. amounts in 6 × 0.75 inch pyrex test-tubes). On autoclaving the pH fell to about 5.0 and was always readjusted to 7.2 with calculated amounts of sterile 0.05 N NaOH before using. The cultures grown for about 7 days on Czapek agar were inoculated into this pectin medium and the growth was allowed to continue for 15 and 30 days at room temperature (25—30°C) at the end of which the culture filtrate from each was tested for the utilisation of pectin. Subsequent work was done with cultures stored on pectin agar.

For studying the effect of $CaCO_3$ on pectin decomposition 1.0 % $CaCO_3$ was incorporated into the above medium. These experiments were carried out using 50 ml. medium in 250 ml. pyrex conical flasks.

Tests for pectolytic activity in the preliminary screening:—Tests made for determining deesterification or demethylation (methylesterase activity) and glycosidic hydrolysis of pectin were similar to those described by Bell and Etchells (1956). Briefly stated, they are as follows:

(*a*) To 1 ml. of the culture filtrate was added 1 ml. of 0.1 M $CaCl_2$ solution. The formation of a calcium pectate gel was taken as evidence for demethylation of the pectin molecule by the species grown in it. Control tubes of uninoculated medium, under similar conditions, gave no evidence of gel formation.

(*b*) If no gel formation occurred in the above test, the pectin, if any, in the culture medium was demethylated by the addition of a few drops of 1 N NaOH. If on standing no gel formation occurred (absence of pectin), the culture grown in it was considered to be capable of causing glycosidic hydrolysis of pectin. If, on the other hand, gel formation was evidenced the culture was regarded as incapable of bringing about both demethylation and glycosidic hydrolysis of pectin. Appropriate controls were always run.

(*c*) The glycosidic hydrolysis when observed under (*b*) above was always confirmed by the observation of the loss of alcohol precipitability (on addition of two volumes of 95 % ethanol) in contrast to the gel formation observable in the uninoculated controls.

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* Micronutrient solution contained per 100 ml: $ZnSO_4.7H_2O$, 1.1 g; $MnSO_4.H_2O$, 0.5g, $CoSO_4$, 0.005 g; H_3BO_3, 0.005 g; Na_2MoO_4, 0.2 g; $CuSO_4.5H_2O$, 0.0007g.

Test for pectin methylesterase activity:—This test was performed on a few streptomycete cultures with a view to confirm the enzymatic nature of the demethylation brought about by these organisms. The test was considered essential in as much as certain cultures in this group could attain such high pH conditions as to cause demethylation of the pectin molecule. The method followed for performing the test was similar to the one described by Kertesz (1937). It consisted of adding 5 ml. of the culture filtrate of the organisms grown for 5 to 7 days in 50 ml. of the above described medium to 15 ml. of the pectin substrate (purified pectin 0.5 g; NaCl 0.56 g; phenol 0.2 g; bromocresol purple 1 ml; distilled water to make 100 ml) and titrating the acid produced against 0.02 N NaOH. That the acidity produced in this test was the result of pectin methylesterase activity was verified by testing whether the pectin in the reaction mixture could form a gel or not in the presence of calcium ions.

Test for pectin polygalacturonase activity:—The presence of the enzyme in culture filtrates of streptomycetes was measured by estimation of the reducing groups released by the hypoiodite method. To 40 ml. of a 0.5 % polygalacturonic acid (Eastman Kodak) substrate adjusted to pH 7.5 were added 10 ml. of the culture filtrate and the enzyme reaction allowed to continue for 24 to 48 hours. For testing acetone powder (crude enzyme preparation made here) 5 ml. of a 1 % suspension were added to 95 ml. of the 0.5 % substrate. Sodium benzoate and toluene were used to inhibit the growth of contaminating micro-organisms.

Pectolytic activity of streptomycetes:—From Table I it will be seen that of the 150 cultures screened 145 showed distinct pectolytic activity. Of these as many as 103 could break up the pectin molecule by glycosidic hydrolysis, 14 could bring about demethylation and 28 were observed to be capable of causing both these changes. It is clear that the decomposition of pectin by glycosidic hydrolysis would involve the elaboration of a polygalacturonase-like enzyme. That such an enzyme was, in fact, produced in this instance was confirmed in a few cases by testing for the enzyme by estimation of reducing groups released. The strains tested, *viz.*, *Streptomyces viridochromogenes, S. coelicolor, S. antimycoticus* were found positive. Actually, *S. viridochromogenes* showed the presence of maximum amount of this enzyme within 7 days while *S. coelicolor* and *S. antimycoticus* were slower in its elaboration.

While there was no doubt about the ability of the species to effect glycosidic hydrolysis, the demethylating activity, as determined in the preliminary screening (Bilimoria and Bhat, 1960) was open to question in that demethylation could also be brought about, as has been mentioned before, by the alkalinity which actinomycete cultures sometimes attain on incubation. To obviate this possibility cultures filtrates of *S. chromogenes, S. lavendulae, S. longisporus, S. antimycoticus* and *S. viridochromogenes* were tested for this enzyme by Kertesz' method. It is interesting to record here that while this enzyme could

not be detected in the case of *S. viridochromogenes* due to high glycosidase activity, in the other slow pectin decomposers it was possible to detect pectin

TABLE I

Streptomycetes decomposing pectin*

Name and number of cultures bringing about:		
Demethylation of pectin	Glycosidic hydrolysis of pectin	Both demethylation and glycosidic hydrolysis of pectin
14 cultures	103 cultures	28 cultures
S. anulatus	*S. albidoflavus*, *S. alboflavus*	*S. achromogenes*
S. canescens	*S. albosporeus*, *S. albus*	*S. antimycoticus*
S. citreus	*S. antibioticus*, *S. aureofaciens*	*S. aurantiacus*
S. flavovirens	*S. cacaoi*, *S. californicus*	*S. bikiniensis*
S. noursei	*S. coelicolor*, *S. erythraeus*	*S. chromogenes*
(3 strains) and	*S. exfoliatus*, *S. fasciculus*	*S. circulatus*
seven other	*S. fimicarius*, *S. flavochromogenes*	*S. diastaticus*
unidentified	*S. flavogriseus*, *S. fradiae*	*S. felleus*
species	*S. gedanensis*, *S. glaucus*	*S. flavus*
	S. griseoflavus, *S. griseoluteus*	*S. fulvissimus*
	(2 strains), *S. halstedii*	*S. gelaticus* (2 strains)
	S. lavendulae, *S. lipmannii*	*S. globisporus*
	S. olivaceus, *S. pluricolor* (2 strains)	*S. griseus*
	S. purpurascens, *S. resistomyficus*	*S. longisporoflavus*
	S. rimosus, *S. roseoflavus*	*S. longisporus*
	S. rutgersensis, *S. tanashiensis*	*S. longissimus*
	S. venezuelae, *S. verne*	*S. netrotropis*
	S. vinaceus, *S. violaceoniger*	*S. rochei* (2 strains)
	S. violaceus, *S. viridans*	*S. virgatus*
	S. viridochromogenes	*S. virginiae*
	S. viridoflavus	and 6 other unidentified species.
	and 62 other unidentified species.	

* Of the 150 cultures tested 5 only failed to decompose pectin.

methylesterase activity. That this change was due to presence of free carboxyl groups and not other causes was confirmed by the fact that the demethylated pectin in the reaction mixture acquired the property to form a gel with calcium ions.

Influence of $CaCO_3$ on pectolytic activity:—Since demethylation of the pectin molecule resulted in a considerable fall in pH and actinomycetes as a group prefer alkaline pH for growth, it was considered worthwhile to study

pectin decomposition in a medium containing excess of $CaCO_3$ (1 %). The results recorded are presented in Table II. It will be seen that incorporation

TABLE II

Effect of $CaCO_3$ on pectin decomposition by streptomycetes

Species name and designation	Incubation period in days	Percentage decomposition in 0.5 % pectin medium Without $CaCO_3$	With $CaCO_3$
S. lavendulae (CF.20)	3	7.4	84.2
	6	32.8	97.6
S. longisporus (CF.21)	3	Nil	60.2
	6	4.2	99.8
S. chromogenes (CF.66)	3	Nil	73.7
	6	15.3	97.9
S. viridochromogenes (CF.68)	1	69.7	86.1
	2	78.0	96.7
S. antimycoticus (CF.71)	3	Nil	63.8
	6	35.1	98.6
S. griseoluteus (CF.51)	4	Nil	51.7
S. griseoluteus (CF.26)	7	Nil	93.6

of $CaCO_3$ resulted in a distinct enhancement in the rate of pectin decomposition. Some of the streptomycete cultures, namely, *S. lavendulae*, *S. longisporus*, *S. chromogenes*, *S. antimycoticus* and two strains of *S. griseoluteus*, which were either negative or possessed only low pectolytic power displayed the ability and/or a markedly enhanced capacity in the presence of $CaCO_3$. It is interesting to note, however, that the initially active *S. viridochromogenes* did not derive as much benefit from $CaCO_3$ as did the others when the concentration of pectin in the medium was restricted to 0.5 %, as was indeed the case in all the experiments reported in the table. When, however, the concentration of pectin in the medium was raised to 2 %, as was always done in most of the quantitative studies, the influence of both $CaCO_3$ and the increased concentration of pectin on its growth and ability to decompose pectin became obvious. For example, repeated attempts at culturing of this strain in 2 % pectin without $CaCO_3$ resulted in securing rather poor growth, not to emphasise that the pectin it could decompose under the conditions was as low as 5.6 % in five days and rose upto only 14.1 % in 14 days. When at this stage the pH of the culture was measured it was revealed that the pH was rather too low (pH was 4.45 at the end of 5 days and 4.35 at the end of 14 days) to permit normal growth and/or consequential breakdown of pectin. Incorporation of $CaCO_3$

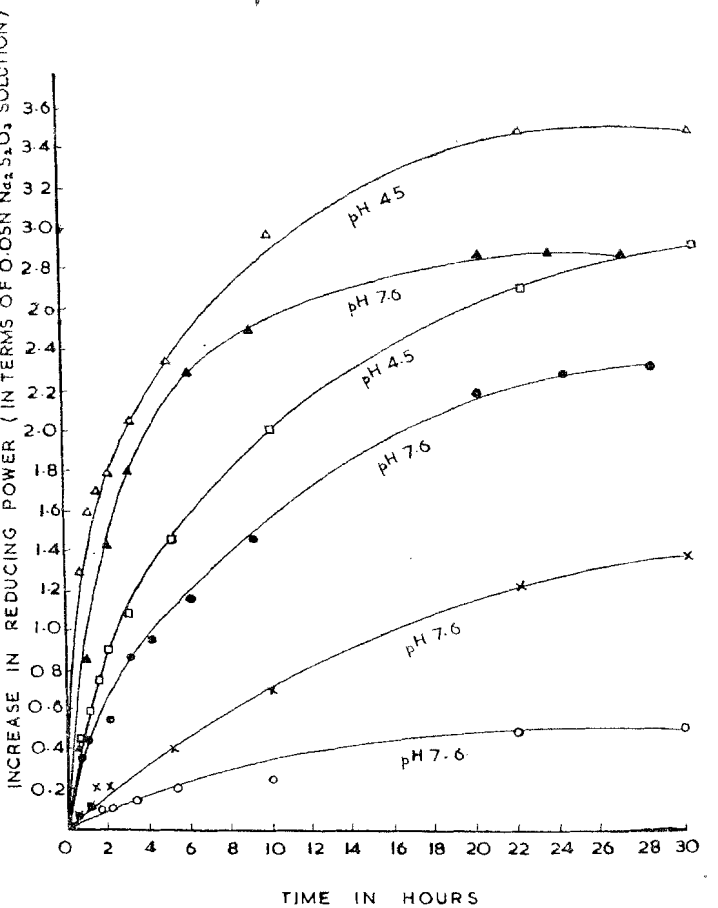

FIG. I

Effect of pH on rate of decomposition of pectic substances by a crude enzyme preparation of *S. viridochromogenes*.

▲, ● Action of the streptomycete preparation on pectic acid and pectin resp. at pH 7.6.

△, × Action of "Pectinase" (N. B. C.) on pectic acid at pH 4.5 and 7.6 resp.

□, ○ Action of "Pectinase" on pectin at pH 4.5 and 7.6 resp.

in the medium under the circumstance was observed not only to enhance its growth, which became abundant, but also its capacity to decompose pectin which then reached a level of 98.5 %.

Additional evidence of the beneficial effect of $CaCO_3$ on pectin decomposition by this strain was derived from the culture filtrates obtained from 2% pectin media with and without $CaCO_3$. Whereas the culture in the former medium (with $CaCO_3$) was accompanied by copious elaboration of pectin polygalacturonase, in the latter (without $CaCO_3$) the culture filtrate from the scanty growth actually failed to show the presence of the enzyme. On the other hand, in the 0.5 % pectin medium no significant difference was evidenced between the cultures grown in the presence of $CaCO_3$ or the rate at which pectin was decomposed by them. Examination of the cultures revealed that the pH had not fallen down in this instance to the extent necessary to suppress growth as was observed in the 2 % pectin medium. In fact, in 2 % pectin media the pH reached a very low level due to the growing culture (which produced acids) as well as due to the alkali consumption—a phenomenon observable in pectin molecules.

Preparation of acetone powder:—The extracellular pectolytic enzyme (s) elaborated by *S. viridochromogenes* was precipitated in powder form with acetone. The activity of this preparation when compared with a commercial pectinase (Nutritional Biochemicals Corporation) showed one important difference (Figure I). Whereas the commercial preparation showed a distinctly higher activity at pH 4.5 the streptomycete preparation at this level was completely inactive, but showed activity at pH 7.5. However, like the commercial pectinase this acetone preparation brought about a rapid loss (within a few minutes) of alcohol precipitability of the polygalacturonic acid in the substrate used for the polygalacturonase test. Both enzymes resulted in an increase in the reducing groups in the reaction mixture of the above enzyme test.

Discussion

The above results seem to justify the conclusion that pectolytic activity among streptomycetes is of widespread occurrence. The high incidence of pectolytic activity among cultures not even initially isolated from pectin enrichments or maintained on pectin media is significant indeed when it is viewed against the failure met with in their isolation from liquid enrichments (containing pectin as the sole source of carbon). No doubt claims have been made for the successful isolation of pectolytic actinomycetes on soil-extract-pectin agar by direct inoculation of soil suspensions (Wieringa, 1947) but the present observation that such a high degree of pectolytic activity is obtaining among streptomycete cultures isolated and maintained on routine media makes it unnecessary, if not altogether impractical, to employ enrichment culture techniques for their isolation. Moreover, it is important to note that not all the soil

isolates made from liquid enrichments could decompose pectin (when tested by the calcium pectate method) a contradiction which is bound to present difficulties till our knowledge of the nature of the pectin molecule and mode of action of the different pectic enzymes is further increased. From all this it appears that polymers like pectin cannot be, as yet, as successfully employed in the enrichment culture methodology as other simpler chemicals of known structure and thus offers a challenge to future workers in this field.

The need for production of pectolytic enzymes for industrial purposes has long been recognized. Indeed, Waksman (*loc. cit.*) as early as 1953, had suggested that actinomycetes could be an important source for pectolytic enzymes. But in the face of their immediate utilisation for the production of antibiotics, cyanocobalbamin and certain other enzymes (Waksman, 1950, 1953) might have resulted in their receding to an obscure group with respect to these enzymes. The present finding suggests that these micro-organisms could form important sources of pectic enzymes (perhaps as a bye product of the antibiotic industry) and thus gain another lead over other types. Although the activity of streptomycete pectin glycosidase at an alkaline pH does not, as yet, warrant its use in the wine and fruit juice industries, none the less the acetone precipitated enzyme obtained by us using *S. viridochromogenes* compares favourably with commercial sample of pectinase and holds out promise of its future exploitation in other industries. At any rate, the present study suggests that a comparative information on the pectic enzymes from different classes of micro-organisms would go a long way in removing the confusion that prevails in the nomenclature of pectic enzymes. For, despite persistent efforts, it is still not clear whether the pectic enzymes bringing about glycosidic hydrolysis constitute one group of different enzymes or represent one and the same enzyme operating at different pH optima depending upon the sources whether fungal, actinomycetal or bacterial. The possibility that the differences in reactions reported in the literature could only be the result of different potencies used has often been suggested and has, in fact, not yet been ruled out. It is hoped that a study designed at clarification of the mechanism of pectolytic breakdown by enzymes elaborated by different groups of micro-organisms would provide a clue to the many intriguing and fundamental questions for which there are as yet no answers.

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