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Utilization of lignocellulosic biomass for the production of omega-3 fatty acids by the marine microalgae *Crypthecodinium cohnii*

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Polyunsaturated fatty acids (PUFAs), especially those with very long chains (LC-PUFAs) such as eicosapentaenoicacid (EPA) and docosahexaenoic acid (DHA), have been widely recognized as important bioactive compounds that can be used in the *food* and *nutraceutical* industry. Microalgae are oleaginous microorganisms that have attracted attention, as they are able to accumulate high amounts of LC-PUFAs, when growing in heterotrophic cultures, supported by a carbon source (Santos-Sánchez *et al* 2016). At present, industrial production of heterotrophic microalgae is hampered by the high costs of glucose. Using lignocellulosic biomass as a substrate is a promising strategy for developing a sustainable bio-economy. Despite the challenges of the process, concerning the presence of inhibitors and the heterogeneity (hexoses/pentoses) of sugars in the biomass fractions, different substrates, such as rice straw hydrolysates (Joe *et al* 2015) have been successfully used in heterotrophic and mixotrophic microalgae cultivations for the production of lipids. The heterotrophic micro alga *Crypthecodinium cohnii* is an interesting source for DHA production and for research on DHA biosynthesis due to its unique fatty acid composition. *C. cohnii* can accumulate relatively high amounts of lipid (>20%) with 30–50% DHA of the fatty acids and no other polyunsaturated fatty acids present above 1% (de Swarf *et al* 2003).

In the present study, *C. cohnii* was used as a model microalgae organism towards the study of the efficient production of omega-3 fatty acids from lignocellulosic biomass. Microalgal cells were grown in standing cultures on standard medium containing 9 g/L glucose, 18.7 g/L sea salts and 2 g/L yeast extract with the pH of the medium set at 6.5, at 23°C in the dark. The standing cultures were used as inoculum for precultures. Shaken flask cultivations (precultures and shaken flask experiments), containing 25 ml medium in 50-ml shake flasks, were carried out at 27°C, pH 6.5, 160 rpm in a linear shaker. The cultures were inoculated with 10% (*v/v*) inoculum and incubated for 184h at 27ºC. At the end of cultivation, cells were harvested, washed, lyophilized, and weighed in order to determine the biomass concentration. For the extraction of the lipids, dried cells were mixed with a chloroform: MeOH 2:1 mixture and left overnight at room temperature. When lipids were extracted into the liquid phase, dH_2O was added at a volume equal to 20% of that of the organic mixture. After centrifugation, the lower phase was washed twice MeOH: dH_2O 1:1 mixture. After evaporation of the solvent, the total lipids were calculated gravimetrically. For the preparation of fatty acids methyl esters (FAME), the dried lipids were diluted in chloroform and mixed with MeOH: HCl 92:8. The tubes were incubated at 60ºC for 15 min, prior to the addition of $CaCl_2$ 5% (w/v). The methyl esters were extracted from the mixture with hexane and analyzed with GC-MS.

First, preliminary experiments were conducted using glucose as a carbon source in order to evaluate the effect of different process parameters, such as the initial concentration of carbon and nitrogen source and the feeding strategy, on the growth and the accumulation of fatty acids. At concentrations above 25 g/L, glucose inhibits growth of *C. cohnii* (de Swaaf *et al* 1999) as well as lipid accumulation in shake-flask cultures. As shown in **Table 1**, the lipid content of cells grown on glucose was 41.1% when yeast extract was added at a concentration of 2 g/L, whereas cells grown on 4 g/L nitrogen source contained less, 28%. In general, lipid accumulation in microorganisms is stimulated by an excess of a carbon source and a limitation in one (or more) of the other nutrients, especially nitrogen. The DHA content of the lipids was quite similar (37.08% and 40.39% respectively). The results showed that both growth and lipid accumulation are affected when initial glucose concentration is higher, so a fed-batch strategy was chosen for the case of lignocellulosic biomass-derived sugars.

For the experiments with lignocellulosic biomass, mild oxidative organosolv pretreated biomass was used as a substrate for the production of highly-concentrated glucose streams able to support the growth of *C. cohnii*. Organosolv lignocellulosic biomass was treated with aqueous solutions of organic solvents (ethanol, acetone, tetrahydrofuran), at different operation conditions (temperature and reaction time) under an overpressure of O_2 that was crucial in depolymerizing and removing the lignin fraction. Enzymatic hydrolysis of produced cellulose-rich fractions was performed using Cellic® CTec2 (Novozymes), a cellulase complex consisting of cellulases, hemicellulases and β-glucosidases to degrade cellulose and residual hemicellulose to fermentable sugars.

Table 1. Biomass and total fatty acid (TFA) production by *C. cohnii* cells in fed-batch cultivation in shake flasks, using either pure glucose (*Glc*) or biomass-derived sugars (*BDS*), under different conditions of nitrogen source supply (*YE*: yeast extract).

Glc (g/L)	YE (g/L)	Additional feeding (96h)	Biomass (g/L)	TFA (g/L)	DHA (g/L)	TFA (% of dry biomass)	DHA (% of TFA)
25	2	25 g/L Glc	15.02 ± 1.28	6.15 ± 0.18	2.28 ± 0.15	41.1 ± 2.3	37.08 ± 1.33
25	4	25 g/lt Glc	20.78 ± 2.59	5.78 ± 0.39	2.35 ± 0.36	28 ± 1.6	40.39 ± 3.49
25	2	25 g/L Glc/2g/L YE	16.08 ± 1.42	4.81 ± 0.15	2.07 ± 0.01	30.2 ± 3.6	43.19 ± 1.4
50	4	-	17.68 ± 0.97	5.09 ± 0.17	2.06 ± 0.05	28.8 ± 0.6	40.59 ± 2.29
25*	4*	25 g/L BDS*	9.23 ± 0.73	1.13 ± 0.01	0.46 ± 0.01	22.6 ± 0.2	40.31 ± 0.37

**lignocellulosic biomass-derived sugars were used as carbon source*

The results showed that *C. cohnii* was able to grow on glucose and other sugars produced from the enzymatic hydrolysis of lignocellulosic biomass, although the biomass and the total lipid production was lower compared to that pure glucose feeding. This is an indication that not only the carbon source but also the medium composition is important for the accumulation process in *C. cohnii*. Among the factors that could possibly affect the growth and metabolism of microalgae cells is the presence of other sugars except for glucose, such as xylose, and the presence of phenolic compounds. Lipid accumulation and DHA content of the lipids was similar to that retained with glucose, reaching 22.6% and 40.31% respectively. Although more experiments need to be conducted in order to optimize the whole process, the overall results of this work demonstrate that there is a potential of lignocellulosic biomass to be utilized for the production for value-added products, such as DHA and other omega-3 fatty acids, through environmentally friendly bioconversion processes.

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