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Accumulation of phycocyanin in heterotrophic and mixotrophic cultures of the acidophilic red alga *Galdieria sulphuraria*

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Abstract

The relationship between light intensity, nitrogen availability and pigmentation was investigated in mixotrophic and heterotrophic cultures of the unicellular red alga *Galdieria sulphuraria* 074G, a potential host for production of the blue pigment, phycocyanin (PC). During the exponential growth phase of batch cultures, *G. sulphuraria* 074G contained 2–4 mg phycocyanin per g dry weight. In carbon-limited and nitrogen-sufficient batch cultures grown in darkness, this value increased to 8–12 mg g^{-1} dry weight during the stationary phase, whereas the phycocyanin content in nitrogen-deficient cells decreased to values below 1 mg g^{-1} dry weight during stationary phase. Light intensities between 0 and 100 μmol photons m^{-2} s^{-1} had no influence on phycocyanin accumulation in mixotrophic cultures grown on glucose or fructose, while light stimulated phycocyanin synthesis in cultures grown on glycerol, in which the phycocyanin content in stationary phase was increased from 10 mg g^{-1} dry weight in darkness to 20 mg g^{-1} dry weight at a light intensity of 80 μmol photons m^{-2} s^{-1}. At higher light intensities, less phycocyanin accumulated than at lower intensities, irrespective of the carbon substrate used. In carbon-limited continuous flow cultures grown on glucose or glycerol at a dilution rate of 0.63 day^{-1}, corresponding to 50% of the maximum specific growth rate, the highest steady-state phycocyanin content of 15–28 mg g^{-1} dry weight was found at 65 μmol photons m^{-2} s^{-1}. In contrast to the apparent glucose repression of light-induced PC synthesis observed in batch cultures, no glucose repression of the light stimulation was observed in continuous flow cultures because the glucose concentration in the culture supernatant always remained at limiting levels. Despite the fact that *G. sulphuraria* 074G contains less phycocyanin than some other microalgae and cyanobacteria, the ability of *G. sulphuraria* 074G to grow and synthesize phycocyanin in heterotrophic or mixotrophic cultures makes it an interesting alternative to the cyanobacterium, *Spirulina platensis* presently used for synthesis of phycocyanin.
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1. Introduction

The blue pigment phycocyanin (PC), a light harvesting pigment that is found only in cyanobacteria, Cryptophyceae and Rhodophyceae [1], is among the most important substances produced uniquely in cyanobacterial and microalgal cultures. PC has found usage as a fluorescent marker in diagnostic histochemistry [2,3] and as a dye in foods and cosmetics [4]. PC may also have potential as a therapeutic agent [5–7]. PC is a phycobiliprotein, composed of an α- and a β-subunit with 1 (α) and 2 (β) phycocyanobilin groups covalently attached (see, e.g. [8]).

Commercially, PC is produced in phototrophic cultures of the cyanobacterium *Spirulina* (*Arthrospira*) *platensis* using sunlight as energy source. Since these cultures depend on externally supplied light, they are difficult to scale up without loss of productivity. As the surface area to volume ratio decreases at increasing scale and the light paths inside the cultures get longer, self-shading increases and dark, unproductive zones develop [9].

PC is also found in the unicellular, acidophilic red alga *Galdieria sulphuraria*, which has been shown to be a candidate for a PC production process [10]. *G. sulphuraria*

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contains PC and minor amounts of a second phycobiliprotein, allophycocyanin. Compared to the established PC production processes using *S. platensis*, a production process involving *G. sulphuraria* may offer several advantages. Some strains of *G. sulphuraria* grow well heterotrophically and at least partly retain their photosynthetic apparatus, including PC, when grown in darkness [11,12]. Production of PC in these strains can therefore be carried out heterotrophically without the need for external light sources, or mixotrophically, in which case only low light intensities would be needed. The natural habitat of *G. sulphuraria* is hot, acidic springs, so the optimal growth conditions are found at temperatures above 40 °C and at pH 1–3. In addition, this alga is able to utilise many different carbon sources [11,13].

G. sulphuraria is a member of the Cyanidiales, a group of single-cell organisms of similar looking morphology, and *G. sulphuraria* has in the past also been referred to under the name of the related species, *Cyanidium caldarium*. However, recent classifications place *G. sulphuraria* and *C. caldarium* into two separate genera [14,15], where *C. caldarium* is unable to grow heterotrophically [14].

Only limited information about PC synthesis by *G. sulphuraria* in different growth conditions is available. Mixotrophy, nitrogen availability and light intensity are conditions that often have considerable effects on pigmentation in microalgae and cyanobacteria. In *G. sulphuraria* and related species, glucose represses PC synthesis [16–18], while it is also one of the carbon substrates that supports rapid growth [11]. Nitrogen limitation may result in almost complete depletion of phycobiliproteins in other photosynthetic microorganisms and halts PC synthesis in *C. caldarium* [19], a close relative to *G. sulphuraria*. In addition to their role in photosynthesis, phycobiliproteins may also function as intracellular nitrogen storage compounds which can be mobilised during nitrogen deficiency [20–22]. Light influences pigment synthesis both positively and negatively in photosynthetic microorganisms. Low light intensities generally stimulate synthesis of pigments while pigment synthesis is repressed at high light intensities [23]. In *C. caldarium*, light induces synthesis of photosynthetic pigments [16,19,24], but there is still no quantitative data on the effect of light intensity on pigmentation. In this paper, we have investigated the effects of nitrogen limitation, carbon limitation (nitrogen sufficiency) and light intensity on the accumulation of PC in a *G. sulphuraria* strain which contains a significant amount of PC when grown heterotrophically in darkness.

2. Material and methods

2.1. Strain and growth media

G. sulphuraria strain 074G was kindly supplied by Dr. Wolfgang Gross, Freie Universität Berlin. Stock cultures were maintained under constant light (30–50 μmol photons m^{-2} s^{-1}) by sequential transfer into photoautotrophic batch cultures and grown at room temperature, pH 2 in a defined growth medium [11] with no organic carbon substrates. The same growth medium, but with various concentrations of the nitrogen source, $(NH_4)_2SO_4$, and supplemented with organic carbon sources (glucose, fructose or glycerol) was used in heterotrophic and mixotrophic growth experiments.

2.2. Batch cultures

Heterotrophic, mixotrophic and phototrophic batch cultures were grown in 500 mL conical flasks containing 100 mL culture. Flasks with heterotrophic cultures were wrapped in aluminium foil to exclude light and placed in a shaking incubator at 42 °C and 200 rpm. Mixotrophic and phototrophic cultures were stirred by magnetic stirring at 400 rpm and placed in a cabinet, which contained eight fluorescent light bulbs (4–15 W) and excluded other (external) sources of light. The cabinet could accommodate eight culture flasks and the light intensity on the culture flasks (0–200 μmol photons m^{-2} s^{-1}) was controlled by the number and power of the fluorescent light bulbs turned on. The temperature in the cabinet was increased above ambient temperature by heat from the fluorescent tubes. When the temperature, which was continuously measured in one of the culture flasks, increased above 42 °C, two fans were turned on to blow room temperature air through the cabinet. At low light intensities, a 40 W heating element was installed inside the cabinet. Temperature was controlled within at 42 ± 0.1 °C. Cultures were grown at pH 2 with 5 g L^{-1} glucose, fructose or glycerol as carbon sources and 0.198–1.98 g L^{-1} $(NH_4)_2SO_4$ as nitrogen source, to give molar C:N ratios between 5:1 and 50:1.

2.3. Continuous flow cultures

Heterotrophic and mixotrophic continuous flow cultures were grown in a 3 L Applikon BTS 05 bioreactor (Applikon, The Netherlands) containing 2.5 L culture. The bioreactor consisted of a cylindrical glass jar with a diameter of 13 cm fitted with a top plate of stainless steel. The bioreactor was placed in a cabinet equipped with eight fluorescent tubes (Osram 15W/31-830) and equipped with a Pt100 temperature sensor and autoclavable pH and oxygen electrodes (Mettler Toledo). The temperature was maintained at 42 °C and the culture was stirred at 500 rpm and aerated with 3 L min^{-1} air. The exit gas was passed through a condenser at 4 °C to reduce evaporation. Depending on the number of fluorescent tubes turned on, the average light intensity on the reactor wall was between 0 and 395 μmol photons m^{-2} s^{-1}. When changing the light intensity, it was changed in the opposite direction of the previous change in order to avoid a continuous selection pressure for mutants with a different tolerance towards light compared to the original strain. The flow rate of growth medium

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