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A pilot-scale bioprocess to produce amphidinols from the marine microalga *Amphidinium carterae*: Isolation of a novel analogue

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ABSTRACT

Marine dinoflagellate microalgae belonging to the genus *Amphidinium* are a key source of an interesting group of polyketide metabolites with potent bioactivities, wide-ranging functional diversity and stereochemical complexity, but low natural availabilities. The feasibility of a microalgae dinoflagellate-based sustainable bioprocess for producing amphidinols (APDNs) by photoautotrophic culture of *Amphidinium carterae* in a pilot-scale LED-illuminated bubble column photobioreactor (PBR) was therefore investigated. A fed-batch culture mode with pulse feeding strategy provided a growth pattern strongly limited by the availability of phosphate content in the culture medium that stimulated the production of cellular APDNs. Since *A. carterae* was found to be much more shear-sensitive than other shear-tolerant non-dinoflagellate microalgae, the culture height and air flow rate were established to ensure the absence of damaging levels of hydrodynamic stress. The biomass capacity yielded by the PBR at the end of the culture (0.540 g d.w. L^{-1} equivalent to 1.70×10^6 cell mL^{-1}) corresponded to that estimated stoichiometrically from the experimentally determined biomass P-molar formula (C_{329} O_{126} H_{732} N_{69} S_3 P_1) and the total phosphorus and nitrogen balances. The downstream processing section was initially conceived to recover APDNs excreted by cells into the supernatant. A dry APDN-enriched extract concentration of 49 mg per liter of supernatant was obtained. This purification process led to partitioning of the extract into several fractions and sub-fractions thereof. Only two sub-fractions were studied, yielding thereof highly pure (> 95% pure) luteophanol D and lingshuiol A, and a new, roughly purified (> 80% pure) APDN, namely amphidinol 20. The percentages of luteophanol D, lingshuiol A and amphidinol 20 by dry weight of the APDN-enriched extract obtained were 1%, 0.39% and 0.31%, respectively, thus representing a concentration in the culture supernatant of 490, 191 and 152 μg L^{-1}, respectively.

1. Introduction

The potential medical and commercial significance of bioactive substances from marine dinoflagellate microalgae has been comprehensively reviewed [1–3]. From the perspective of a hypothetic pilot-scale bioactive substance production bioprocess, the engineering and operation of photobioreactors (PBRs) and downstream processing of the primary extracts produced to obtain a purified bioproduct are relevant aspects that need to be addressed in detail. For example, a consistent production of certain high-value microalgal metabolites requires artificial illumination of PBRs. In this regard, illumination using light-emission diodes (LEDs) has recently been demonstrated to be particularly attractive for reliably culturing shear-sensitive dinoflagellate microalgae in pilot-scale bubble column PBRs [4]. Despite this, the few reported studies concerning LED-based illumination have mostly focused on small PBRs and no attention has been given to scale-up of these systems.

Any metabolite bioproduction process must be inexpensive and consistently achieve a satisfactory level of productivity under well-defined culture conditions. The titer of the target bioproduct in the production of bioactive substances from dinoflagellates is often low [1], thus increasing the cost of downstream processing due to the need to handle large volumes of broth and compromising the feasibility of the bioprocess in question. Concentration of a target compound in the broth

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could therefore become a criterion for selecting producer species. In this respect, among marine dinoflagellates, the production of amphidinolides (APDLs) and amphidinols (APDNs) (henceforth referred to as APDs indistinctly) is remarkable in species of the genus *Amphidinium* [5,6]. APDLs are a family of macrolides with a particularly high antitumor activity that currently comprise over 40 members and exhibit a vast variety of backbone skeletons ranging from 12 to 29 carbons, with more than half of them containing an odd-numbered lactone ring [7]. This structural variety is increased by additional traits such as the presence of at least one *exo*-methylene group and vicinally located one-carbon branches [6,8–11]. In general, the best titers of these macrolides reported for laboratory-scale batch cultures of *Amphidinium* have been reported to be around 40 $\mu g\,L^{-1}$, with biomass (d.w.) yields of about 0.1 $g\,L^{-1}$ (calculations performed using data from [8]). However, all attempts to reproduce these titers in large-scale cultures failed [12].

APDNs are also a representative type of secondary metabolite produced by the genus *Amphidinium* and first reported in 1991 from cultures of *Amphidinium klebsii* collected at Ishigaki in Okinawa [13]. In contrast to APDLs, this family embraces a growing set of open long-chain compounds eliciting potent antifungal and hemolytic activities [6,8,13–15]. These compounds are defined as a polyhydroxy-polyene and are characterized by the presence of two tetrahydropyran rings separating the polyol and polyene moieties. This group currently comprises 19 analogues, with a total of approximately 30 members considering the amphezonol and colopsinol types, some of which exhibit some modifications in the two tetrahydropyran rings [6,13,16]. In addition to the bioactivities mentioned previously, APDNs also exhibit strong toxicity against diatoms [17], including allelochemical effects on epiphytic microbes in marine ecosystems, and even in terrestrial pathogenic bacteria such as those belonging to the *Mycoplasm* genus. As a result, the use of enriched APDNs to avoid the growth of these bacteria in cell or tissue cultures has recently been patented [18]. The aforementioned toxicity against microorganisms is believed to arise from the ability of APDNs to interact with the phospholipid bilayer, thus resulting in membrane permeabilization [19–26]. Furthermore, it has been proposed that this activity is independent of the membrane thickness and that the pore size is dependent on the polyhydroxyl region, which remains on the surface of the membrane [22]. A better comprehension of this phenomenon and its mechanism would allow us to gain an understanding of the mode of action of these antifungal activities, thus helping in the development of drugs for to treat AIDS-related diseases and those commonly found in transplant patients [21].

Given the diverse functionality, stereochemical complexity, low natural availability and the marked bioactivities shown by these secondary metabolites from the *Amphidinium* genus, they have been considered to be challenging targets for total synthesis [2,27]. However, the synthetic methodologies used to obtain them are difficult to apply industrially due to their length and complexity [2]. This study reports on the feasibility of a microalgae dinoflagellate-based sustainable bioprocess for producing APDs from photoautotrophic culture of the shear-sensitive marine microalga *Amphidinium carterae* in a pilot-scale LED-illuminated bubble column. The culture mode was fed-batch with a pulse feeding strategy. The average irradiance and macronutrients (i.e. nitrogen and phosphorus) in the culture volume were monitored off-line and the growth kinetics was interpreted in terms of these variables. The downstream processing section, comprising a combination of separation operations, was conceived to separately recover APDs excreted by cells into supernatants (APDs previously reported to have been extracted from biomass), with the general aim of establishing a culture methodology and chromatography protocols for the isolation of APDs with a focus on setting up pipelines that can be adapted to industrial applications, thereby resulting in a sustainable source of these compounds that meets their industrial demand as active substances.

Of the APDs previously reported to be produced by *Amphidinium carterae*, we report excellent isolated yields of the APDNs luteophanol D [28], lingshuiol A [29] and a new analog amphidinol 20, thus confirming the biosynthetic flexibility of the amphidinol pathway. These metabolites are excreted by the cells into the extracellular media and are found in the supernatants obtained from the downstream process applied, thus resulting in a simple and high-performance procedure that could be suitable for the commercial development of APDs as active substances.

2. Materials and methods

2.1. Upstream processing

2.1.1. The microalga

The marine microalgal dinoflagellate *Amphidinium carterae* (strain ACRN03) was used. This alga was obtained from the Culture Collection of Harmful Microalgae at the IEO (Vigo, Spain). *A. carterae* is considered a shear- and bubble-sensitive microalga [30]. Inocula were grown in flasks at 21 ± 1 °C under a 12:12 h light–dark cycle. Four 58 W fluorescent lamps were used for illumination and the irradiance at the surface of the culture flasks was 60 $\mu E\,m^{-2}\,s^{-1}$. A modification of K medium prepared using filter-sterilized (0.22 μm Millipore filter; Millipore Corporation, Billerica, MA, USA) Mediterranean seawater was used to grow the cultures. The composition of the modified K medium was as follows: $NaNO_3$, 882 μM; NH_4Cl, 50 μM; NaH_2PO_4, 10 μM; TRIS, 1 mM; $Na_2EDTA{\cdot}2H_2O$, 90 μM; Fe-Na-EDTA, 14.6 μM; $MnCl_2{\cdot}4H_20$, 0.9 μM; $ZnSO_4{\cdot}7H_2O$, 0.08 μM; $CoSO_4{\cdot}7H_2O$, 0.05 μM; $Na_2MoO_4{\cdot}2H_2O$, 0.03 μM; H_2SeO_3, 0.01 μM; Thiamine, 0.7 μM; Biotin, 2.1 nM; B12, 0.37 nM.

2.1.2. Photoacclimation experiments

To take into account a possible effect of photoacclimation on the interpretation of results from the PBR culture, a set of experiments comprising static batch cultures conducted in vertically arranged T-flasks (60 mL working volume, 5.6 cm culture high) located on an incubator tray and illuminated frontally was carried out on a small scale. The light source was based on multicolor LEDs similar to those used to illuminate the PBR culture as detailed below in subsection 2.1.4. Parallel LED strips were attached to a flat reflective plastic (PVC) cover. The LED control unit allowed different illumination regimens to be selected. A sinusoidal diel variation pattern of outdoor irradiance was imposed using the following equation [4]:

$$I_o(t) = \begin{cases} I_{o\max} \sin\left[\pi \frac{t - t_{sr}}{t_{ss} - t_{sr}}\right] \text{ if } t_{sr} \le t \le t_{ss} \\ 0 \text{ otherwise} \end{cases} \quad (1)$$

where I_{omax} is the maximum irradiance occurring at midday on the surface of T-flasks; t_{sr} is the time of sunrise (6 h); and t_{ss} is the time of sunset (18 h), with a dark period of 12 h. The walls of the incubator were painted black to avoid reflections that might modify the irradiance distribution at the surface of the cultures. The selected culture volume provided a high surface to volume ratio, a relatively low culture thickness and absence of carbon dioxide limitation during exponential growth and absence of hydrodynamic stress. As such, light attenuation by the cell suspension is minimum and the irradiance impinging on the culture, $I_o(t)$, could be assumed to be similar to the average irradiance inside the culture, I_{ave} (t), at any time.

The daily mean irradiance (Y_{ave}) perceived by cells was calculated by averaging $I_o(t)$ over 24 h. Different Y_{ave} values (from 0 to 424 $\mu E\,m^{-2}\,s^{-1}$) were obtained by varying I_{omax} from 0 to 1500 $\mu E\,m^{-2}\,s^{-1}$ as a result of varying the distance from the T-flask surface to the light source. In a first set of experiments, cells from the same original inoculum, obtained as described above in subsection 2.1.1, were cultured in the T-flasks in batch mode at different levels of Y_{ave}. Cells grown at each Y_{ave} were subcultured (i.e. a culture fraction was transferred to a fresh growth medium) at the same Y_{ave} twice more. Irradiance was measured using a 4π sensor (Biospherical instruments Inc., mod. QSL-100, San Diego, CA, USA), and all experiments were