

## PEROXIDASE PRODUCTION BY CELL SUSPENSION AND HAIRY ROOT CULTURES OF HORSERADISH (*ARMORACIA RUSTICANA*)

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The potential of cell suspension and 'hairy root' cultures of horseradish for production of peroxidase was assessed and the factors limiting production in culture were examined. Peroxidase activity is closely related to growth in both cell suspension cultures and 'hairy root' cultures of horseradish. Under the growth conditions used in the present study however, cell suspension cultures produce twice the total activity of 'hairy root' cultures, and up to 8% of this activity is released into the culture filtrate at high specific activity. The growth and peroxidase activity of cell suspension cultures are limited by both phosphate and sucrose and the rate of oxygenation of the culture is critical for maximising growth rate, biomass accumulation and peroxidase activity. The isoelectric focussing banding pattern of peroxidase from the 'hairy root' cultures was identical with commercially available horseradish peroxidase whilst that from cell suspension cultures differed with an additional two bands at high pI.

*Key words:* peroxidase; cell suspension; 'hairy root'; horseradish; *Armoracia rusticana*

### Introduction

Studies on the production of fine chemicals by plant cell cultures have traditionally concentrated on the secondary metabolites used by the food and pharmaceutical industries. Recently however, the attractions of producing valuable enzymes from plant tissue cultures have become evident, and the production of peroxidase from cultures of horseradish has been reported by several workers [1-3].

The enzyme is currently extracted from the roots of field-grown horseradish plants, grown mostly in the tropics, and consequently its production suffers from the uncertainty of the climate. Extraction is also hampered by the coarse, fibrous nature of the plant material and the high levels of acrid allyl isothiocyanates present.

Plant peroxidases are however produced in undifferentiated cells maintained in culture to specific and

total activities which may surpass that of the root [4]. The ease of handling of the cultures, the possibility that a proportion of the enzyme may be secreted, the suppression of secondary metabolism, and the possibility of an inherently high level of productivity in cell suspension cultures suggest that these cultures could provide an attractive system for commercial scale extraction of peroxidase.

In horseradish, production of peroxidase has been demonstrated in callus [2], and in cell suspension culture [1,3]. The latter workers suggested that peroxidase production could be enhanced by callus screening to obtain high-producing lines. One important conclusion from their work was that the slow rate of cell division in culture is a major barrier to commercial exploitation of the technique. However, the authors did not establish the constraints to biomass growth. Furthermore, they did not investigate production in "hairy root" cultures, a fast growing and potentially productive system of culture [5]. This would be an attractive approach to the production of horseradish peroxidase since the enzyme is produced in the root in the intact plant and transfor-

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mation of horseradish by *Agrobacterium rhizogenes* to produce "hairy root" cultures has already been reported as a micropropagation system [6], thus facilitating its exploitation.

## Materials and Methods

All materials were obtained from Sigma Chemical Company Ltd. (Poole, U.K) except for Murashige and Skoog Plant Salts Mixture which was obtained from Flow Labs (Irvine, Scotland) and Biorad Protein Reagent which was obtained from Bio-rad Laboratories (Caxton Way, Watford). Isoelectric focusing reagents were obtained from Pharmacia.

Seeds of horseradish were obtained locally from Mackey Seeds, (Dublin, Ireland). These were surface sterilised in 70% Ethanol for 20 s followed by a 10% solution of Domestic Bleach (Domestos) for 10 min, washed in three changes of sterile distilled water and germinated on Murashige and Skoog medium [7] with 3% sucrose (designated RM) solidified with 0.7% Difco Bactoagar. After emergence of the second pair of leaves the nodal cuttings were transferred to fresh medium, and subcultured at intervals of eight weeks.

Callus was raised by culturing leaf pieces of nodal cuttings on solidified RM supplemented with 5 mg/l 2,4-dichlorophenoxy acetic acid and 0.1 mg/l kinetin (designated 5D). These were subcultured at intervals of four weeks and friable pieces introduced into liquid 5D medium to initiate cell suspensions. These were subcultured at a dilution of 1 part suspension: 3 parts fresh 5D medium every week. For assessment of growth kinetics, nutrient uptake and the effect of the volumetric mass transfer coefficient ( $k_1a$ ) on growth, suspensions in late exponential phase were vacuum filtered and subcultured into 50 cm<sup>3</sup> 5D at a final concentration of 25g/l fresh weight.

'Hairy root' cultures were produced essentially as described previously [8] by inoculation of the petiole of nodal cuttings with a suspension of *Agrobacterium rhizogenes* at 10<sup>7</sup>/cm<sup>3</sup>. *A. rhizogenes* strain LBA 9402 harbouring the binary vector pBin 19 [9] was the kind gift of Dr. John Hamill (Food Research Institute, Colney Lane, Norwich, U.K). Control cultures in which the bacterial suspension was filter sterilised through a 0.22  $\mu$ M pore Milipore filter

prior to inoculation did not produce callus or roots from the site of infection nor were roots ever observed on petioles under any other conditions. 'Hairy root' cultures were established by excising the site of infection together with 1cm on either side of the wound and incubating this in liquid RM medium on an orbital shaker. 500 mg/l Ampicillin was used for three subculture cycles to prevent growth of the bacteria after which the Ampicillin could be removed. 'Hairy root' cultures were characterised by a rapid growth and did not exhibit a strong geotropism.

The growth of cultures was monitored by fresh weight (determined from vacuum-filtered cells), protein (measured by the micro-assay according to Biorad) and packed cell volume (determined as previously described [10]).

To assay peroxidase, the culture filtrate was removed by vacuum filtration and assayed directly. To assay peroxidase that was cell-associated, 500 mg fresh weight of vacuum-filtered cells plus 500 mg of acid washed sand was rapidly ground for 60 s in a mortar with 10 cm<sup>3</sup> of 10 mM phosphate buffer (PH 6.2) containing 150 mM NaCl (PBS). The extract was then made up to 50 cm<sup>3</sup> with PBS and centrifuged at 1000 *g* for 10 min to remove cell debris. The supernatant was decanted and assayed. Peroxidase was assayed at 20°C as previously described [11] using 0.125 mM dimethoxy benzidine and 0.8 mM hydrogen peroxide in PBS. Activity was determined by monitoring the change in absorbance at 410 nm upon addition of 10  $\mu$ l of enzyme solution.

Isoelectric focussing of peroxidase was performed as previously described [12] with the modification that a 1% agarose gel (Zeroelectro-osmosis grade) was used instead of acrylamide. Cell extracts and culture filtrates were centrifuged at 10000 *g* for 20 min, the supernatant made up to 60% v/v acetone, and proteins precipitated overnight at 4°C. The proteins were pelleted by centrifugation at 10000 *g* for 20 min. The supernatant was discarded, the pellet washed once with PBS, and then resuspended in PBS. The peroxidase activity of each sample and standard was then measured and each diluted with PBS to give a final concentration of peroxidase of 5  $\mu$ g/cm<sup>3</sup>. These were applied to the isoelectric focussing gel in strips of Whatman Number 1 filter paper for 30 minutes after which the filter paper

wicks were removed. The calculated loading of peroxidase per lane was 200 ng. Peroxidase isozymes were visualised by using an agarose overlay containing 2.5 mM  $H_2O_2$  and 1.75 mM dimethoxy benzidine.

Carbohydrate was determined by the phenol/sulphuric acid method [13], phosphate by the phosphomolybdate method [14], Ammonium by Nessler's reagent [15] and Nitrate by nitration of brucine [16]

The Volumetric Oxygen Mass Transfer Coefficient ( $k_{1a}$ ) was determined as previously described [17] with the modification that oxygen depletion was by purging with nitrogen. At set time intervals after the onset of aeration, medium was carefully decanted and the percentage oxygen determined using a Clark type oxygen electrode.

## Results

Previous research had shown that peroxidase production in culture was related to growth [1]. Figure 1 shows that growth, as measured by fresh weight and protein accumulation, is closely associated with peroxidase production, in both cell suspension and 'hairy root' cultures. However, the maximum total activity per litre in cell suspension cultures of  $1.2 \times 10^6 \Delta A/\text{min/l}$  is almost twice that of the 'hairy root' cultures at  $0.64 \times 10^6 \Delta A/\text{min/l}$  and the specific activity of the enzyme from cell suspension cultures is also higher than that from the 'hairy root' cultures at  $1200 \Delta A/\text{min/mg protein}$  and  $600 \Delta A/\text{min/mg protein}$  respectively. The growth rate of the cell suspensions is however slower than the 'hairy root' cultures with mean cell doubling times through the exponential phase of culture of 5.5 days and 4 days, respectively, and the final biomass accumulation is only two thirds that of the 'hairy root's.

Peroxidase is released into the culture filtrate from both 'hairy root' and cell suspension cultures (Fig. 2). Release from 'hairy root' cultures probably results from the initial cut injury prior to subculture as there is little change in activity throughout the growth cycle and the specific activity of peroxidase in the culture filtrate matches that of peroxidase which is cell-associated. In contrast, in the cell suspension cultures, peroxidase is released continuously over the growth cycle to up to 8% of the total

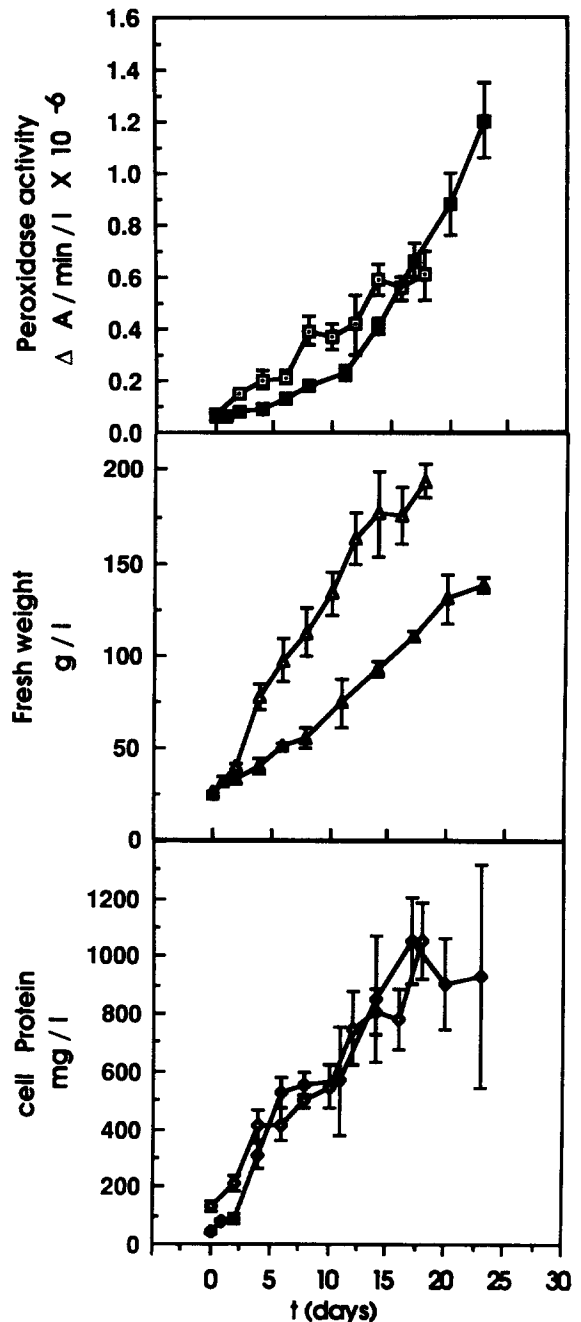
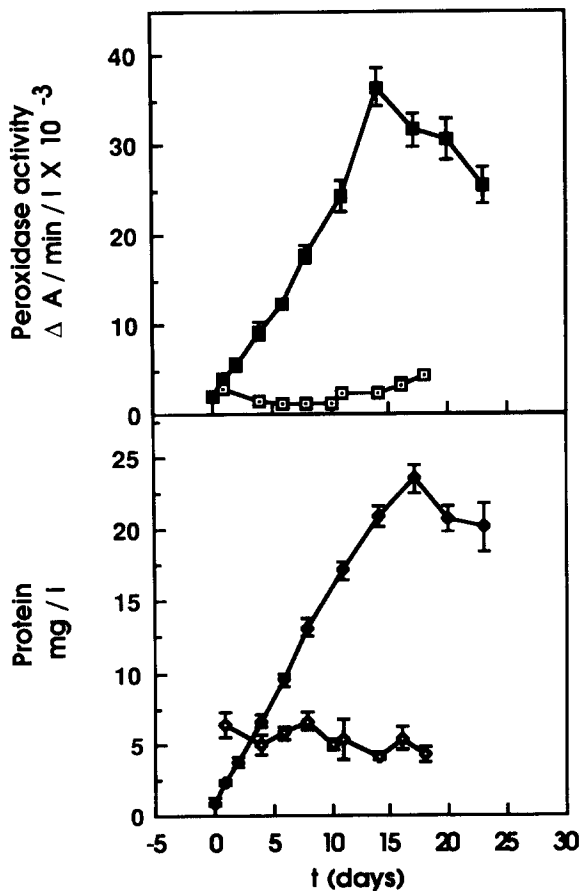


Fig. 1. The increase in peroxidase activity ( $\blacksquare$ ,  $\square$ ), fresh weight ( $\blacktriangle$ ,  $\triangle$ ) and protein ( $\blacklozenge$ ,  $\lozenge$ ) with time of culture in cell suspension cultures (closed symbols) and 'hairy root' cultures (open symbols). Each point is the mean of at least five replicates and is shown with the standard error of the mean.

activity in the culture and at a relatively high specific activity of  $2500 \Delta A/\text{min}/\text{mg}$  protein.

Given that peroxidase activity is related to growth, it is important to maximise both the final biomass accumulation and the specific growth rate in the cultures. This objective was addressed in several additional studies on the growth of cell suspension cultures.

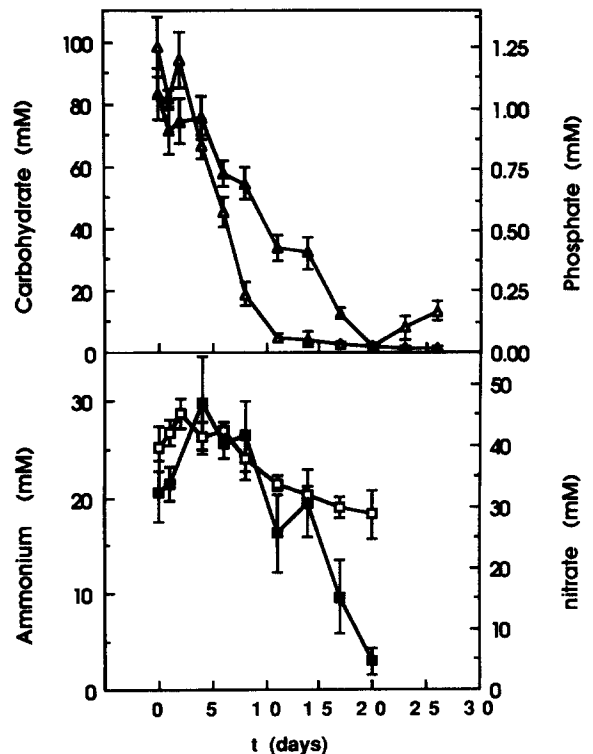
Given sufficient space, cell suspension cultures will grow until limited by the exhaustion of critical nutrients. Previous research has shown that phosphate [18], carbohydrate [19], nitrate [20], and ammonia [21] may all limit growth either singly or in combination [22,23]. The concentration of these



**Fig. 2.** The peroxidase activity and protein content of the culture filtrate of cell suspension cultures and 'hairy root' cultures. Symbols are the same as in Fig. 1. Each point is the mean of at least five replicates and is shown with the standard error of the mean.

four nutrients in the culture filtrate of cell suspensions throughout the growth cycle are shown in Fig. 3. This reveals that of the nutrients examined, only phosphate and carbohydrate are exhausted from the culture filtrate prior to the cessation of growth and are therefore capable of limiting growth. It must however be borne in mind that both these nutrients may be sequestered by the plant into forms that may or may not be capable of supporting further growth of the culture [18,19]. Ammonium is also approaching exhaustion by the end of the growth cycle and although not directly limiting growth, may quickly become limiting once the growth limitation by other nutrients is released.

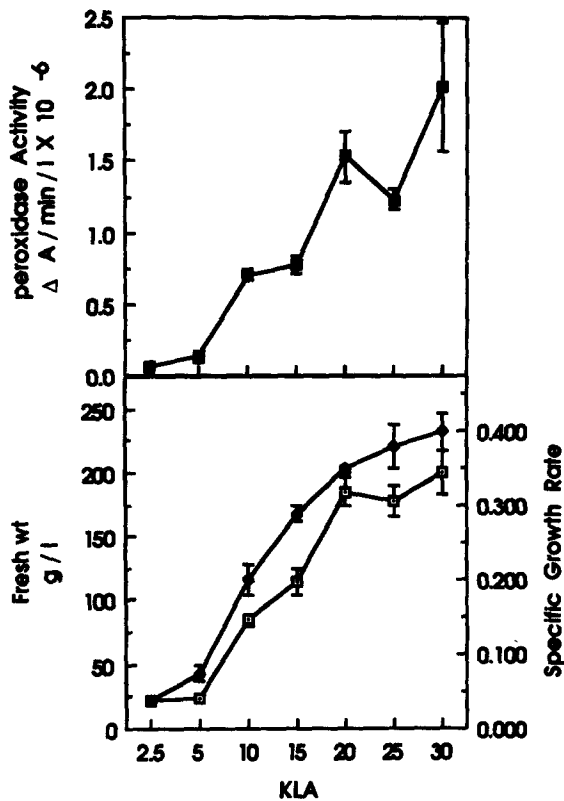
The factors limiting the growth rate of cell suspension cultures are not well understood, however aeration of the cultures plays a part, and growth is related to the volumetric oxygen mass transfer coefficient ( $k_1a$ ) at low oxygenation rates [17]. To



**Fig. 3.** The concentration of nutrients in the culture filtrate throughout the growth cycle:  $\Delta$  phosphate,  $\blacktriangle$  soluble carbohydrate,  $\square$  nitrate,  $\blacksquare$  ammonium. Note that stationary phase was reached on day 20 in these cell suspensions.

permit experimental variation of the  $k_1a$  in shake flasks, the relationship between  $k_1a/h$  and culture volume in 25 cm<sup>3</sup> Erlenmeyer flasks was established at a constant shaker speed of 110 rev./min on an orbital shaker. Over the range 10–200 cm<sup>3</sup> there was a linear relationship between  $\log_{10} k_1a$  and culture volume according to the following relationship:  $\log_{10} k_1a/h = 1.8 - 0.00613 \text{ culture volume (cm}^3\text{)}$ . The correlation coefficient for the relationship ( $r$ ) was 0.988.

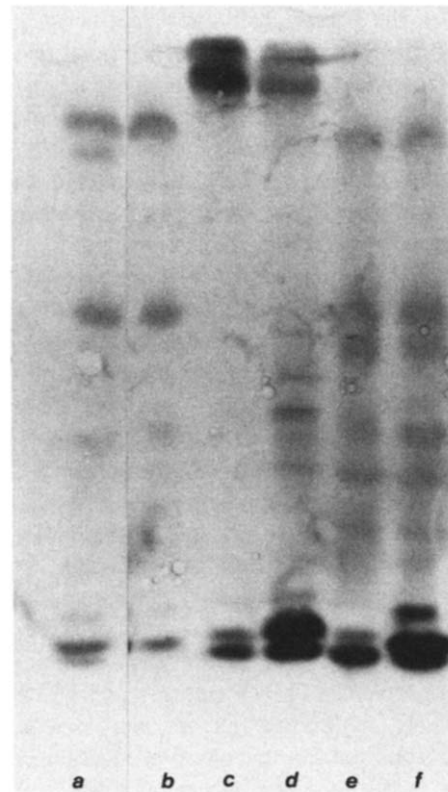
This relationship was then used to vary  $k_1a$  in shake flasks. Growth was monitored daily by measurements of packed cell volume, and at the end of the experiment by fresh weight. The results of this experiment (shown in Fig. 4) show that the specific



**Fig. 4.** The effect of the volumetric oxygen mass transfer coefficient ( $k_1a$ ) on growth and peroxidase activity of cell suspension cultures. (◆) specific growth rate (SGR) in exponential phase of the culture (cm<sup>3</sup> packed cell volume/cm<sup>3</sup> packed cell volume/day), □ fresh weight per litre of the culture at 1 day into stationary phase, ■ peroxidase activity of the culture per litre at 1 day into stationary phase. Each point is the mean of at least five replicates and is shown with the standard error of the mean

growth rate of the cultures, the total biomass accumulation in stationary phase, and the peroxidase activity of the cultures are highly dependent on the  $k_1a$  between 2.5/h and 20/h, and hence on the rate of oxygenation of the cultures. Clearly, the rate of oxygenation of cell suspension cultures can be one of the key constraints on growth and peroxidase activity. Analysis of the specific growth rate, fresh weight, and total peroxidase activity of the cell suspensions used in the experiments into growth kinetics are all consistent with growth at a  $k_1a$  of 12/h. It would therefore appear that these cultures were grown under sub-optimal conditions.

The isozyme complement of these cultures is shown in Fig. 5. The enzyme from the cell extract



**Fig. 5.** A Zymogram of peroxidases obtained in culture. (a) Cathodic, isozymes (Sigma Type XII) (b) Crude horseradish peroxidase (Sigma). (c) Cell extract from cell suspension cultures (d) Culture filtrate of cell suspension cultures. (e) Cell extract from 'hairy root' cultures. (f) Culture filtrate of 'hairy root' cultures. The bands are developed along a linear pH gradient from pH 3 at the bottom to pH 10 at the top. Samples and standards were diluted so that the same total peroxidase was applied per lane

and culture filtrate of 'hairy root' cultures closely resembles that of the crude commercially available peroxidase. The enzyme from cell cultures and the culture filtrate of cell cultures contain some of the same isozymes as peroxidase from horseradish root with the addition of two extra bands at high pI.

## Discussion

Both 'hairy root' and cell suspension cultures are attractive systems of culture for the production of peroxidase, although under the particular conditions of culture used in the present study, the latter were more productive in terms of total peroxidase activity per litre of culture, peroxidase secretion into the culture filtrate, and specific activity. A more detailed investigation of the horseradish 'hairy root' system is still needed to evaluate its commercial potential. The most fruitful line of approach would probably be enhancement of growth rate and final biomass yield since most evidence [5] suggests that 'hairy root' cultures produce similar amounts of products to roots of the intact plant, as was found for peroxidase in our cultures of horseradish.

The present report involves a more detailed evaluation of horseradish cell suspension cultures for peroxidase production. Peroxidase may be produced in cell suspension cultures to a level greatly in excess of that found in the intact plant [24]. Both callus cultures of horseradish [2] and the cell suspensions used in these experiments showed enhanced peroxidase production over roots, yet were not derived from callus cell lines which had been overtly selected for enhanced production of peroxidase, in contrast to cell suspensions reported previously [3]. Clearly, there are factors other than overt cell selection which have contributed to the increased accumulation of peroxidase in cell cultures and which may be exploited to produce further increases in yield. The reasons for the enhanced production of peroxidase in culture are not known but there are likely to be a number of contributing factors.

Undifferentiated cells proliferating in an uncontrolled manner such as callus and cell suspension cultures resemble the cells generated at wounds in many respects, and it is known that peroxidases may be specifically induced as part of a wound response to many injurious agents [24,25]. These cells

are also in a radically different environment to the cells of the root, both in terms of their nutrient supply and in the relative size of the extracellular space and it is likely that components of the culture medium may stimulate production. For example, the secretion of peroxidase by suspended cells is known to be under the control of the calcium concentration in the culture filtrate [26] and the relatively high levels of calcium supplied may increase secretion and synthesis of peroxidase. This effect will be compounded by the greatly increased extracellular space of cultured cells in relation to the cells of the root and it has been suggested [2] that this may relieve feedback inhibition on enzyme production. The energy requirements for this active secretion are likely to be met by the abundant supply of both oxygen and carbohydrate supplied to the cultures.

It would be very advantageous from the point of commercial extraction of the enzyme to obtain its release into the culture filtrate. Extraction and purification of the enzyme would be simplified, and extraction of the culture filtrate would allow conservation of the biomass during harvesting of the product and hence allow biomass re-use. The secretion of peroxidase into the culture filtrate of cell suspension cultures is reported to be an active process and to be regulated by the concentration of extracellular calcium [26]. There may therefore be a basis for controlled secretion of a major proportion of the total peroxidase in the culture. The increase in specific activity of the enzyme in the culture filtrate in our suspension cultures suggests that active secretion of the enzyme must have occurred, however leakage of peroxidase into the culture filtrate from dead and dying cells must also contribute to this activity. Given that levels of viability in cell suspension cultures may be as low as 65% [27] and that the peroxidase present in dead and dying cells is released, a considerable portion of total culture peroxidase may be leaked into the culture filtrate. The importance of this leakage is underlined by the fact that the proportion of peroxidase released into the culture filtrate in all studies on horseradish culture so far undertaken [1-3] is within this limit. It is therefore important in experiments to investigate peroxidase secretion that cell viability should also be examined.

These observations suggest that a number of ap

proaches may be fruitful in the maximisation of peroxidase production in cell suspension cultures of horseradish. Cell selection, ideally at the level of the individual cell, for high yielding and fast growing cell lines; optimisation of the nutrient regime to relieve nutrient limitation on growth and peroxidase synthesis; optimisation of the  $k_{1a}$  of the culture to maximise the specific growth rate of the culture, the peroxidase activity, and the total biomass accumulation; elicitation, to induce a controlled wound response and hence a controlled production and release of peroxidase. These investigations are currently underway in our laboratory.

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