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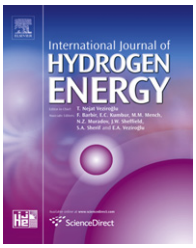

Enhanced hydrogen production by means of sulfur-deprived *Chlamydomonas reinhardtii* cultures grown in pretreated olive mill wastewater

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ABSTRACT

Under sulfur-deprived conditions, the metabolism of *Chlamydomonas reinhardtii* switches to the photoproduction of hydrogen. This process is sustained by both photosystem II-driven water splitting and by the fermentation of stored carbohydrates. We investigated the possibility of using diluted pretreated olive mill wastewaters (OMW), which contain organic acids and sugars, as a substrate on which to grow *Chlamydomonas*, in order to obtain suitable biomass to produce hydrogen. The cells grown on a mixture of pretreated OMW and TAP (tris-acetate-phosphate) (50% dilution) were found to be richer in carbohydrates and exhibited a greater production of hydrogen (150 ml H_2 l^{-1} culture), compared to the control cells (100 ml H_2 l^{-1} culture). In these cultures, the hydrogen production process was characterized by a shorter aerobic phase and a longer hydrogen-production period. The results offer a useful perspective for the utilization of olive mill wastewaters, which constitute an environmental problem, particularly in Mediterranean areas, and for increasing the output for hydrogen production with *Chlamydomonas*.

1. Introduction

The world's declining fossil fuel reserves require the development of alternative clean and renewable resources of energy, and the production of photobiological hydrogen is considered to be promising in this respect [1,2]. Hydrogen has several desirable characteristics. It is a high-energy carrier, does not pollute, and yields only water after combustion. These characteristics make hydrogen a suitable fuel for the future [3–5].

A selected group of photosynthetic organisms has evolved the ability to harness light energy to drive H_2 gas production from water. Among these, the green microalga *Chlamydomonas reinhardtii* under anaerobic conditions is capable of H_2 photoproduction [1,6]. The process is sustained by both the photosystem II (PSII)-driven water splitting, and the fermentation of stored carbohydrates which provide H^+ and e^- to hydrogenase (H_2ase), the synthesis and functioning of which is very sensitive to the presence of oxygen [7,8]. Anaerobiosis is attained by down-regulating the activity of PSII by means of sulfur starvation, until the rate of photosynthesis crosses that of respiration [6].

In order to optimize the bio-hydrogen production process in *C. reinhardtii*, different strategies have been suggested, such as an optimization of the light, pH regime, medium composition [9–12] and addition of sulphate to the medium during hydrogen production [13–15]. Moreover, the utilization of mutant strains with reduced light harvesting antenna complexes and an increased electron and proton supply have been studied [16,17]. It has been reported a relevant increase

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in the rate and duration of hydrogen production under sulfur starvation in a *C. reinhardtii* mutant capable of a high starch accumulation and blocked in state 1 condition [17]. Recently, a consistent increase in hydrogen production was obtained with D1 protein *C. reinhardtii* mutants [18,19].

The possibility of improving the biomass and output rates of hydrogen production has been reported in *C. reinhardtii*, by introducing an alternative route to supply H^+ and e^- to the hydrogenase enzyme utilizing an alternative source [20]. In particular, the authors reported an increased H_2 production in a *C. reinhardtii* mutant by facilitating access to glucose. It has also been reported that *C. reinhardtii* cells were capable of accumulating large amounts of starch following nitrogen starvation, particularly when they were grown under mixotrophic and heterotrophic conditions [21].

The versatility in utilizing organic substrates as a carbon source makes *C. reinhardtii* and other microalgae suitable for testing their growth in wastewater which are usually characterized by high organic matter content [22].

Olive mill wastewater (OMW) is the by-product of the production of olive oil. It has a dark brownish color and a strong odor, and is considered to be one of the most greatly pollutant agricultural wastes. The fresh organic matter content in OMW causes agricultural and environmental concerns in olive oil-producing countries because of its effects on soil microflora, status and fertility and groundwater contamination [23]. The toxicity of OMW is also due to its high content of phenolic compounds in a wide range of molecular weights (MW), from low-MW substituted phenols to complex high-MW phenolic compounds [24–27]. During the production of olive oil, large quantities of phenols are released along with the wastewater, depending on their partition coefficient. Previous studies have reported the utilization of olive mill wastewaters for the growth of the microalgae [28–31], and for photobiological hydrogen production [32] since these wastewaters are enriched with large amounts of organic constituents, such as sugars, volatile acids, polyalcohols and fats.

We have devised and patented a new system of OMW depuration, which consists of the biofiltration of crude OMW using two vegetable matrices, the first one of which is made with the biomass of the *Azolla caroliniana* fern and the other one, with activated carbon (Patent n. FI2006A000155). By using this system, it is possible to remove almost totally the phenolic compounds thus obtaining clean water which still contains the main short-chain organic acids and monosaccharide sugars that can sustain the growth of microalgae.

In this paper we have investigated the possibility of utilizing diluted pretreated OMW as a substrate for growing *C. reinhardtii*. Cell-grown in OMW has been found to be rich in carbohydrate content, which constituted a source for hydrogen production under conditions of sulfur starvation.

2. Materials and methods

2.1. Pretreatment of olive mill wastewaters

The OMW was centrifuged and subjected to filtrations using two differently packed vegetable matrices (*A. caroliniana* and granular activated carbon), which removed most of the pollutant substances, particularly polyphenols, by means of absorption [33]. The chemical composition of OMW before and after the treatment is shown in Table 1.

In order to standardize the composition of the OMW used in the experiments, the treated OMW was diluted to a concentration of organic compound (COD) of 5000 mg kg^{-1}. For this purpose, the treated OMW was usually diluted by about 50%. Furthermore, such a dilution ratio made it possible to further reduce both the color of the wastewater, and the initial COD between 11.000 and 12000.

2.2. Olive mill wastewater analysis

Table 2 shows the chemical composition of the treated OMW used in this study. The treated OMW showed the presence of a considerable amount of sugars, such as glucose and fructose, which were detected at concentrations of 340 mg l^{-1} and 130 mg l^{-1}, respectively. Among the organic acids, citric, acetic and malic acids were predominant. In addition, appreciable amounts of lactic acid and succinic acid could also be detected. Among the alcoholic compounds, mannitol was the most abundant. The total nitrogen was very low 7.01 mg l^{-1}, while phosphorus contents reached 42.94 mg l^{-1}. The presence of polyphenol was reduced to 10.41 mg l^{-1}. Among heavy metals the highest value was found for Ni (65.81 μg l^{-1}), while the amounts of Pb, Cr, Hg, and Cu, was in the order of few μg l^{-1}. However, OMW was characterized by a high concentration of COD, in particular sugars (glucose and fructose) and acetic acid, which could be used profitably for the photomixotrophic growth of *Chlamydomonas*.

Table 1 – Chemical composition of the olive mill wastewater, before and after the treatment with *Azolla caroliniana* and Granular Activated Carbon [33].

Compounds	Before the treatment	After the treatment
	mg l^{-1}	
Chloride	593.4 ± 27.2	254.6 ± 11.6
Nitrate	3.4 ± 0.18	<0.5
Sulphate	72.5 ± 3.5	62 ± 3.0
Orthophosphate	926.3 ± 46.2	159.3 ± 7.9
Total *P*	428.6 ± 18.3	98.6 ± 4.3
Total *N*	785.9 ± 35.8	16.2 ± 0.7
K	16.8 ± 0.8	4.6 ± 0.2
Ca	648.3 ± 32.0	341.3 ± 16.9
Mg	180.3 ± 9.1	47.1 ± 2.4
	μg l^{-1}	
Cd	<0.5	<0.5
Cr	33.2 ± 1.2	10.2 ± 0.4
Fe	6535 ± 280	2975 ± 127
Hg	<0.5	<0.5
Ni	925.3 ± 36.0	142.2 ± 0.2
Pb	7.8 ± 0.2	5.2 ± 0.1
Cu	335.2 ± 11.2	1.9 ± 0.1
	mg Kg^{-1}	
Polyphenols	4005 ± 185	24.0 ± 1.1
	mg l^{-1} O_2	
COD	52,500 ± 2700	11,620 ± 598
BOD5	16,250 ± 800	3620 ± 178
pH	3.6	4.6