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An optimal culture medium for growing *Karlodinium veneficum*: Progress towards a microalgal dinoflagellate-based bioprocess

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ABSTRACT

In addressing a potential bioprocess for bioactives production from microalgae, the choice of a proper culture medium is a key factor. In the present work, an optimal culture medium for producing the marine dinoflagellate microalga *Karlodinium veneficum* was formulated using a stochastic search strategy based on genetic algorithm. The optimized formulation had 25 components including the macronutrients, trace elements and vitamins. With this medium, the final attainable cell concentration during batch growth was enhanced by 120% relative to the control culture grown in the L1 medium. The final titer of the bioactive karlotoxins measured using the hemolytic activity of the culture filtrate was enhanced by 190% relative to the L1 control. These results are a promising basis for further development of photobioreactor culture strategies for microalgal dinoflagellates.

1. Introduction

The marine dinoflagellate microalgae are often associated with the production of bioactive secondary metabolites with biotechnological significance [1,2]. *Karlodinium veneficum* is one such dinoflagellate. It is a producer of karlotoxins (KmTxs), a group of toxins with hemolytic, cytotoxic and ichthyotoxic activities [3–5]. KmTxs are a potential basis for design of drugs for treatment of heart disease and some cancers [6]. Production of KmTx for investigational use requires cultivation of *K. veneficum* as a pure culture. As toxin productivity and growth of diverse dinoflagellates are highly influenced by nutrition [7], development of suitable culture media formulations for them becomes a key factor for addressing a dinoflagellate-based bioprocess. The L1 medium that has been traditionally used to grow many dinoflagellates was originally developed for growing diatoms and has been shown to be inadequate for highly productive culture of dinoflagellates [8]. For example, based on the published elemental composition of the marine phycoplanktons, including dinoflagellates [9], the nitrogen (N) and phosphorous (P) contents of the biomass are 11.9% and 1.6% by weight, respectively. The L1 medium contains 12.4 mg/L of N and 1.1 mg/L of P [10]. Therefore, the maximum dinoflagellate biomass concentration that the L1 medium can support considering the available nitrogen is 104 mg/L. Similarly, based on the available P, the maximum biomass concentration that L1 can support is lower, at 68 mg/L. It is therefore stoichiometrically impossible to obtain a biomass concentration exceeding about 70 mg/L using the L1 medium. This work reports on the use of a genetic-algorithm-based search strategy for the development of a culture medium for growth and KmTx production by *K. veneficum*. *K. veneficum* and its bioactives have been reviewed in the literature [5,6].

A genetic-algorithm-based approach has been previously shown to be quite successful for formulating a culture medium of the dinoflagellate *Protoceratium reticulatum* [8,11]. Genetic algorithm (GA) based methods are superior to the conventional statistical experimental designs that have been commonly used for developing microbial culture media. A GA-based stochastic search is able to efficiently explore a large experimental space and has proven effective in improving the productivity of many biological processes [12].

2. Materials and methods

2.1. Algal culture and growth conditions

The marine microalgal dinoflagellate *K. veneficum* (strain K10) obtained from the Culture Collection of Harmful Microalgae of IEO (Vigo, Spain) was used as pure cultures. This dinoflagellate was initially isolated by the Institute of Marine Sciences, Barcelona, Spain, as *K. veneficum* ICMB 265. The source was the Ebro River delta, Spain. The microorganism was partly sequenced and the relevant sequence was deposited with the GenBank (*K. veneficum* strain ICMB 265; nucleotide sequence accession number jf906081). This strain was observed to produce

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hemolytic toxins, presumed to be karlotoxins (KmTxs), as hemolytic KmTxs commonly occur in strains of *K. veneficum* [4,5]. Inocula were grown at 18 ± 1 °C in a 12:12 h light–dark regimen. The L1 medium prepared in natural Mediterranean Sea water was used in all experiments. The medium was filter sterilized (0.22 μm Millipore filter; Millipore Corporation, Billerica, MA, USA) prior to use. The medium was inoculated using exponentially growing cells. The cultures were acclimatized to the light level of the relevant experiments by being exposed to this level during exponential growth for several dilutions of the culture volume.

Polystyrene multiwell plates (24-wells; Corning®) were used to grow the cells for the optimization experiments. The fill volume of each well was 2.8 mL at an initial concentration of 3×10^4 cells mL^{-1}. The plates were held on an incubator tray and identically illuminated by an overhead bank of 58 W cool daylight fluorescent lights. The average irradiance at the surface of the plates was 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The irradiance was measured using 4π sensor (QSL-2101; Biospherical Instruments, San Diego, CA, USA). The optimization experiments were in triplicate.

Growth was quantified as cell counts. Thus, 200 μL samples of the culture were collected and the cells were counted using a flow cytometer (CellLabQuanta SC, Beckman Coulter). The long-term effects of a new medium on cell growth and KmTx production were characterized by growing the cells in the best medium formulation of each generation of the experiments. T-flasks (75 cm^2) were used as culture vessels. The inoculation level and the other conditions (illumination regime, temperature) were as specified above for the multiwell plates. The fill volume was 100 mL. The effect of subculturing was assessed using the final selected medium formulation. The T-flask experiments were carried on in duplicate, unless stated otherwise.

2.2. Selection of potential medium components

Twenty-five components that are found in some of the common seawater-based dinoflagellate culture media (i.e. ES, West&McBr (ES), ESNW, f/2, K, L1, MNK, PC, Pro99, SN, von Stosch (Grund), Walnes; [10]) were assessed. The concentrations of the components ranged from nil to the maximum value used in the above referenced collection of media (see Fig. 1). The components assessed were: $NaNO_3$, urea, Na_2 β-glycerophosphate·$5H_2O$ ($C_3H_7Na_2O_6P \cdot 5H_2O$), $Na_2HPO_4 \cdot 2H_2O$, $NaSiO_3 \cdot 9H_2O$, Na_2CO_3, $Na_2EDTA \cdot 2H_2O$, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, $FeCl_3 \cdot 6H_2O$, Fe–Na-EDTA·$3H_2O$, $CuSO_4 \cdot 5H_2O$, $MnCl_2 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $NiSO_4 \cdot 6H_2O$, H_3BO_3, $Na_2MoO_4 \cdot 2H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, H_2SeO_3, Na_3VO_4, K_2CrO_4, thiamine HCl, biotin, vitamin B12, and citric acid·H_2O ($C_6H_8O_7 \cdot H_2O$).

2.3. Genetic algorithm

A genetic algorithm (GA) is an iterative search method that imitates the biological process of natural selection to arrive at an optimal solution. A genetic algorithm based search involves a cycle of four stages. The process begins by creating a population of individuals (experiments). Each experiment is represented by its set of properties encoded in a "chromosome". The two subsequent stages evaluate these experiments or individuals to select the best ones. These are then interbred to generate a new population, or generation. The fitness of the individuals in each generation is assessed in terms of the value of an objective function. The cycle terminates either when a certain number of generations are reached, or the individuals in a given generation have attained a certain improved level of fit relative to the initial population. The functioning of genetic algorithms is further discussed by Muffler et al. [13].

The computer software Galop (Institute of Biotechnology, Jülich, Germany) was used for the genetic algorithm calculations. The keep-best roulette wheel selection method was used to determine a string's probability for reproduction and breeding. The breeding policy stipulated that a parent with better chromosomes produces more descendants

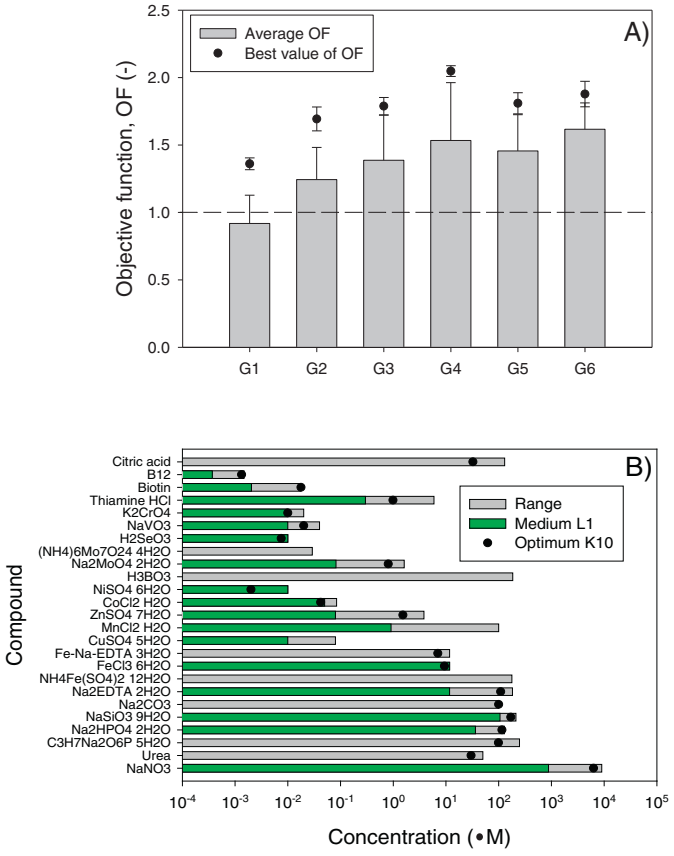

Fig. 1. (A) Optimization sequence of the genetic algorithm. The bars represent the average values of the objective function (O.F.) for all individuals of a given generation. The corresponding standard deviations are shown. Circles denote the best value of the objective function within a given generation. (B) Composition of the tested media. The gray bars denote the possible range of concentration of the specified media constituents. The concentrations generally used in the L1 medium for growing dinoflagellates are denoted by green bars. The black circles denote the best concentrations found in the generation 6.

with the same chromosome. The crossover probability was set at 95% and the mutation rate was fixed at 1%. The starting point, or the initial population, was randomly generated by the genetic algorithm. Each parameter in the chromosome was specified by a binary bit string of a given length. This length was determined on the basis of the different ranges of concentrations of the different media components. An individual string consisted of 3–4 bits, to give a chromosome of 81 bit length. The maximum number of individuals (i.e. experiments) that could be handled in a single generation was 70 cultures, each with a different medium composition and each composition assessed in triplicate. The duration of the cultures was 10 days in multiwell plates and at least 15-days in T-flasks. The cells were counted every three days in multiwell plates and every two days in T-flasks. A total of 420 experiments were carried out in this study

The biomass productivity relative to the control culture grown in the L1 medium was used as the objective function (O.F.) for evaluating each chromosome. The objective function is calculated as follows:

$$O.F. = \frac{N_{exp.f} - N_{exp.0}}{N_{L1.f} - N_{L1.0}}. \quad (1)$$

In Eq. (1) $N_{exp.f}$ is the final cell concentration (cells mL^{-1}) attained in a given medium formulation; $N_{exp.0}$ is the initial cell concentration in the same formulation; $N_{L1.f}$ is the final cell concentration obtained in the L1