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Enhancement of fermentative hydrogen production from *Spirogyra* sp. by increased carbohydrate accumulation and selection of the biomass pretreatment under a biorefinery model

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In this work, hydrogen (H_2) was produced through the fermentation of *Spirogyra* sp. biomass by *Clostridium butyricum* DSM 10702. Macronutrient stress was applied to increase the carbohydrate content in *Spirogyra*, and a 36% (w/w) accumulation of carbohydrates was reached by nitrogen depletion. The use of wet microalga as fermentable substrate was compared with physically and chemically treated biomass for increased carbohydrate solubilisation. The combination of drying, bead beating and mild acid hydrolysis produced a saccharification yield of 90.3% (w/w). The H_2 production from *Spirogyra* hydrolysate was 3.9 L H_2 L^{-1}, equivalent to 146.3 mL H_2 g^{-1} microalga dry weight. The presence of protein (23.2 ± 0.3% w/w) and valuable pigments, such as astaxanthin (38.8% of the total pigment content), makes this microalga suitable to be used simultaneously in both food and feed applications. In a *Spirogyra* based biorefinery, the potential energy production and food-grade protein and pigments revenue per cubic meter of microalga culture per year was estimated on 7.4 MJ, US $412 and US $15, respectively, thereby contributing to the cost efficiency and sustainability of the whole bioconversion process.

[**Key words:** Microalgae; *Spirogyra* biorefinery; *Clostridium butyricum*; Dark fermentation; Microalga pigments]

Hydrogen (H_2) has been suggested as the energy carrier of the future, mainly due to its high conversion efficiency and, most importantly, its non-polluting nature (1). At present, most H_2 is produced thermochemically from fossil fuels, by steam reforming and coal gasification. It can also be produced from biomass, by pyrolysis or gasification, and electrochemically from water (1). Compared to these H_2-producing processes, the biological route has several advantages, such as being energy saving and environmentally friendly (2).

Dark fermentation, in particular, may use lignocellulosic biomass such as agricultural and agro-industrial residues as feedstock for the production of second generation (2G) biofuels. However, microalgae may present an alternative to this type of biomass feedstock, for the production of third-generation biofuels (3). Microalgae display higher photosynthetic efficiencies, higher yields and growth rates, and fewer requirements for land cultivation. They can be grown in non-arable terrain that is arid and barren, unlike traditional lignocellulosic biomass. Moreover, their growth is not inhibited by low quality water, including wastewater. Microalgae also require lower amounts of water than some terrestrial crops, thereby minimising the risk of depleting freshwater sources (4). Depending on the genera and species, algae can produce and accumulate different types of carbohydrates, lipids and other complex molecules that are suitable for the production of biofuels and bioproducts. Several examples of microalgae can be mentioned that have been studied for biodiesel (5–7), bioethanol (8) and H_2 production (9–11), possibly within a biorefinery framework (12).

Spirogyra sp. microalga possesses one of the highest capabilities of sugar accumulation, up to 64% of the algal dry weight (dry wt.) (13). This microalga is a member of the Zygnemataceae family which comprises filamentous, unbranched green algae (14). *Spirogyra* sp. is usually found in bodies of stagnant water, ditches or slowly flowing freshwater habitats all over the world (14). The cell wall of *Spirogyra* sp. has two layers: the outer wall is composed of pectose while the inner wall is mainly cellulose. The pectose changes to pectin which dissolves in water making the filament slimy to the touch. Depending on the lifecycle, *Spirogyra* sp. cell walls produce this large mucilaginous layer on the outside, to facilitate adhesion between cells and contact surfaces. The chloroplasts of the microalga also contain pyrenoids, intracellular structures around which starch usually accumulates (15). Moreover, the main pigment present in *Spirogyra* sp. is astaxanthin which, it is claimed, acts as a protective agent against oxidative stress damage (16). This carotenoid pigment is a potent radical scavenger and singlet oxygen quencher. Increasing evidence indicates that it even surpasses the antioxidant benefits of β-carotene, vitamin C and vitamin E and therefore has many industrial applications (17). The protein content in microalgae biomass is usually well balanced with essential amino acids present, and has been mainly used in the feed industry (18).

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