MICROBIAL DECOMPOSITION OF PECTIC SUBSTANCES

II. The role of yeasts in the process with particular reference to a polygalacturonase producing *Cryptococcus laurentii*

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ABSTRACT

A survey of the ability of yeasts to decompose pectic substances has revealed the presence of an exocellular polygalacturonase active at pH 4.5 in a marine strain of *Cryptococcus laurentii*, a strain of *Cryptococcus albidus*, a culture of *Pullularia pullulans*, and two unidentified yeast cultures. The capacity to decompose pectin has also been shown in four strains of *Cryptococcus diffluens*, three of *Saccharomyces kluyveri*, one each of *Cryptococcus laurentii* (terrestrial), *Pullularia* sp., *Saccharomyces bayanus*, *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae* (mutant strain), *Saccharomyces marxianus*, *Saccharomyces turbidans*, and several unidentified cultures, but the presence of the requisite enzyme(s) among these remains to be demonstrated. The nature of the *Cr. laurentii* (marine) polygalacturonase (whether constitutive or inducible) and some factors affecting its production have been considered.

INTRODUCTION

Pectic substances and pectic enzymes are of considerable interest to the scientist and to the technologist alike in that little is understood about their structure and formation notwithstanding the fact that they have long been in use in the food and brewing industries. The interest of this laboratory in investigations on pectic substances stems from a desire not only to understand better the microbiological process of their degradation but to explore the whole range of micro-organisms with a view to select, if possible, those species which can serve well the purpose intended for their industrial exploitation. In a previous paper (Bilimoria and Bhat, 1961) the role of soil streptomycetes in the decomposition of pectin and the characteristics of the enzymes elaborated by one of the species, *viz.*, *S. viridochromogenes*, were reported. The purpose of this communication is to present the results of an examination of yeasts for their ability to decompose pectin and to point out that although yeasts, as a class, cannot be regarded as efficient in their action on this substrate of universal distribution, they contain a few species which are highly active and which hold promise of their utilisation in industry. That yeasts in general are not pectolytic has previously been observed by Luh and Phaff (1951) and by Bell and Etchells (1956).

MATERIALS, METHODS AND RESULTS

Yeast Cultures: One hundred and forty-nine yeast cultures representing twelve genera and thirty-five species along with a large number of mutants derived from two species were obtained from different laboratories and examined for their pectolytic activity. The principle of enrichment culture technique was also exploited for isolating fresh cultures and of the thirty odd isolates obtained this way, twenty proved to be pectolytic and were therefore maintained for a detailed study. The cultures examined were derived from both terrestrial and marine environments. They were repeatedly examined for their power to attack pectin employing media which contained besides 0.5 % pectin-mineral solution,

(i) Yeast extract in both 0.1 % and 0.3 % concentrations
(ii) Liver extract (aqueous) 0.1 % yeast extract
(iii) Malt extract
(iv) 1.0 % peptone – 0.1 % yeast extract
(v) Mixture of ten members of the vitamin B family.

Repeated tests in media of different compositions were necessitated by the fact that many of the pectolytic yeasts displayed poor action on pectin and it was hoped that changes in composition of the medium would bring out the activity more clearly, a circumstance well documented in other studies (Potter and McCoy 1952; 1955). However, supplementation of the basal medium did not result in any enhancement of activity as ascertained by methods previously described (Bilimoria and Bhat, 1961) for the purpose; neither did shaking of cultures help to enhance activity in the case of the weakly positive cultures. On the contrary, it appeared to have an adverse effect on pectin decomposition by them. Table I summarises the results of the screening work.

From a glance at the results in Table I, it will be seen that 36 of a total of 149 cultures tested could attack pectin. Out of these 36 positive cultures, only *Cryptococcus laurentii* (both marine and terrestrial), *Cryptococcus diffluens*, *Cryptococcous albidus*, *Pullularia pullulans* and isolates obtained by the enrichment method were able to rapidly break down pectin, whereas the remaining cultures, though positive, showed action only on prolonged incubation. In fact, most of these weakly positive cultures displayed only poor polygalacturonase (PG) activity. Contrastingly, *Cr. laurentii* (marine), *Cr. albidus*, *Pullularia pullulans*, and isolate numbers 25 and 171 displayed high PG activity. *Cr. laurentti* was the most active among all of these and, as such, was taken up for a detailed study of its enzyme (s) system.

This marine cryptococcus showed its ability to elaborate within 24 hours (shake cultures) a powerful polygalacturonase in the sense its culture filtrate could readily release reducing groups from pectic acid (see Figure I). However, the culture filtrate did not seem to contain pectin methylesterase when tested by the method of Kertesz (1937). Nonetheless it was possible to derive clear

TABLE I

Species of yeast cultures tested for pectolytic activity and number of strains found positive (indicated in brackets)

Terrestrial yeasts :

Candida utilis, 3 (Nil); *Cryptococcus albidus*, 1 (1); *Cryptococcus diffluens*, 4 (4); *Cryptococcus laurentii*, 1 (1); *Hansenula saturnus*, 1 (Nil); *Lypomyces lipoferus*, 2 (Nil); *Pullularia pullulans*, 1 (1); *Rhodotorula glutinis*, 1 (Nil); *Saccharomyces bayanus*, 1 (1); *Saccharomyces carlsbergensis*, 1 (1); *Saccharomyces cerevisiae* and mutants, 47 (1); *Saccharomyces cerevisiae* (distillery yeast) and mutants, 14 (Nil); *Saccharomyces cerevisiae* var. *ellipsoideus*, 2 (Nil); *Saccharomyces italicus*, 1 (Nil); *Saccharomyces kluyveri*, 3 (3); *Saccharomyces ludwigii*, 1 (Nil); *Saccharomyces marxianus*, 1 (1); *Saccharomyces turbidans*, 1 (1); *Schizosaccharomyces octosporus*, 1 (Nil); *Schizosaccharomyces pombe*, 1 (Nil); *Schwanniomyces occidentalis*, 1 (Nil); *Torulopsis pulcherrima*, 1 (Nil); *Torulopsis utilis*, 2 (Nil); *Zygosaccharomyces barkeri*, 1 (Nil); *Zygosaccharomyces priorianus*, 1 (Nil); Unidentified cultures, 27 (17);

Marine yeasts :

Candida guilliermondi, 1 (Nil); *Candida melibiosi*, 1 (Nil); *Candida tropicalis*, 7 (Nil); *Cryptococcus laurentii*, 3 (1); *Debaryomyces hansenii*, 1 (Nil); *Debaryomyces kloeckeri*, 1 (Nil); *Debaryomyces nicotianae*, 1 (Nil); *Debaryomyces subglobosus*, 1 (Nil); *Pullularia* sp., 1 (1); *Saccharomyces fructuum*, 1 (Nil); *Saccharomyces rosei*, 1 (Nil); *Torulopsis candida*, 1 (Nil); *Torulopsis famata*, 2 (Nil); *Torulopsis glabrata*, 1 (Nil); *Torulopsis* sp., 2 (Nil): Unidentified cultures, 3 (3).

chemical evidence for its power to degrade pectin by the calcium pectate method (Kertesz 1951) inasmuch as this organism could decompose 56% of the pectin within a 66 hour period, and as high as 81% within 78 hours from the 2.0% pectin medium in which it was tested. Indeed, its activity compares favourably with other pectolytic organisms studied so far (Potter and McCoy, 1952, 1955; Betrabet and Bhat, 1958; Bilimoria and Bhat, 1961) and justifies the conclusion that certain strains of this species, *e.g.*, the one under study, may actually be those associated in the decomposition of pectic substances under marine environs. That the enzyme elaborated by this strain was adaptive was ascertained by manometric and other methods. It is pertinent to refer here that the enzyme obtained by Luh and Phaff (1951) from *Saccharomyces fragilis* was reported to be constitutive and it may be mentioned that unlike the present culture *S. fragilis* failed to degrade pectin as tested by the calcium pectate method.

Preparation of active Cr. laurentii enzyme powder: The polygalacturonase enzyme from this marine yeast was prepared and harvested in the form of an acetone powder by the method detailed before (Bilimoria 1961 ; Bilimoria and Bhat, 1961) for obtaining *Streptomyces viridochromogenes* enzyme (*s*) with this difference that whereas for the streptomycete preparation stationary cultures were preferred, in the present instance shaking of the culture was resorted to for the rapid elaboration of the enzyme. Shaking of *S. viridochromogenes* cultures, it is interesting to recall, resulted in a slight depression on the enzyme production (Bilimoria, 1961), whereas shaking with *Cr. laurentii* cultures enhanced enzyme formation so much so that its elaboration was complete within 24 hours. Prolonged shaking, however, resulted in a fall in enzyme concentration (see Figure I). It should here be emphasized that 50 % acetone precipitated powder represents the endocellular rather than the exocellular polygalacturonase and that for the precipitation of the latter ammonium sulphate at 90 % saturation has been found suitable.

NATURE AND CHARACTERISTICS OF *Cr. laurentii* POLYGALACTURONASE

1. *Action on polygalacturonic acid and pectin*: It has already been mentioned earlier that the strain of *S. fragilis* studied by Luh and Phaff (1951) could not degrade pectin, whereas *Cr. laurentii* could do so readily. Hence, it was considered necessary to test both the culture filtrate and the acetone powder made therefrom for their respective action on polygalacturonic acid and pectin employing the release of reducing groups as an index of enzyme action. The results presented in Figure II clearly bring out that pectic acid, and not pectin, is the natural substrate of this yeast enzyme. In other words, the cryptococcal enzyme is similar to Pectinol 100D (a pectinase manufactured by Rohm and Haas Co.) studied by Jansen and MacDonnell (1945) in that its attack on pectin is restricted to only those glycosidic bonds joining two galacturonic acid molecules with free carboxyl groups, a circumstance which results in restricted hydrolysis of the pectin molecule. The acetone preparation of *S. viridochromogenes*, on the other hand, could readily attack both pectic acid and pectin by virtue of the presence in it of the enzyme pectin methylesterase although the acidity produced even in the buffered substrate had the effect of slowing down of the reaction.

2. *Optimum pH of activity*: The influence of pH on the enzyme of *Cr. laurentii* was then determined. For this purpose, the acetone powder as well as the yeast culture filtrate were employed. In either case it was observed that the optimum pH of this polygalacturonase was around pH 4.5 (see Figure III). In this respect the yeast enzyme resembled the commercial pectinase (Nutritional Biochemicals Corporation) but differed widely from the streptomycete preparation which showed its maximum activity above pH 8.0 (unpublished data).

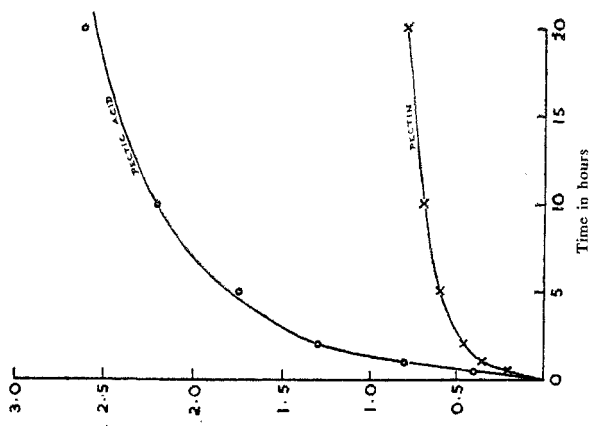

FIG. II
Action of *Cr. laurentii* pectinase on pectin and pectic acid

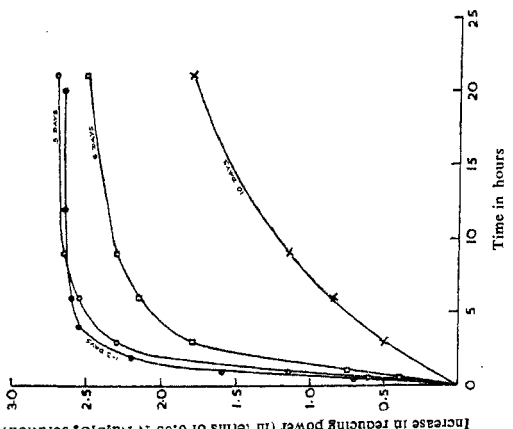

FIG. I
Polygalacturonase activity of *Cr. laurentii* culture filtrates at different intervals of growth (shake cultures)

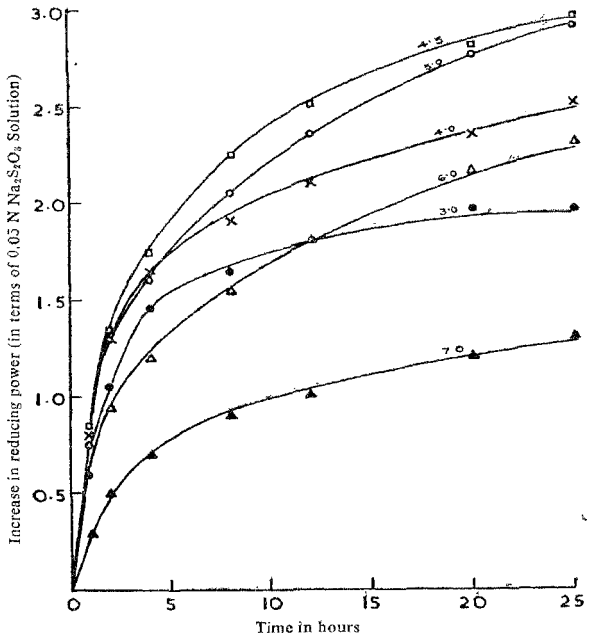

FIG. III
Effect of pH on activity of *Cr. laurentii* pectinase

3. *The nature of Cr. laurentii polygalacturonase*: It was of considerable interest to ascertain if the cryptococcal polygalacturonase was a constitutive or inducible enzyme inasmuch as another yeast polygalacturonase, derived by Luh and Phaff (1951) and Phaff and Demain (1956) from *S. fragilis*, was found to be constitutive. For this purpose, the polygalacturonase producing culture of *Cr. laurentii* was cultured for several generations on a pectin-free medium, *viz.*, glucose-nutrient agar. This glucose adapted culture was then subcultured into (i) pectin-mineral medium, (ii) galacturonic acid-mineral medium, and (iii) glucose-mineral medium and the culture filtrate from each was tested for PG activity. The results of stationary and shake cultures are presented in Figures IV and V. It is clear therefrom that incorporation of pectin or galacturonic acid in the medium was essential for inducing profuse polygalacturonase

production and that in the absence of either of these the enzyme formed was conspicuously low. The possibility that the weak activity observed in the glucose medium (particularly in stationary cultures) could have been due to the induction of the enzyme by a metabolite resultant of growth of the yeast in the medium and not due to the constitutive nature of the enzyme cannot

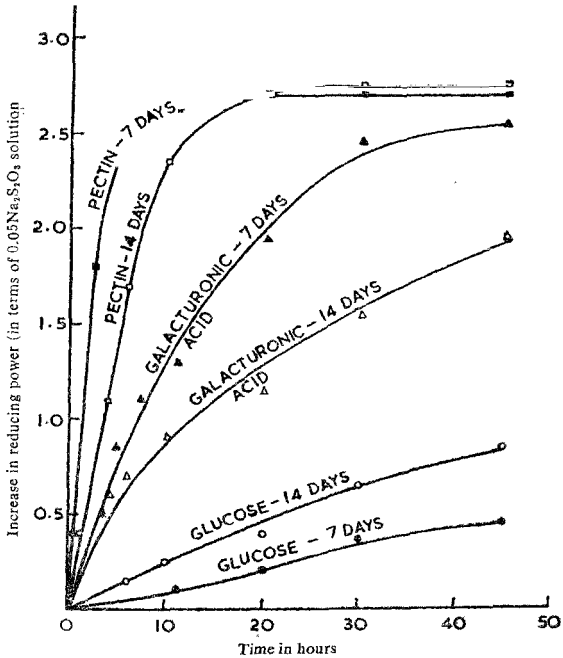

FIG. IV

Polygalacturonase activity of 7 and 14 day culture filtrates of glucose adapted *Cr. laurentii* grown on glucose, galacturonic acid and pectin media (stationary cultures)

altogether be ruled out from the existing information on the subject. Moreover, tests made for polygalacturonase activity by growing glucose adapted *Cr. laurentii* in (i) pectin-mineral medium and (ii) glucose-pectin-mineral medium showed (Figure VI) that the presence of glucose in the medium actually had an adverse effect on enzyme production whether in shake or stationary cultures.

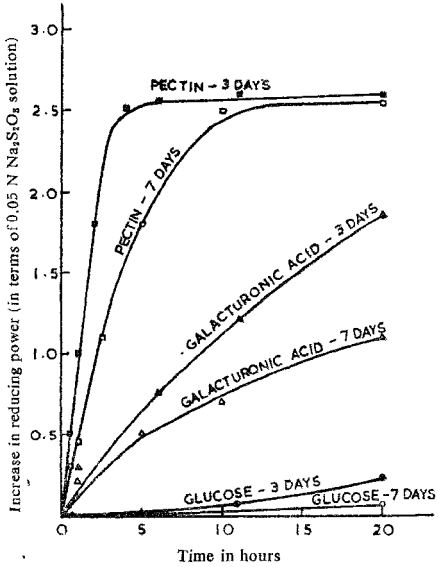

FIG. V

Polygalacturonase activity of 3 and 7 day culture filtrates of glucose adapted *Cr. laurentii* grown on glucose, galacturonic acid and pectin media (shake culture).

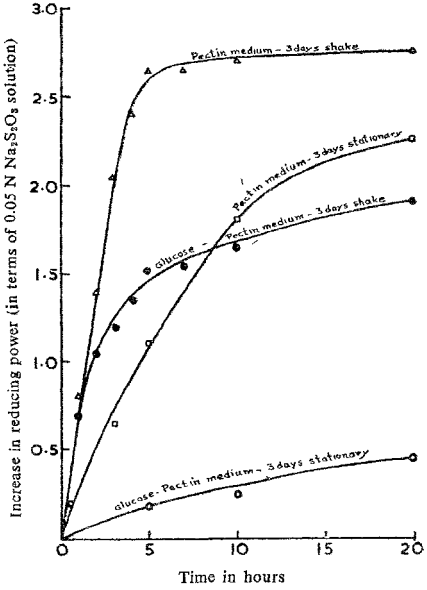

FIG. VI

Effect of incorporating glucose in pectin medium on polygalacturonase produced by *Cr. laurentii*.

Further evidence for the adaptive nature of cryptococcal polygalacturonase was derived from manometric experiments. For these, the yeast, subcultured repeatedly for several generations in glucose medium, was inoculated into (i) glucose-mineral medium and (ii) pectin-mineral medium, and, after growth had occurred for 18-24 hours, the cells were harvested, washed twice with distilled water, and tested for their oxygen uptake at 30°C in the Warburg

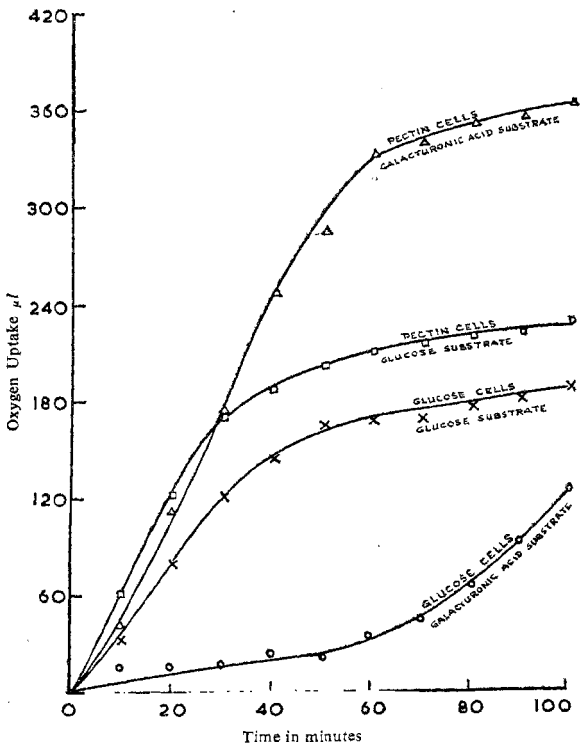

FIG. VII

Oxygen uptake from the oxidation of glucose and galacturonic acid by washed intact cells of *Cr laurentii* grown in glucose and pectin Media

apparatus employing the technique described by Umbriet *et al.* (1957). Glucose and galacturonic acid were the substrates tested in these experiments. From the graph (Figure VII) it would be clear that the pectin grown cells showed an immediate response to oxygen in the presence of both glucose and galacturonic acid, whereas the glucose grown cells displayed a distinct lag phase before utilising the latter substrate, *viz.*, galacturonic acid. From all this it is reasonable to conclude that the enzymes elaborated by *Cr. laurentii* are adaptive in nature with respect to galacturonic acid and pectin. Needless to mention that the enzyme for glucose was constitutive.

DISCUSSION

The survey of pectolytic activity among yeasts presented in this paper would appear to support the observations of Luh and Phaff (1951) and Bell and Etchells (1956) that pectolytic activity is not widespread among yeasts. At the same time it has brought to bear that a closer examination of a large collection of these micro-organisms (particularly members of the genus *Cryptococcus*) under suitable conditions could lead to the revelation of newer species possessing this ability. As far as the authors are aware this report presents the first evidence of pectolytic activity in *Cr. laurentii* and *S. kluyveri* species. In fact, Wieringa (1956) could not detect any pectolytic activity in *Cr. laurentii* he had tested.

In so far as the nature of the cryptococcal polygalacturonase is concerned the experimental evidence adduced is in favour of referring the enzyme as inducible rather than as constitutive in character despite the finding that the presence of glucose in the medium permitted, to an extent, its elaboration. In the presence of pectin, on the other hand, the enzyme elaboration was at once profuse and rapid and this observation is in agreement with that made by Phaff (1947), Mills (1949), and Nagel and Vaughn (1961) in their studies on *Penicillium chrysogenum*, *Pseudomonas prunicola* and *Bacillus polymyxa* respectively. Dingle and Solomons (1952), however, reported on the lack of inducer activity of pectin in the medium used by them to study polygalacturonase activity of *Aspergillus aureus*, *Aspergillus niger* N.R.R.L. 326, *Aspergillus nidulans*, and *Syncephalastrum racemosum*, but considering the complexity of the medium used (consisting of an extract of bran and a protein hydrolysate) their finding is not surprising. Our observation on a culture of *Aspergillus niger* (maintained in this laboratory) showed that the presence of either pectin or galacturonic acid had the effect of inducing copious formation of polygalacturonase in contrast to the low activity witnessed in the glucose medium free from pectin or galacturonic acid. By and large, the highly pectolytic micro-organisms so far studied, viz., *A. aureus*, *A. niger*, *A. nidulans*, *S racemosum* (Dingle and Solomons), *S. fragilis* (Luh and Phaff) and *Cr. laurentii* and *A. niger* studied in this laboratory have all been observed to be able to produce detectable quantities of polygalacturonase in media devoid of pectic substances. Weakly pectolytic

cultures studied in this laboratory, on the other hand, could produce only traces of enzyme even in a medium containing pectin. Under the circumstances can the pectolytic enzyme elaborated copiously in pectin media and sparsely in glucose media be termed truly constitutive? We are inclined to believe that the pectic enzymes produced by *Cr. laurentii*, for example, are inducible and are similar in nature to the inducible, exocellular chitinase described by Reynolds (1954) in a *Streptomyces* sp. and to the inducible polygalacturonase produced by *Streptomyces viridochromogenes* (unpublished data). It is pertinent to refer to the "Unitary Hypothesis" of enzyme formation in this connection and if it is taken to mean that all enzymes are inducible, then the difference between "constitutive" and "adaptive" enzymes altogether vanishes and significance would be attached only to the extent to which enzyme formation occurs. In the meantime, the presence of a powerful polygalacturonase in a marine species in the present instance has opened up a new field for investigation on the subject.

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