Original Paper

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Continuous marenin production by agar-entrapped Haslea ostrearia using a tubular photobioreactor with internal illumination

Abstract The marine diatom Haslea ostrearia was immobilized in a tubular agar gel layer introduced into a photobioreactor of original design with internal illumination for the continuous synthesis of marenin, a blue-green pigment of biotechnological interest. Marenin was produced for a long-term period (27–43 days) and the volumetric productivity was maximum (18.7 mg day⁻¹ l⁻¹) gel at the highest dilution rate (0.25 day⁻¹) and lowest agar layer thickness (3 mm). Heterogeneous cell distribution in the agar layer revealed diffusional limitation of light and nutrients. However, the 3 mm gel thickness led to a more homogeneous cell distribution during incubation and to an increase of the whole biomass in the agar gel layer.

Introduction

The pennate diatom Haslea ostrearia is able to synthesize and excrete a hydrophilic, blue-green pigment named marenin, which is responsible for the greening of the oyster’s gills. More recently, an anti-proliferative effect has been shown in the lung cancer model (Carbonnelle et al. 1999), with a prospect of pharmacological development.

In order to produce this pigment for the greening of oysters at the industrial level, batch free-cell cultures are currently performed, using 6-m³ tanks under non-axenic and partially controlled incubation conditions (Robert 1989). However, to standardize marenin production in terms of quantity and quality, efficient closed systems are required to allow more careful control of levels of nutrients such as nitrogen (Neuville and Daste 1972) and lighting (Schubert et al. 1995).

In the natural environment, the number of pigmented cells increased during the migration of algae from the planktonic to the benthic compartment and became maximum during the benthic stage (Robert 1983) where algal cells were immobilized in their own exopolysaccharides (Rincé et al. 1999). Artificial immobilization is a suitable technique to reproduce this natural phenomenon and seems the more adapted to microalgae, particularly to Haslea ostrearia cells, which are very sensitive to disturbance (Yang and Wang 1992; Rouillard 1996). Several microalgal species have been entrapped in various transparent polymer matrices for exometabolite production, and immobilized-cell techniques depend on the matrix composition. Polyurethane foams (Bailiez et al. 1988) and some cations used in alginate beads (Tamponneau et al. 1985) are particularly toxic for the majority of microalgae, while polysaccharides extracted from seaweed, such as sodium alginate (Hertzberg and Jensen 1989; Kannapiran et al. 1997), are suitable for diatoms, although phosphate in media from seawater has led to partial dissolution of the alginate gel (Brouers and De Jong 1988). Travieso Cordoba et al. 1995. In a previous work (Lebeau et al. 1999), Haslea ostrearia was successfully entrapped in agar gel discs for long-term marenin production. About 8 × 10¹⁰ cells l⁻¹ gel were reached, whereas the free-cell concentration did not exceed 1 × 10⁹ cells l⁻¹ medium, and the average specific productivities of immobilized cells were higher than those of free cells.

In studies dealing with immobilized microalgal cells, standard bioreactors derived from those devoted to non-
photosynthetic cells have been used, either packed-bed or fluidized-bed (Santos-Rosa et al. 1989; Traviesto et al. 1992; Vilchez & Vega 1995). However, the hazards related to the limited transfer of light and nutrients into the matrices in which the microalgal cells are immobilized require suitable photobioreactors. To overcome these problems, the few photobioreactors devoted to immobilized microalgal cells are characterized by a high surface exchange between algal cells and nutrients and light (Wang et al. 1991; Chetsuomon et al. 1993; Kaya et al. 1996) and/or an illumination device (Junter et al. 1989; Burgess et al. 1993).

In the present study, we made use of a bioreactor prototype dedicated to immobilized photosynthetic cells (Junter et al. 1989) – previously used for hydrogen gas production (Planchard et al. 1989) – for the production of marenin. *H. ostrearia* cells were entrapped in a layer of agar gel and cultured in continuous mode. To optimize the production of marenin, we tested the influence of the gel layer thickness (3 mm and 6 mm), the culture medium (F/2 and F/20) and the dilution rate (0.025 day$^{-1}$ and 0.25 day$^{-1}$).

### Materials and methods

Algal strain and maintenance medium

The study was performed using an axenic strain of *H. ostrearia* Simonsen isolated from oyster pond waters of the Bouin district (Vendée, France). The cells used (strain HO1, ISOmer, Nantes, France) to maximize marenin production were characterized by an average modal length of 70 μm. The algal cultures were maintained by weekly transfer to fresh ES 1/3 medium (Table 1). The pH of the medium was set to 7.8, the incubation was at 15 °C and light (horticultrual neon lighting, 36 W) was provided at $3 \times 10^{16}$ quanta cm$^{-2}$ s$^{-1}$ with a 14/10-h light/dark cycle.

Preparation of inocula

Algal precultures were performed applying a two-step procedure in the same culture conditions as for cell maintenance. Cells from the clone pool were first precultured for about 6 days in 250-ml Erlenmeyer flasks filled with 150 ml maintenance (ES 1/3) medium. Then the flask contents were inoculated in 2 l Erlenmeyer flasks containing 1 l (ES 1/3) medium. Algal inocula were collected by centrifugation (4000g, 6 min, 15 °C) from cultures in the exponential growth stage after incubation of these larger flasks for 6 days. Cell suspensions with concentrations ranging between

<table>
<thead>
<tr>
<th>Table 1 Composition of ES 1/3 and F/2 media</th>
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<td><strong>ES 1/3 medium:</strong></td>
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<tr>
<td>Enrichment solution (see below)</td>
</tr>
<tr>
<td>Stock metasilicate solution (see below)</td>
</tr>
<tr>
<td>Vitamin solution (see below)</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
</tr>
<tr>
<td>Filtered seawater (salinity adjusted to 28 g/l with distilled water)</td>
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<tr>
<td>Adjust pH to 7.8. Sterilize at 121 °C for 20 min</td>
</tr>
</tbody>
</table>

**Enrichment solution**

- Na$_3$NO$_3$ 350 mg
- Sodium glycerophosphate (C$_3$H$_5$O$_7$PNa$_2$·6H$_2$O) 50 mg
- Tris(hydroxyethyl)aminomethane (Tris buffer) 500 mg
- Fe-EDTA solution (see below) 2.5 ml
- Trace metal solution (see below) 25 ml
- Distilled water 100 ml

**Fe-EDTA solution**

- Fe(NH$_4$)$_2$(SO$_4$)$_2$·6H$_2$O 3.51 g
- Na$_2$EDTA 3.50 g
- Distilled water 500 ml

**Trace metal solution**

- H$_3$BO$_3$ 570 mg
- FeCl$_3$·6H$_2$O 24.5 mg
- MnSO$_4$·H$_2$O 62.15 mg
- ZnSO$_4$·7H$_2$O 11 mg
- CoSO$_4$·7H$_2$O 2.4 mg
- Na$_2$EDTA 500 mg
- Distilled water 500 ml

**Stock metasilicate solution**

- Na$_2$SiO$_3$·5H$_2$O 10.607 g
- Distilled water 500 ml

**Vitamin solution**

- Thiamine 500 mg
- Biotin solution (20 mg/100 ml water) 1 ml
- Vitamin B$_{12}$ solution (20 mg/100 ml water) 1 ml
- Distilled water 500 ml

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<tr>
<th><strong>F/2 medium:</strong></th>
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<tr>
<td>Vitamin solution (see below)</td>
</tr>
<tr>
<td>Filtered seawater (salinity adjusted to 28 g/l with distilled water)</td>
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<tr>
<td>Major nutrient solution</td>
</tr>
<tr>
<td>Sterilize at 121 °C for 20 min</td>
</tr>
</tbody>
</table>

**Major nutrient solution**

- Na$_3$NO$_3$ 7.5 g
- Distilled water 100 ml
- FeCl$_3$·6H$_2$O 315 mg
- CuSO$_4$·5H$_2$O (98 mg/100 ml water) 1 ml
- ZnSO$_4$·7H$_2$O (220 mg/100 ml water) 1 ml
- CoCl$_2$·6H$_2$O (100 mg/100 ml water) 1 ml
- MnCl$_2$·4H$_2$O (1800 mg/100 ml water) 1 ml
- Na$_2$MoO$_4$·2H$_2$O (63 mg/100 ml water) 1 ml
- Distilled water 95 ml
- Sterilize at 121 °C for 20 min

**Trace metal solution**

- Vitamin solution 436 mg
- Thiamine – HCl 10 mg
- Biotin (5 mg/100 ml water) 1 ml
- Vitamin B$_{12}$ (5 mg/100 ml water) 1 ml
- Distilled water 98 ml