Short Communication

Composition of the Spent Cell Culture Medium. I. Time-course of Ethanol Formation and the Excretion of Hydrolytic Enzymes into the Medium of Suspension-cultured Cells of *Lupinus polyphyllus*

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**Summary**

Cell suspension cultures of *Lupinus polyphyllus* form about 100 mmol/l (0.4 %) ethanol when entering the stationary growth phase. 50–70 % of the ethanol produced is released into the culture medium which occupies ca. 50 % of the total volume. The culture medium also contains various hydrolytic and oxidative enzymes. The activities of extracellular peroxidase, sparteine oxidase, acid phosphatase, esterase, and protease increase during the active growth phase and decrease during stationary growth, when ethanol formation starts.

**Key words:** Cell culture, conditioned medium, ethanol formation, hypoxia, *Lupinus polyphyllus*.

**Introduction**

The *in vitro* culture of microbial and higher cells is widely applied in modern biology. The culture medium not only provides nutrients for the cultured cells but often functions as a storage compartment for excreted products. Whereas the spent medium of microbial and animal cell cultures has been intensively analyzed, relatively few detailed studies on the composition of the culture fluid, i.e. the so-called «conditioned medium», of suspension-cultured plant cells have been reported.

The spent plant cell culture media may contain variable amounts of primary and secondary products (Berlin et al. 1984; Böhm 1982; Ojima et al. 1984; Rennenberg 1976) as well as remarkably high activities of various hydrolytic and oxidative enzymes (Berlin and Barz 1975; Barz and Köster 1981; Gamborg and Eveleigh 1968; Gaspar et al. 1983; Olson et al. 1969; Oleson and Fahrlander 1982; Sticher et al. 1981; Pospisil et al. 1983; Van Huystee and Turconi 1973; Wink 1984a). Thus, the media have a composition similar to plant vacuoles (Matile 1975, 1978; Marty et al. 1980). We have therefore postulated (Wink 1984b), that the plant

cell culture medium represents an extracellular storage and lytic compartment, and may therefore play a similar role as that attributed to plant vacuoles (Matile 1975, 1978, 1984; Marty et al. 1980). This view is in accordance with the hypothesis that vacuoles and the extracellular space of plant cells constitute the lytic compartment (Matile 1975).

In this communication we report on the time-course of the release of hydrolytic enzymes into the culture medium in relation to the formation of ethanol, which is produced by densely grown cell suspension cultures of *Lupinus polyphyllus*.

**Materials and Methods**

1. Plant material. Cell suspension cultures were kept under standard conditions as described in Wink et al. 1980, 1983 and Wink 1984 a. 300 ml Erlenmeyer flasks were filled with 50 ml cell culture medium, containing 6% sucrose and were closed with aluminum foil.

2. Enzyme assays and enzyme extraction

At intervals cell culture media were separated from the cells by filtration followed by centrifugation (10 min, 20,000 xg). The media were adjusted to pH 7 and stored at -70 °C. The respective cells were frozen in liquid nitrogen and stored at -70 °C. About 0.5 g frozen cells were homogenized in 2 ml 0.1 M acetate buffer (pH 5.5). The homogenate was centrifuged at 17,000 xg for 10 min and the supernatant employed in the enzyme assays. The pellet fraction which contains cell wall-bound hydrolytic enzymes was not analyzed.

a) Peroxidase. Conditions: 4 ml 0.1 M phosphate buffer (pH 7), 0.3 µg guaiacol, 0.08 µg H₂O₂, 50 µl medium or 20 µl cell extract. The reaction was followed photometrically at 470 nm.

b) Acid phosphatase. Conditions: 2 ml 0.1 M acetate buffer (pH 5.5), 100 µg p-nitrophenylphosphate, 500 µl medium or 100 µl cell extract. After 20 min the reaction was terminated with 500 µl 1 M NaOH and the absorbance measured at 405 nm. A blank control containing buffer instead of enzyme was always assayed and used to correct the enzyme data.

c) Esterase. Conditions: 1 ml 0.1 M phosphate buffer (pH 7), 100 µg p-nitrophenylacetate, 500 µl medium, 200 µl cell extract. Incubation and determination as in b.

d) α-Galactosidase. Conditions: Same as in c, except p-nitrophenyl-α-D galactopyranoside as substrate.

e) Protease. Conditions: 2 ml medium (pH 7), 5 mg azocoll. Incubation at 30 °C for 180 min. After centrifugation the absorbance was read at 520 nm.

f) Sparteine oxidase. Conditions: 2 ml enzyme (pH 7), 50 µg sparteine, 125 µg H₂O₂. After an incubation at 30 °C for 60 min the reaction was terminated by adding 1 ml 6 M NaOH. The solution was added onto an Extrelut column (filled with 1.7 g Extrelut, Merck, Darmstadt) and sparteine was eluted from the column with 30 ml Cl₂CH₂. After evaporation of the solvent, sparteine was analyzed by capillary GLC (Wink, 1984 a; Wink et al., 1980, 1983). The decrease of sparteine was taken as a measure for sparteine oxidation (Wink, 1984 a).

Ethanol determination. About 0.5 g cells were homogenized in 1.5 ml 1.0 M HCl. After centrifugation for 10 min at 17,000 xg, the supernatant was directly employed for the enzymatic ethanol determination. Assay conditions: 2 ml 62 mM glycine/pyrophosphate buffer (pH 9), 73 mM semicarbazide, 1.8 mM NAD⁺, 10 mM glutathione 100 µl alcohol dehydrogenase (10 U), 5 or 30 µl medium or cell extract. After an incubation of 30 min at 20 °C, the reaction was completed and the increase of absorbance was recorded at 365 nm.

Protein was determined by the Lowry-method (Cooper 1981).

Results and Discussion

1. Formation of ethanol in cell suspension cultures

Higher plants are obligate aerobes which can, however, tolerate anaerobic conditions for certain periods of time (Davies 1980). Depending on the plant species either ethanol, lactate, malate, or succinate accumulate under anaerobic conditions with ethanol formation as the most frequent process. Plant cells cultured in vitro are also able to form ethanol under anaerobic condition, as indicated by in vivo NMR studies (Wray et al. 1985).

Table 1: Ethanol in conditioned media of suspension-cultured plant cells.

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Days after transfer</th>
<th>Ethanol mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lupinus polyphyllus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>heterotrophic strain</td>
<td>22</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>26</td>
</tr>
<tr>
<td>green mixotrophic strain I</td>
<td>8</td>
<td>33</td>
</tr>
<tr>
<td>strain II</td>
<td>8</td>
<td>48</td>
</tr>
<tr>
<td><em>Lupinus luteus</em></td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td><em>Lupinus mutabilis</em></td>
<td>25</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>31</td>
</tr>
<tr>
<td><em>Lupinus hartwegii</em></td>
<td>31</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>46</td>
</tr>
<tr>
<td><em>Laburnum alpinum</em></td>
<td>10</td>
<td>37</td>
</tr>
<tr>
<td><em>Cytisus canariensis</em></td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td><em>Spinacia oleracea</em></td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td><em>Conium maculatum</em></td>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

According to Tab. 1 ethanol formation is a common event in plant cell suspension cultures, although hardly documented in the literature. Ethanol was found to be present in both the cells and the cell culture medium of the 8 species studied. The highest concentration found was about 160 mmol/l medium (= 0.7 %) in densely grown cultures of *Lupinus polyphyllus*.

This culture was selected to study the time-course of the formation and release of ethanol into the culture medium (Fig. 1). Ethanol formation obviously depends on the oxygen tension of the environment as it does in intact lupin seedlings (Young et al. 1977). It is markedly higher in flasks sealed with a rubber stop-cock as compared to flasks closed with aluminum foil or silicon foam stoppers. But even in ordinary cultures closed with aluminum foil or silicon foam (Technomara), oxygen supply becomes obviously limited when the culture has reached a high cell density (Fig. 1, 2). The ethanol concentration in the medium is usually 40–100 % higher than in the corresponding cells (ethanol concentration was determined on a cell weight basis) (data not shown). Consequently the total amount of ethanol per flask is usually higher in the medium than in the cells (Fig. 2). The release of ethanol into the external medium

Fig. 1: Ethanol formation in cell suspension cultures of *Lupinus polyphyllus* in relation to growth and aerobic conditions. All culture flasks were started with the same amount of cells (A, B: 3 g/50 ml; C: 5 g/50 ml) and at each interval indicated, a culture was taken, and cells and medium processed separately. Culture flasks were closed with aluminum foil (A), with Tecnomara silikon foam (B), or with a rubber stop-cock (C).

seems to be a common feature of plant cell suspension cultures and was also observed in seedlings kept under anaerobic condition (Literature in Davies 1980). It is not clear whether the release of ethanol into the medium is due to diffusion or to a carrier-mediated transport.

Lupin cells were even able to grow in flasks sealed with a rubber stop-cock (Fig. 1). The growth rate (1 g/day) was reduced by 50% as compared to cells cultured under standard conditions, i.e. less tight stoppers (2 g/day). This observation suggests that anaerobic glycolysis, which is indicated by the increased formation of ethanol, provides sufficient energy at least for a slow growth of suspension cultured cells.

Under all conditions tested, ethanol formation was evident in lupin cell suspension cultures indicating that anaerobic conditions may regularly occur in cell suspension cultures. In many laboratories cell suspension cultures are assayed for the production

Release of ethanol and enzymes in suspension-cultured *Lupinus* cells

![Graph showing the release of ethanol and enzymes over time.](image)

Fig. 2: Time course of the excretion of hydrolytic or oxidative enzymes into the culture medium by suspension-cultured lupin cells in relation to growth and ethanol formation. All culture flasks were inoculated with 3 g cells/50 ml medium. At intervals culture flasks were harvested and processed as described in Materials and Methods. Protein contents in the medium (mg protein/flask) are given in the box in which the time-course of protease is shown. C = cells, M = medium.

of economically important secondary metabolites and often with rather limited success (Zenk, 1982). The influence of hypoxic conditions on secondary metabolism has not been studied in detail but will probably be of importance. It should be recalled that the biosynthesis of some secondary compounds is restricted to specific organs,
e.g. tropane alkaloids are formed in roots, lupin alkaloids in leaf chloroplasts which are characterized by a different oxygen supply.

2. Release of enzymes into the cell culture medium

We could recently show that the medium of suspension-cultured lupin cells contains hydrolases, such as glucosidases, nucleases, proteases, phosphatases, esterases, and oxidative enzymes, e.g. peroxidase and «sparteine oxidase» (Wink, 1984 b).

A representative time-course experiment on the activity of a few selected enzymes (phosphatase, esterase, galactosidase, peroxidase, protease, and «sparteine oxidase») in cells and medium is demonstrated in Fig. 2. Except for galactosidase, the other 5 enzymes showed maximal activity in the cell culture medium, when the culture entered the stationary growth phase. Afterwards a marked decrease of enzyme activity took place (exception: α-galactosidase), which was inversely correlated with the increase of ethanol formation. The subsequent decrease of enzyme activity might be due to increased proteolytic activity, since the amount of protein shows a similar time-course (Fig. 2). A re-uptake of the proteins by the cells seems less likely but cannot be ruled out. Whether the hypoxic conditions and subsequent ethanol formation during the late growth phase determine the kinetics of enzyme excretion into the medium has yet to be established.

Alkaloid accumulation in these cultures shows an initial maximum 2–3 days after transfer of the cells into fresh medium (Wink and Hartmann, 1982; Wink, 1984 a). The subsequent decrease of the alkaloid content coincides with the increase of «sparteine oxidase» in both the medium and the cells, which provides further evidence for our observation that alkaloid degradation regulates alkaloid production by lupin cells (Wink, 1984 a, 1985).

The results of these studies indicate that the culture fluid is an important element of suspension-cultured cells, which stores metabolites and active enzymes and might play the role of a dynamic extracellular lytic compartment (Wink, 1984 b).

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References


Release of ethanol and enzymes in suspension-cultured *Lupinus* cells
