RELEASE OF PEROXIDASES
BY CULTURED POTATO CELLS

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SUMMARY

Electrophoretic analysis of peroxidase isoenzymes and other proteins present in the culture medium of potato (*Solanum tuberosum* L. cv. Bintje) cells in suspension culture was carried out by isoelectric focusing and starch gel electrophoresis. The results showed that during the exponential growth phase, the number of peroxidase isoenzymes as well as their activities increased. In addition, several other proteins have increased in the culture medium.

The high specific activity of medium peroxidase as compared to that of cellular peroxidase and differences in the release of various peroxidase isoenzymes suggest that the peroxidase was released by specific secretion.

1. INTRODUCTION

The release of peroxidase from cultured plant cells into the surrounding culture medium has been reported for several species (MISAWA & MARTIN 1972; GAMBURG et al. 1977; MÄDER et al. 1981; STEPHAN & VAN HUYSTEE 1981; HIRSCH & FORTUNE 1984). The predominant part of the peroxidase in the medium appears to be the result of an active secretion (STICHER et al. 1981; KEVERS et al. 1982; VAN HUYSTEE & LOBARZEWSKI 1982). Further, it is known that the release of peroxidase is dependent on several endogenous and exogenous factors, such as culture age (OLSON et al. 1969), temperature (DE JONG et al. 1968) and growth hormone balance (ARNISON & BOLL 1976).

Several physiological studies have been made on the peroxidases of potato, especially in relation to wound healing (BOCHERT 1974; BRINKMAN and SMINIA 1977; THOMAS & DELINCÉE 1979) and disease resistance (FEHRMANN & DIMOND 1967; WEAVER et al. 1971). However, investigations on the properties of peroxidases in potato cell suspension cultures have not been reported. Because of the presumed involvement of peroxidases in growth and differentiation processes (GASPAR et al. 1982), it is important to gain further knowledge on peroxidase activity of *in vitro* cultures.

The present work was undertaken in the framework on growth and plant regeneration from cells of 'Bintje', the most important potato cultivar in The Netherlands. Isoelectric focusing and starch gel electrophoresis were employed to study the release of peroxidases from potato cells into the liquid culture medium. In addition, the patterns of total proteins were studied to obtain a greater insight into the release of proteins in suspension cultures.

2. MATERIAL AND METHODS

2.1. Cell cultures
The cell suspension culture of potato (*Solanum tuberosum* L. cv. 'Bintje') con-sisted of a mixture: a culture that has been initiated and maintained by subcultur-ing weekly for three years by Dr. Ch. H. Hänisch ten Cate of this Institute (GIL-ISSEN et al. 1983) and another line derived from this culture. The latter was sub-cultured every two weeks. The data presented in this article concern the first subculture of the mixed cell population.

2.2. Preparation of extracts
The cell cultures from 2 or 3 flasks (replicates) were mixed thoroughly and fil-tered through a Büchner funnel. The cells were homogenized with 4% NaCl in distilled water in a Potter Elvehjem homogenizer at 0 °C. The crude extracts were centrifuged at 18,000 g for 30 min and the resulting supernatants were used for protein and peroxidase assays or starch gel electrophoresis. For isoelec-tric focusing of cellular proteins and peroxidases, cells were extracted with ace-ton of –15 °C and the subsequently obtained aceton powders extracted with 4% NaCl. After centrifugation, the supernatants were dialyzed overnight against 0.05 M phosphate buffer, pH 7.0, at 2 °C.

The culture filtrates were centrifuged at 18,000 g for 30 min and used without further treatments for starch gel electrophoresis. For isoelectric focusing the culture filtrates were desalted on PD-10 columns (Pharmacia), lyophylized and dissolved in 0.05 M phosphate buffer, pH 7.0, the final concentrations of the peroxidases and proteins being 5 and 50 times higher than those of the medium.

2.3. Electrophoresis
Starch gel electrophoresis was carried out as described previously (BREDEMEIJER 1974) with the following modifications: the gels contained 12% starch and were dried at 4 °C during 30 min.

Isoelectric focusing was performed on thin-layer polyacrylamide gel plates containing Ampholine carrier ampholytes in the pH range 3.5–9.5 (L.K.B. Swe-den).

Peroxidase isoenzyme bands were visualized by staining with benzidine and hydrogen peroxide as described previously (BREDEMEIJER 1974). Protein staining was carried out with the Bio-Rad silver stain according to the Bio-Rad instruc-tions.

2.4. Protein and peroxidase assays
The protein content was also measured according to the instructions for the Bio-Rad protein assay with bovine serum albumin (Merck) as a standard.

Peroxidase activity was measured by following the increase in absorbance at 436 nm and 25 °C which is due to oxidation of guiaiacol in the presence of hydrogen peroxide and enzyme. The reaction mixture contained 0.2 ml of 1.25% guiaiacol, 0.1 ml of extract, 2.65 ml of 0.05 M acetate buffer at pH 5.0, and

Table 1. Relative changes in total peroxidase activity in a suspension culture of potato cells. Cellular peroxidase activity and fresh weight at day 0 are fixed as 1.

	CELLS		CULTURE MEDIUM	
day	peroxidase activity	fresh weight	peroxidase activity	% of total activity
0	1.0	1.0	0.5	33
4	1.2	1.4	0.3	20
10	4.5	4.0	1.8	29
13	4.8	6.0	2.8	37
18	10.1	10.1	3.5	26

0.05 ml of H_2O_2 solution (0.1 ml perhydrol from Merck in 50 ml distilled water). The number of enzyme units was calculated according to the method described in the information sheet on peroxidase (Boehringer, Mannheim).

2.5. Cytological analyses

The staining of viable and non-viable cells with fluorescein diacetate (FDA) and Evan's Blue respectively was carried out according to the procedures of WIDHOLM (1972) and GAFF & OKONG'O-OGOLA (1971) with some modifications: the cell samples stained with FDA were subsequently stained with Evan's Blue.

For determination of the mitotic division cell samples were fixed in ethanol: acetic acid (3:1 v/v). The mitotic index was expressed as the percentage of nuclei undergoing mitosis in the total nuclei scored in a sample (KING & STREET 1978). The number of nuclei analysed for a given period ranged from 600–1200.

3. RESULTS

3.1. Growth and viability of cells in suspension culture

The data on the growth of cells expressed as relative change in fresh weight during 0–22 days are shown in *fig. 1*. The growth curve consisted of a lag phase, an exponential phase and a stationary phase. After 18 days, the culture reached a stationary phase showing about an eight-fold increase in fresh weight.

The mitotic index was 1–2% during the first days after subculture. It increased to a maximum value of 3.5% on day 4 and later decreased to 0% after 11 days. Thus, the maximum mitotic activity occurred prior to the exponential growth phase of the cells, indicating that the exponential growth was caused by cell enlargement.

The staining tests for viability of cells with fluorescein diacetate and Evan's Blue indicated that there were about 20% non-viable cells during the exponential growth phase. During the stationary phase the proportion of non-viable cells increased rapidly, reaching about 40% at day 22.
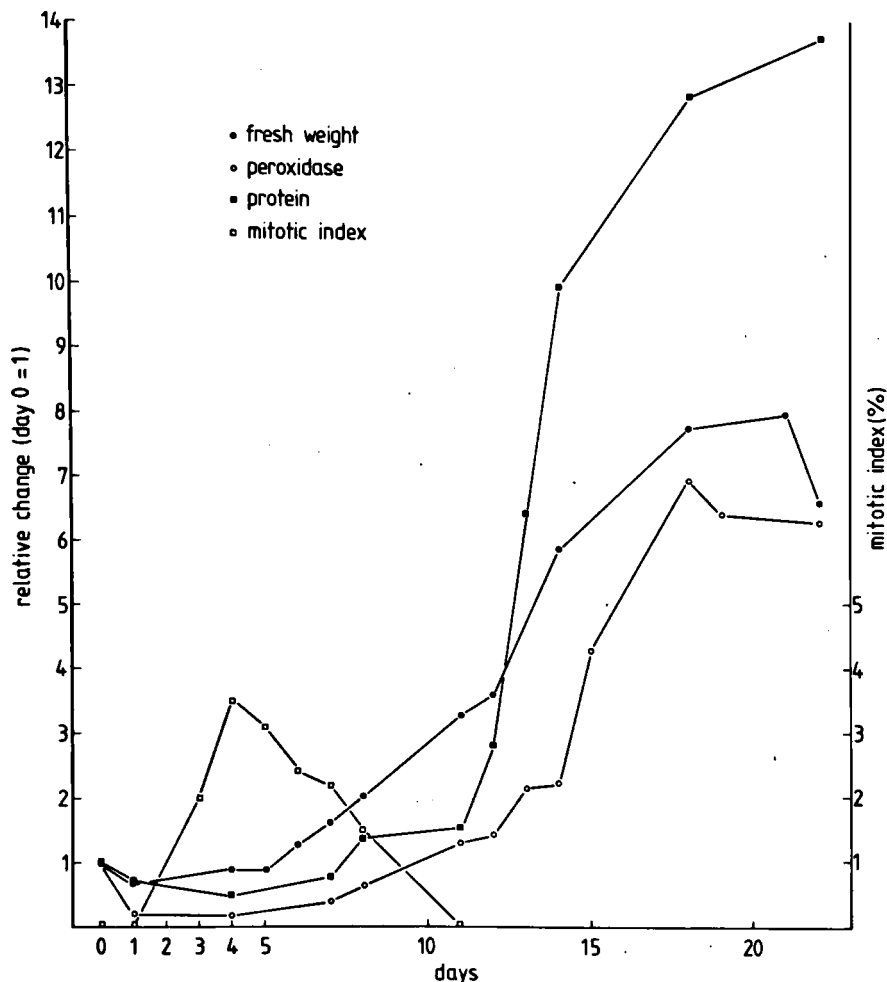
Fig. 1. Changes in fresh weight, mitotic index, and in protein and peroxidase activity of the culture medium during the growth cycle of potato cells in suspension culture.

3.2 Total protein and peroxidase activity in the culture medium

The relative increase in peroxidase activity of the cells during the culture period was similar to that of the fresh weight of the cells (*table 1*). Consequently, the peroxidase activity on the basis of fresh weight changed only slightly during the growth cycle.

    The protein content and peroxidase activity of the culture medium were low during the first days after subculture showing a minimum level at day 4 (*fig. 1*). Afterwards, they increased rapidly, especially during the cell expansion phase, i.e., after cell division decreased to 0%. After 18 days when cells reached
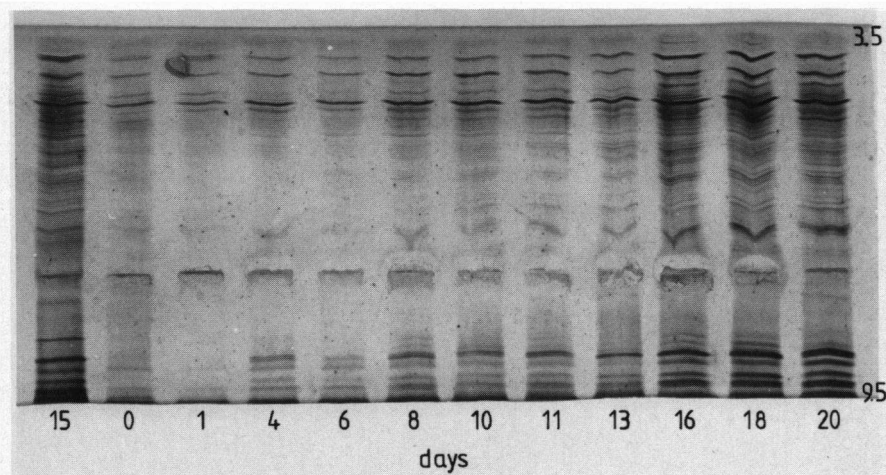
Fig. 2. Isoelectric focusing (pH 3.5 to 9.5) of proteins of the culture medium during the growth cycle of potato cells in suspension culture. The initial day 15 is from the preceding cycle.

the stationary phase, the peroxidase activity decreased while there was a further increase in protein content.

The peroxidase activity in the medium ranged from 20 to 37 per cent of the total peroxidase activity in the culture (*table 1*). The specific activity of medium peroxidase was high. At day 18, it amounted to 67.7 U/mg protein for the medium and only 1.4 U/mg protein for the cellular fraction (data not shown).

3.3. Protein patterns of the medium
The proteins in the culture medium were subjected to isoelectric focusing in a pH range from 3.5 to 9.5. The patterns revealed several protein bands distributed over the whole pH range (*fig. 2*). The staining intensity of the protein bands showed a sudden decrease upon subculture, i.e. on day 0 because of the dilution of the old medium by fresh medium. The increase in the total amount of protein in the medium observed during the exponential growth phase (*fig. 1*) was reflected by an increase in the amounts of all the individual proteins (*fig. 2*). At the beginning of the stationary phase the protein bands showed no further increase in density (*fig. 2*). However, when the cultures became brown (day 21), the amounts of proteins in the medium increased again.

3.4. Peroxidase isoenzyme patterns of the medium
The peroxidase isoenzyme patterns obtained by isoelectric focusing are shown in *fig. 3*. The activity of the isoenzymes strongly decreased following inoculation into fresh medium (day 0). During the first days after subculture only slight changes were observed; the activity of some peroxidases decreased. With the onset of growth, the activity of the peroxidase isoenzymes increased steadily during the exponential phase attaining the highest level at the beginning of the
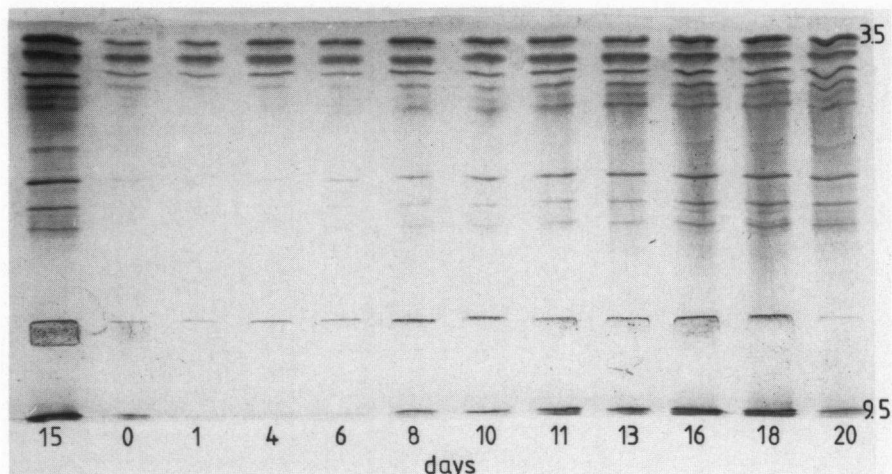
Fig. 3. Isoelectric focusing of peroxidase isoenzymes of the culture medium during the growth cycle.

stationary phase. Afterwards, some peroxidase isoenzymes showed a decreased activity.

It was observed that certain peroxidase isoenzymes had isoelectric points beyond the pH range 3.5 to 9.5. Therefore, starch gel electrophoresis was used to analyse the medium peroxidases. The results are summarized diagramatically in *fig. 4*. Although the peroxidase isoenzymes detected by starch gel electrophoresis cannot be compared as such with those observed after electrofocusing, results obtained by both methods indicated that the activity of all the peroxidase isoenzymes increased steadily during exponential growth of the cells.

In order to investigate the stability of the peroxidases of the culture medium, a cell-free culture filtrate of day 13 was incubated for 4 days at the same temperature as used throughout this study (i.e. 28 °C). There was, however, only a slight decrease in activity of some peroxidases, indicating that the peroxidases released into the culture medium were rather stable.

3.5 Comparison of medium and cellular peroxidases

The comparison of the peroxidase isoenzymes and other proteins present in the culture medium with those in the cells revealed that their release from cells into the medium is a selective process. Various proteins and peroxidase isoenzymes were released at different rates (*figs. 4* and *5*). These results also demonstrate that the electrophoretic mobilities of the peroxidases in the medium were the same as those of the corresponding cellular isoenzymes.

A comparative analysis of the protein and peroxidase patterns revealed that not only peroxidase, but also non peroxidase-proteins were released into the culture medium (*fig. 5*). These proteins may include other enzymes such as amylase and acid phosphatase which are also known to be released by plant cells cultured *in vitro* (STRAUS & CAMPBELL 1963; ARNISON & BOLL 1978).
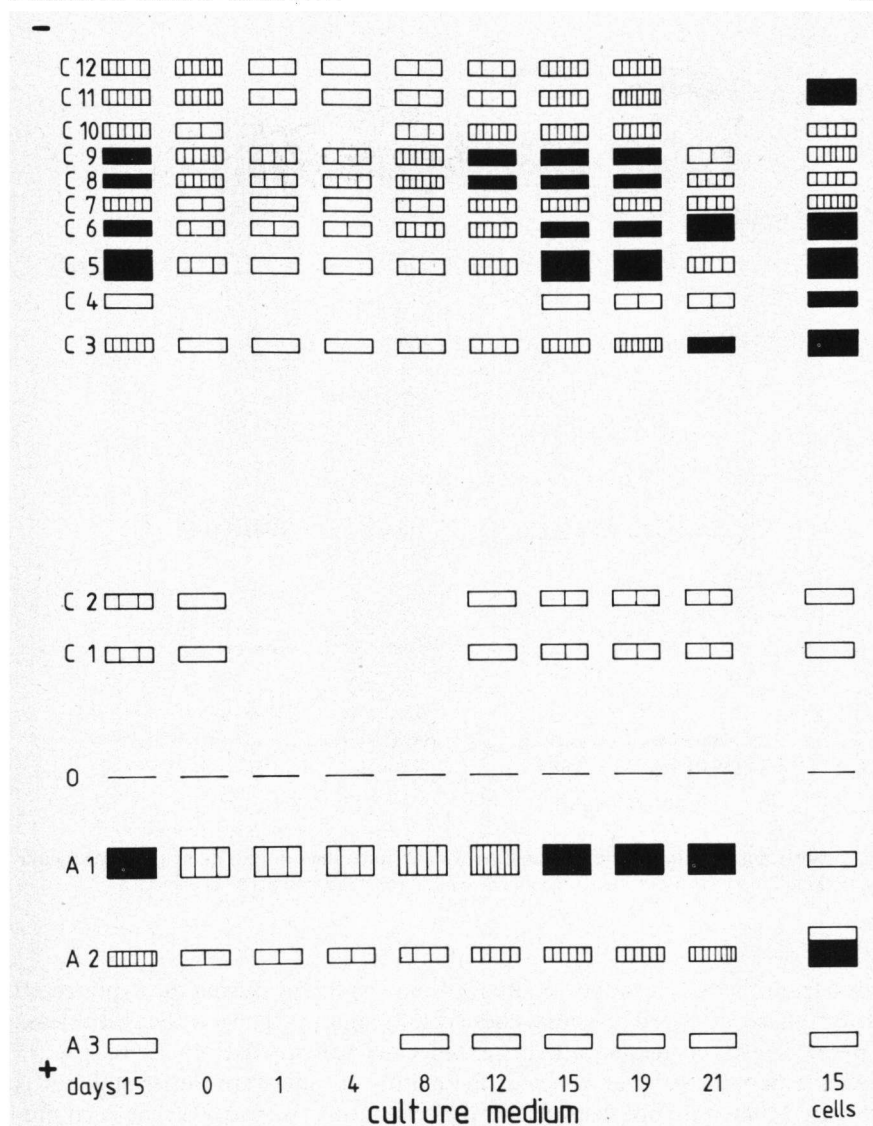
Fig. 4. Starch gel electrophoresis of peroxidase isoenzymes of the culture medium during the growth cycle and of cells at day 15.

4. DISCUSSION

The results obtained in the present study on peroxidase activity in potato cell suspension culture showed that a considerable amount of peroxidases was re-
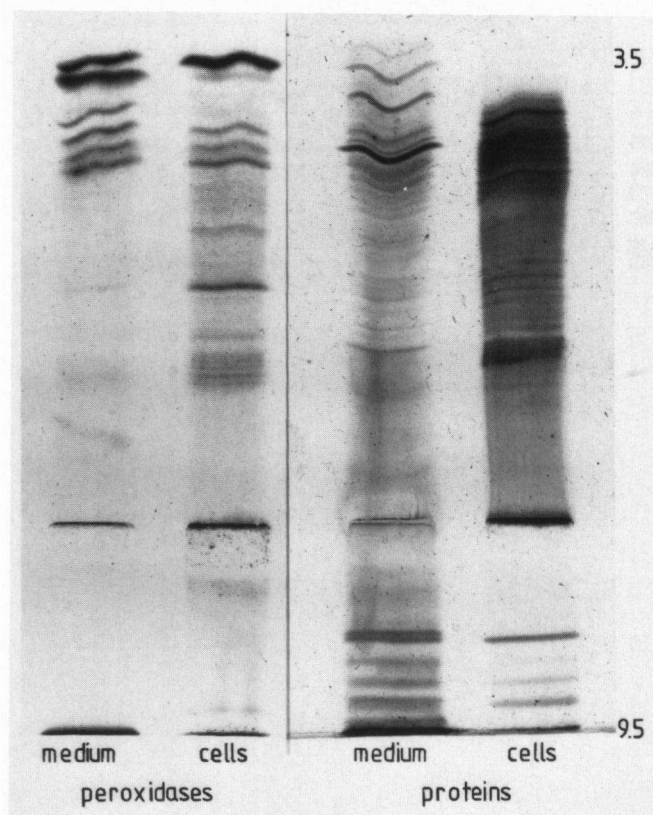
Fig. 5. Isoelectric focusing of proteins and peroxidase isoenzymes of cells and culture medium at day 19. Medium was concentrated 5 × and 66 × for peroxidases and proteins respectively.

leased from the cells into the culture medium, especially during the exponential growth phase, after cell division. These results support the view that the release of peroxidases is correlated with cell expansion (ARNISON & BOLL 1976).

As the peroxidase isoenzymes in the culture filtrate were rather stable it is likely that they were not degraded in the cell culture, similarly as has been presumed for tobacco peroxidases (MÄDER et al. 1981). The stability as well as the continuous release of peroxidases from the cells during growth explain the very high peroxidase activity in the medium at the end of the exponential growth phase. The relatively rapid decrease in activity of some peroxidase isoenzymes during the stationary phase, in spite of their high stability, might be due to absorption by the cells as proposed by MÄDER et al. (1981) or to inhibition by phenolic compounds (LEGRAND et al. 1976). The browning of the culture medium during the stationary phase indeed indicated the release of phenolics into the medium.

In contrast with the results for *Phaseolus vulgaris* (ARNISON & BOLL 1976) and *Silene alba* cell cultures (LEGRAND & DUBOIS (1977), but in accordance with the data for *Beta vulgaris* (KEVERS et al. 1982) the electrophoretic mobilities of the peroxidase isoenzymes detected in the medium of the potato cell culture were the same as those of the corresponding isoenzymes in the cells extracts. The alterations in the electrophoretic mobilities of peroxidase isoenzymes during the release process in other species have been attributed to physico-chemical transformation of the isoenzymes (LEGRAND & DUBOIS 1977; SRIVASTAVA & VAN HUYSTEE 1977). Such modifications do not seem to occur in the potato cell suspension culture. If physico-chemical transformation has taken place, it should have occurred far before the release of the isoenzymes.

The present results are in agreement with the view that a considerable amount of peroxidase in the culture medium is the result of specific secretion (GAMBURG et al. 1977; STICHER et al. 1981). First, the specific activity of peroxidase in the medium was much higher than that of the cellular peroxidase. Second, the various peroxidase isoenzymes were released at different rates. During the stationary phase, one would expect that the release of proteins by active secretion decreases due to senescence (increase in number of dead cells; browning of the culture). Nevertheless, it was observed that the release of proteins including some peroxidases continued suggesting an increase in passive leakage. Attempts to establish the contribution of leakage to the release of proteins by determining the presence of glucose-6-phosphate dehydrogenase (a plasma marker enzyme) in the medium were unsuccessful because no activity could be detected. It is possible that the enzyme, which could be detected only in the cells, was not released or that it was inactivated by medium constituents or by phenolics released from the cells. FIRENZUOLI et al. (1969) have indeed demonstrated that several phenolics inhibit the activity of glucose-6-phosphate dehydrogenease.

The secretion of peroxidases from cytoplasm into cell walls observed in plant tissues (e.g. BIRECKA & MILLER 1974; BREDEMEIJER & BLAAS 1983) has been proposed to be correlated with cell wall synthesis and specific cell wall functions like disease resistance (FRY 1980; GASPAR et al. 1982). The release of peroxidase from the cell walls into the medium of cell cultures in turn might be incidental and without a specific biological function.

Possibly, their release is only a consequence of the fact that the medium forms an artificial continuation of the cells walls. Apart from the question whether or not the presence of peroxidase in the culture medium is incidental, it is clear that this enzyme might influence the properties of the cell culture, e.g. by its IAA oxidase activity (RUBERY 1972).

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