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Biochemical activities in *Chlorella* sp. and *Nannochloropsis salina* during lipid and sugar synthesis in a lab-scale open pond simulating reactor

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ABSTRACT

Chlorella sp. and *Nannochloropsis salina* cultivated in a lab-scale open pond simulating reactor grew well and produced 350–500 mg L^{-1} of biomass containing approximately 40% and 16% of lipids, respectively, while different trends in storage material (lipid and sugar) synthesis were identified for the two strains. In continuous culture the highest biomass and lipid productivity was respectively 0.7 and 0.06 mg L^{-1} h^{-1} at $D = 0.0096\ h^{-1}$, for *Chlorella* sp. and 0.8 and 0.09 mg L^{-1} h^{-1} at $D = 0.007\ h^{-1}$ for *N. salina*. The major polyunsaturated fatty acid (PUFA) in the lipid of *Chlorella* sp. was α-linolenic acid, found at a percentage of 23.0%, w/w, while *N. salina* synthesized eicosapentaenoic acid at a percentage of 27.0%, w/w. Glycolipids plus sphingolipids were predominant and richer in PUFA, compared to neutral lipids and phospholipids.

Activities of some key enzymes, such as pyruvate dehydrogenase (PDC), ATP-citrate lyase (ATP:CL), malic enzyme (ME) and NAD-isocitrate dehydrogenase (ICDH), which are implicated in acetyl-CoA and NADPH biosynthesis, were studied in cells grown in batch and continuous modes. PDC involved in the conversion of pyruvate to acetyl-CoA presented a constant activity in all growth phases. The high ATP:CL activity observed in algal cells, combined with low or zero ICDH activity, indicated the algae ability to generate acetyl-CoA from sugar via citrate. However, the lipogenic capacity of the strains under investigation seemed to be restricted by the low ME activity resulting to limited NADPH synthesis.

1. Introduction

Microalgae can convert solar energy into energy-rich compounds, such as starch and lipids. Although the process of lipid biosynthesis requires much more energy, enabling polysaccharides synthesis more common for most microalgae (Chisti, 2007; Ratledge and Cohen, 2008; Ahmad et al., 2011; Christophe et al., 2012), some of them accumulate lipids (namely Single Cell Oils) in high proportions exceeding in several cases 20% of their dry biomass, and therefore are placed in the group of oleaginous microorganisms (Ratledge, 2004; Ratledge and Cohen, 2008; Christophe et al., 2012). Indeed, some oleaginous microalgae synthesize polyunsaturated fatty acids (PUFAs), and many other bioactive compounds (such as pigments, antioxidants, antibiotics and toxins), so they are considered as competent sources of new products of interest for nutraceutical and pharmaceutical industries. Besides, algal lipids can be considered as feedstock for biofuels (biodiesel) manufacture, although this perspective is less realistic, considering the high cost of oil production when compared to that of plant oils (Ratledge and Cohen, 2008; Meng et al., 2009; Vicente et al., 2009).

During photosynthesis CO_2 is converted to glycerate-3-phosphate (G3P) which is the precursor for the synthesis of many cell molecules, such as polysaccharides and lipids. Lipid synthesis, a process competitive to that of polysaccharides synthesis, occurs after conversion of G3P to pyruvate which is then converted to acetyl-CoA via a reaction catalyzed by the pyruvate dehydrogenase complex (PDC). Acetyl-CoA is then used as precursor for fatty acid synthesis in the plastid. Alternatively, G3P can be converted into polysaccharides, molecules used for structural purposes and, under certain circumstances, as storage material. Energy from sugars is generated during their catabolism, commonly via glycolysis in the cytosol following by citric acid cycle in the mitochondrion. However, under specific growth conditions (i.e. under nitrogen or phosphate limitation) citric acid cycle could be disturbed, due i.e. to the inhibition of isocitrate dehydrogenase (ICDH) enzyme that catalyzes the conversion of isocitrate to α-ketoglutarate. In this case citrate is accumulated in the mitochondrion and then excreted in the cytosol where, in the presence of ATP dependent citrate lyase (ATP:CL), is ceased into acetyl-CoA and oxaloacetate. Again, acetyl-CoA generated from citrate could be used for fatty acid synthesis. Besides acetyl-CoA, a supply of NADPH (generated from NADH via a small cycle in which malic enzyme – ME participates) is also required for the fatty acids synthesis. The biochemical pathway described above, permitting the conversion of polysaccharides to lipids, is common in oleaginous heterotrophs (Ratledge,

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2004) but is never shown in microalgae. In fact, regulation of biosynthesis of storage materials in microalgae, as well as potential inter-conversions connecting the pools of polysaccharides and lipids, are less understood. Several approaches have been recently used aiming to direct carbon flux toward lipid than polysaccharides synthesis (Rawsthorne, 2002; Weselake et al., 2008).

In this paper, lipid and sugars formation during growth of a strain of *Chlorella* sp. (able to accumulate large quantities of lipids) and of a strain of *Nannochloropsis salina* (which in preliminary studies was proved to accumulate relatively low quantities of lipids) was studied. Formation of neutral and polar lipids (phospholipids, glycolipids and sphingolipids), as well as fatty acid composition of individual lipid fractions were studied in detail. The activities of some key enzymes implicated in lipogenesis were determined in cells grown in both batch and continuous culture hoping to get an insight of conceivable metabolic shifts of carbon flux toward sugars or lipid biosynthesis. The experimental results presented in this paper permit to conclude that algal cell, possessing high ATP:CL activity, can generate acetyl-CoA from citrate. However, lipid biosynthesis is probably restricted by the limited synthesis of NADPH, as far as the low ME activity indicates.

2. Materials and methods

2.1. Microorganisms and culture medium

Chlorella sp. and *N. salina* strains were provided from PLAGTON SA (Agrinion, Greece) and BlueBioTech Int. GmbH (Kollmar, Germany), respectively. The strains were maintained in 250 cm^3 flasks containing 100 mL of modified artificial seawater (ASW), under constant illumination of 180 $\mu E\,m^{-2}\,s^{-1}$ and at 25 ± 1 °C. The composition of modified ASW (Boussiba et al., 1987) was (in $g\,L^{-1}$) NaCl (Sigma, Steinheim, Germany), 27; $MgSO_4{\cdot}7H_2O$ (Sigma), 6.6; $CaCl_2$ (Carlo Erba, Rodano, Italy), 1.5; KNO_3 (Merck, Darmstadt, Germany), 1; KH_2PO_4 (Sigma), 0.02; $FeCl_3{\cdot}6H_2O$ (BDH, Poole, England), 0.014; Na_2EDTA (Sigma), 0.019 and 1 $mL\,L^{-1}$ of a microelement solution containing (in $mg\,L^{-1}$) $ZnSO_4{\cdot}7H_2O$ (Merck), 40; H_3BO_3 (Fluka, Steinheim, Germany), 600; $CoCl_2{\cdot}6H_2O$ (Sigma), 1.5; $CuSO_4{\cdot}5H_2O$ (BDH) 40; $MnCl_2$ (Sigma), 400 and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (Sigma), 370. The above medium containing KH_2PO_4 0.02 $g\,L^{-1}$ should be considered as a phosphate limited medium (Pruvost et al., 2011)

2.2. Photobioreactor design and culture conditions

Mono-algal but non-aseptic cultures were performed in ASW medium, in a laboratory scale home-made glass photobioreactor (PBR), resembling to an open pond (Fig. 1). The pond's depth and surface area were 15 cm and 580 cm^2, respectively resulting to a total volume of 8.7 L. The working volume was 5 L. Agitation was achieved by a circulator, in the entryway of which natural saturated with moisture air was provided to the culture with a rate of 0.5 vvm. The reactor was placed in a temperature-controlled room at 25 ± 2 °C. An electronic temperature sensor and a pH electrode (EYELA, Model *FC-10*, *Tokyo Rikakikai* Co. Ltd., Japan) were used to monitor temperature and pH. pH was manually controlled at 8 ± 0.3 using a 0.4 M HCl solution. In preliminary experiments 1 M tris–HCl buffer (pH 8.0) was used to control pH, but this buffer supported bacterial growth. Constant illumination of 120 $\mu E\,m^{-2}\,s^{-1}$ was provided by 8 W fluorescent lambs, which were placed 20 cm above the culture's surface. A transparent glass (50 mm thickness) was placed at the top of the pond to minimize medium evaporation.

PBR was inoculated with 500 mL algal preculture containing 5.5×10^6 cells mL^{-1}. Precultures were performed in 250 cm^3 conical flasks containing 100 mL of ASW and incubated in a rotary shaker working at 100 rpm and $T = 25 \pm 1$ °C under constant illumination of 180 $\mu E\,m^{-2}\,s^{-1}$.

Fig. 1. The open pond simulating photobioreactor designed and used for the current study. Characteristics: depth, 15 cm; surface area, 590 cm^2; total volume, 8.7 L; working volume 5 L; agitation was achieved by a circulator; moisture air supplied at 0.5 vvm; light illumination, 120 $\mu E\,m^{-2}\,s^{-1}$.

Batch cultures were carried out for a period of 25 days. Continuous cultures at different dilutions rates (D, h^{-1}) were carried out by adding fresh medium through a pump (Peripex G2 peristaltic pump IP40, Bioengineering AG, Switzerland) at a constant rate. Fluctuations of flow rate were less than 1.3%. Working volume was kept constant in the reactor, by using an overflow weir connected to a REGLO Analog MS-4/6 ISM 828, ISMATEC, Switzerland pump which was operating at the same rate with that of the inflow pump. Steady-state conditions were obtained after continuous flow of at least four working volumes of medium. Samples were taken at daily intervals and analyzed for microbial mass until constant values.

All cultures, chemical analyses and biochemical determinations were carried out at least in duplicate. The experimental data were treated using OriginPro 8 SR0©, 1991–2007.

2.3. Estimation of the maximum specific growth rate

The maximum specific growth rate (μ_{max}, h^{-1}) of the strains was estimated by fitting of the equation 1 on the experimental data derived from batch cultures.

$$\frac{dN'}{dt} = \mu \cdot N' \quad (1)$$

where N' ($= N/N_0$) is the normalized cell concentration and μ is the specific growth rate calculated using Eq. (2) (Verhulst, 1838).

$$\mu = \mu_{max} \cdot \frac{N'_{max} - N'}{N'_{max}} \quad (2)$$

where N'_{max} is the maximum normalized capacity of the system.

The model was fitted on the experimental data using ModelMaker© 1993. The Runge–Kutta integration method was used and the parameter values were optimized using the least squares method.

Additionally, μ_{max} was calculated by fitting Eq. (3),

$$\ln N = \ln N_0 + \mu_{max} \cdot t \quad (3)$$

on the linear section of the growth curve $\ln N = f(t)$.