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Effect of iron and molybdenum addition on photofermentative hydrogen production from olive mill wastewater

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ABSTRACT

Photofermentative hydrogen production from olive mill wastewater (OMW) by *Rhodobacter sphaeroides* O.U.001 was assessed under iron and molybdenum supplementation. Control cultures were only grown with 2% OMW containing media. The analysis included measurements of biomass accumulation, hydrogen production, pH variations of the medium, and changes in the chemical oxygen demand (COD) of the wastewater. Growth under control and Mo-supplemented experiments yielded about the same amount of biomass (~0.4 g dry cell weight per L culture). On the other hand, Mo addition slightly enhanced the total volume of H_2 gas production (62 mL H_2), in comparison with the control reactor (40 mL H_2). Fe-supplemented cultures showed a significant increase on H_2 production (125 mL H_2), tough having a longer lag time for the observation of the first H_2 bubbles (24 h), compared to the control (15 h) and Mo-supplemented ones (15 h). Fe-added cultures also yielded better wastewater treatment by achieving 48.1% degradation of the initial chemical oxygen demand (COD) value compared to the control reactor having 30.2% COD removal efficiency. Advances described in this work have the potential to find applications in hydrogen industry while attempting an effective management of cheap feedstock utilization.

1. Introduction

Photosynthetic bacteria are one of the most favorable organisms for biological hydrogen production processes, in accordance with their higher substrate conversion efficiencies and capabilities of the utilization of variable substrate sources [1]. Hydrogen production by photosynthetic bacteria (i.e., *Rhodobacter sphaeroides*) occurs under illumination in the presence of anaerobic atmosphere through the breakdown of organic substrates [2], by the process known as photofermentation. However, practical applications for H_2 production cannot tolerate the utilization of expensive synthetic culture media unlike the most laboratory based experiments. An efficient way to overcome the economic restrictions of photofermentative hydrogen production is to combine this photosynthetic process with wastewater utilization. Thus, several studies of the recent literature are focused on the utilization of cheap organic substances such as residual wastes from the

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food and agricultural industry, or wastewaters with high levels of organic compounds [3].

In our previous studies, olive mill wastewater (OMW) has been reported as being a sole substrate source for photobiological hydrogen production [4]. This kind of waste material has severe disposal problems in accordance with its high production potential, mainly throughout the Mediterranean countries. The olive oil production process generates various phases, such as (i) oily phase, (ii) solid residue and (iii) dark colored "vegetable water" which are mainly obtained from the water content of the fruit. OMW is a blend of this vegetable water, various soft tissues resulting from the pulp of olive fruit, and some process water [5,6].

Photofermentative hydrogen production and consumption are known to be derived by the actions of nitrogenase and membrane-bound uptake hydrogenase enzymes, respectively [7–9]. Nitrogenases function on catalyzing the reduction of dinitrogen into ammonia [8,10]. Mo-nitrogenase is a binary enzyme, consisting of two metalloproteins as: (i) Fe-protein and, (ii) Mo–Fe protein [10]. Several researchers investigated the effect of various parameters on the amount of hydrogen production activity, with a reported reduction in the nitrogenase activity in the absence of metal ions such as molybdenum and iron [11,12]. Enhanced supplementation of the substrate source with Mo and Fe metals was also stated to increase the amount of hydrogen production by various *Rhodobacter* species, with an average concentration of 0.1 mM of Fe-citrate [11,13,14]. For the amount of molybdenum, the highest hydrogen productivity was obtained at 16.5 μM of Mo in a study by Kars et al. (2006) [11]. However, its concentration was kept around 0.16 μM by the subsequent researchers [13–15], since 16.5 μM and 0.16 μM molybdenum concentration yielded similar results with a slightly higher yield for the former one. Presence of molybdenum and iron is necessary since they are found in the structure of Mo-nitrogenase [2,8], and moreover Fe is found in several electron carriers of the photosynthetic electron transport system (ETS) such as ferrodoxin [16]. Photofermentation is strongly associated with the ETS, in which the bacteria produce energy [2,17].

Despite the fact that there have been several studies related with the positive effects of increased metal iron concentration on the amount of biological hydrogen production, these studies considerably differ from each other on the aspect of microorganism, substrate source, and type of the fermentation process used for H_2 production (i.e., dark fermentation, photo-fermentation). Thus, direct comparisons among different processes are not straightforward. The majority of "metal-supplemented" studies used either mixed cultures or defined media as a substrate source for biohydrogen production via dark fermentation [18–21]. Other portion of "metal-supplemented" biohydrogen production studies investigated the positive effect of increased metal (i.e., iron) concentration on the amount of photofermentative hydrogen production by using single photosynthetic bacteria [11,13–16]. Within these references, Uyar et al. (2009) [13], Ozgur et al. (2010) [14] and Afsar et al. (2009) [15] used dark fermentation effluents of several plant extracts, whereas Kars et al. (2006) [11] and Zhu et al. (2007) [16] added the metal ions into defined media. Nevertheless, it has never been reported how Mo and Fe metal supplementation would influence the photofermentative hydrogen production from olive mill wastewater. Understanding the effects of supplementary metal to the olive mill wastewater can be beneficial for yielding an efficient biohydrogen production in addition to enhanced wastewater treatment.

Therefore, there is a need to examine the performance and productivity of a culture grown in OMW that has been supplemented by essential metal ions. In this context, we investigated the effect of additional Mo and Fe on the photosynthetic hydrogen production from olive mill wastewater, as well as the COD removal efficiencies of these metal supplemented cultures. Throughout these photofermentative hydrogen production processes; biomass accumulation, pH variation, hydrogen production, and changes in the chemical oxygen demand (COD) values are mainly examined.

2. Materials and methods

Hydrogen production experiments were performed in jacketed glass-column photobioreactors (400 mL total volume, with a working volume of approximately 360 mL), while the temperature was kept around 32 °C by the thermostated water flow through the outer-jackets of the reactors. The photobioreactor liquid was stirred at a rate of 300 rpm with a magnetic stirrer (Heidolph, MR-3000). Light intensity at the outer surface of the reactor was kept at 200 W/m^2, by using a tungsten lamp. For all hydrogen production experiments, argon gas was flashed to create an anaerobic atmosphere. *R. sphaeroides* O.U.001 (DSM 5864) was used as the photosynthetic organism, as details given by Eroglu et al., 2004 [4]. Control reactor for hydrogen production media only contained 2% OMW (dilution was achieved by distilled-water). Main analysis including the cell concentration of the culture, composition of the collected gas, pH of the culture medium measurements were clearly explained in our previous publications [4,6]. Physicochemical properties of the OMW sample were given in our previous paper (Sample ID: OMW-C) [22]. Extensive descriptions of the material and methods were previously described in detail within our previous papers [4,6], and here we only summarize the key points in the following paragraphs.

Currently re-mentioned properties of OMW including color and COD were determined by following the "Standard Methods" of American Public Health Association [23]. By using Elemental Analyzer (C–H–N 600, Leco), elemental quantities of carbon, nitrogen and hydrogen were obtained. The amounts of Mo and Fe were determined by an atomic absorption spectrophotometer (Philips, PU9200X) by following the instructions given in "Standard Methods" [23]. Analysis for the organic substances had been carried out by the HPLC system (Varian ProStar HPLC) in Middle East Technical University's Central Laboratory and the detailed procedure has been explained in one of our previous manuscript [24]. At the end of each experiment, total carbonate/bicarbonate content of the supernatant from the centrifuged cell culture (centrifugation at 10,000×*g* for 15 min @ 4 °C) was analytically calculated by following the titration procedure as explained previously [25].

According to the metal analysis, some amounts of Fe (0.01 mM) and Mo (0.07 μM) were already present in the 2% OMW containing culture media. In order to see the effect of

these ions on H_2 production, their amounts were increased by external supplementation. Throughout these experiments; additional molybdenum and iron was obtained from sodium-molybdate ($Na_2MoO_4 \cdot 2H_2O$), and iron(III)-citrate ($Fe(C_6H_5O_7) \cdot H_2O$), respectively. Amounts of external Fe and Mo supplementations are 0.1 mM and 16.5 μM, respectively and are based on the optimum metal concentration obtained for the same microorganism by Kars et al. (2006) [11].

Three datasets had been investigated for the confirmation of the reproducibility of the data shown in the following tables and figures. Each data has been given in the form of the arithmetic mean and standard deviations of these three measurements, where indicated in the following tables and figures. The data points given in the following figures have been curve-fitted with smooth-lines via KaleidaGraph™ program.

3. Results and discussion

Both Mo and Fe are the two important factors of photobiological hydrogen production studies since they are known to be the part of nitrogenase-enzyme which catalyzes H_2 production [2,8]. In order to investigate the effect of these two metals on the growth and H_2 productivity of photosynthetic bacteria, iron-citrate and sodium-molybdate were independently added into the 2% OMW containing medium at specified concentrations, as previously explained in the section for Material and Methods. For comparison purposes, a control reactor containing 2% OMW as a sole substrate source was operated in parallel to the +Mo and +Fe supplemented ones.

3.1. *Growth and hydrogen productivity under iron and molybdenum supplementation*

Following results compare the patterns of biomass accumulation (Fig. 1), cumulative hydrogen gas production (Fig. 2), and pH values of the growth media (Fig. 3) of the cultures in the photobioreactors under control (Control) and metal supplemented (+Mo, +Fe) conditions.

Biomass accumulation under control and molybdenum supplemented conditions revealed similar trail since both increased rapidly as a function of time and reached a stationary phase around 0.4 g dry cell weight (dcw) per L culture at about 42 h after inoculation (Fig. 1). Under iron supplemented conditions, biomass production has been observed at several stages (Fig. 1, +Fe): (i) there was an initially higher rate of biomass accumulation, which continued for the first 42 h, (ii) after the 42nd h, unlike the control and Mo supplemented ones that enter stationary phase, the biomass accumulation was continued by a declined rate of growth until reaching a stationary phase at about 0.56 g dcw per L culture by the 87th h after inoculation (Fig. 1, +Fe).

Anoxygenic phototrophic bacteria is known to release hydrogen as a mechanism of dissipating excess reducing ions that has been accumulated during the cell growth in [C]/[N] rich anaerobic conditions [16,26]. Hydrogen gas accumulation under Control was initially observed after a lag time of 15 h (Fig. 2, Control). H_2-accumulation increased linearly as a function of time with a rate of about 1 mL H_2 per h until reaching

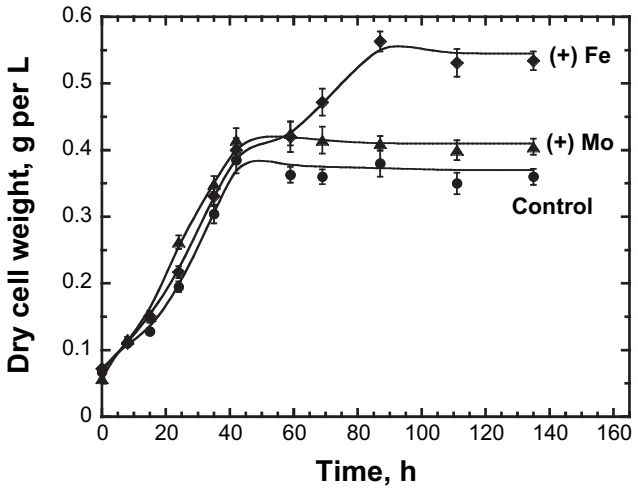

Fig. 1 – Biomass (dry cell weight) accumulation as a function of growth time of *R. sphaeroides* under control (C, circles), molybdenum supplemented (Mo, triangles) or iron supplemented (Fe, diamonds) reactors. Note the prompt and linear increase in biomass accumulation under all reactors until 49th h and prolonged growth for the Fe supplemented cultures between 49 and 87th h after the inoculation.

a total volume of approximately 30 mL at 42 h, and finally 40 mL was collected at about 135 h after inoculation (Fig. 2, Control). Similarly, under +Mo conditions, the lag time for H_2 production was around 15 h (Fig. 2, +Mo). After the initial lag, H_2 production increased linearly as a function of time with a rate of approximately 2 mL H_2 per h until reaching a total volume of approximately 61 mL at 42 h (Fig. 2, +Mo), while having the shortest duration time for hydrogen production (27 h)

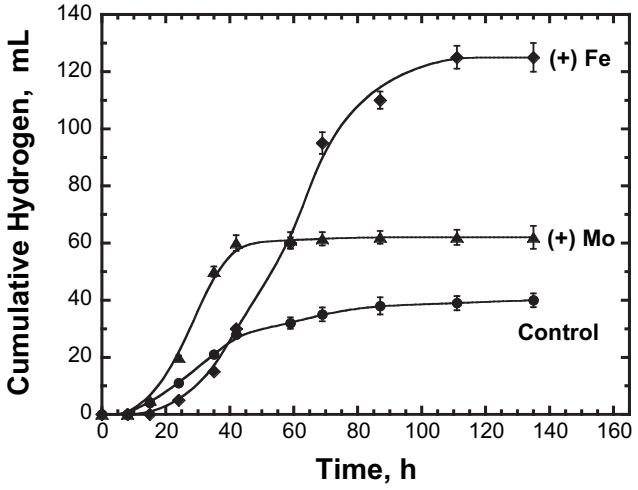

Fig. 2 – Cumulative hydrogen gas production as a function of growth time of *R. sphaeroides* under control (C, circles), molybdenum supplemented (Mo, triangles) or iron supplemented (Fe, diamonds) reactors. There is a 15 h lag in H_2 production under control and Mo supplemented reactors and the pronounced 24 h lag seen under Fe supplemented conditions.

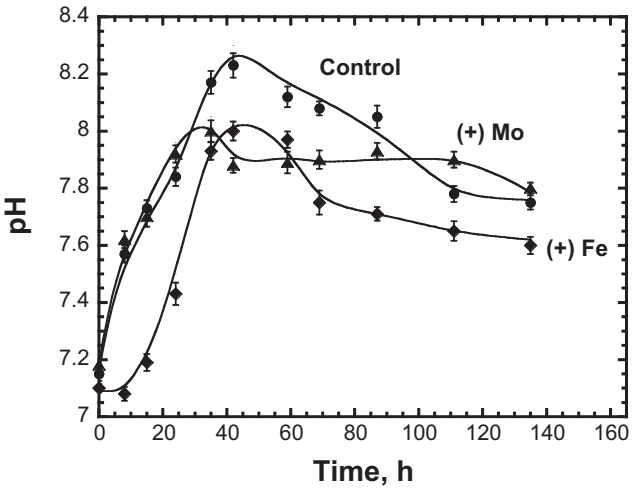

Fig. 3 – The pH of the medium as a function of growth time of *R. sphaeroides* under Control (C, circles), molybdenum supplemented (Mo, triangles) or iron supplemented (Fe, diamonds) reactors. Note the unchanged pH for the first 8 h after the inoculation, for the Fe-supplemented reactor. For all reactors, there is a prompt and linear increase in pH from ~7.1 to around 8–8.2 for the first 49 h and a step-wise decrease until the end of the experiments. Reduction of pH at later stages is more pronounced for the control and the Fe supplemented reactors.

compared to the other reactors. On the other hand, under +Fe conditions, the lag time for H_2 production was extended to 24 h (Fig. 2, +Fe). Under iron-metal supplemented conditions: (i) during the first stage (24–69 h) H_2 accumulation increased linearly as a function of time with a rate of about 2 mL H_2 per h, and reached a total volume of approximately 95 mL at 69 h, (ii) second stage includes the accumulation of hydrogen with a rate of approximately 0.7 mL H_2 per L culture per h, until reaching a total volume of approximately 125 mL at 111 h after the inoculation (Fig. 2, +Fe).

Under growth conditions for the Control reactor, the pH of the media increased linearly from 7.15 (initial pH) to 8.23 between the time intervals of 0–42 h (Fig. 3, Control). After the first 42 h of the Control reactor, pH of the media showed a gradual decrease from 8.23 to 7.75 by the end of the experiment. Similar to the Control, +Mo supplemented conditions observed a pH increase from 7.18 to 8.0 during the first 35 h of cultivation. This was followed by a slow decrease down to the pH of 7.8 by the end of the experiment (Fig. 3, +Mo). The pH values of these two media increased during their exponential phase of growth (Fig. 1), and presumably reflecting the maximum uptake of anion nutrients by the cells [6,14]. Under iron supplemented conditions, pH change has been observed after 8th h. Between the time intervals of 8–42 h, pH of the culture media increased from 7.1 to 8.0 (Fig. 3, +Fe). This was followed by a gradual decrease to 7.6, which started from the 42nd h until the end of the experiment (Fig. 3, +Fe), corresponding to the stage of slower rate of growth for the same time interval (Fig. 1, +Fe).

As indicated before, a pH increase as a function of time during the cell growth reflects the active uptake of anion nutrients by the cell, associated exchange of hydroxide anions for sustaining a balance of electrical charge across the plasma membrane [6,14]. In this view, a continuous pH increase under Control and +Mo supplemented conditions (Fig. 3) reflects the continuous uptake of nutrients in parallel to biomass accumulation until the stationary phase was reached (Fig. 1).

The step-wise pH increase for the +Fe samples (Fig. 3, 0–8 h and 8–42 h) apparently reflects lower nutrient uptake activity during the first time interval (0–8 h), followed by the enhancement of this activity between the time interval of 8–42 h. This initial decrease in the uptake of nutrients appears to be non-limiting for the growth of the cultures, since iron-supplemented cultures yielded similar growth rates with the others until the first time interval of 0–42 h (Fig. 1, +Fe). However, the decrease in the uptake of nutrients might have resulted longer lag time in the hydrogen production pattern of the iron supplemented ones (Fig. 2, +Fe). Changes in the pH values of all media are more clearly evident during the early stages after inoculation and is somewhat reduced at later times. The gradual decline in the pH during stationary phase could be due to the formation of fermentation end products with acidic properties. By the end of the experiments, the pH of the +Mo and Control media was found to be the more alkaline than +Fe media (Fig. 3).

In a study by Yang and Shen (2006), the affect of soluble iron concentration on the fermentative hydrogen production by anaerobic mixed microflora was investigated [27]. They figured out the flocculation of iron compound in the form of $FeCO_3$ when the initial pH of the culture media was around 8.0, and that yielded a decrease in the concentration of soluble iron. Due to such kind of flocculation, hydrogen production as well as the substrate conversion was observed to be enhanced via sorption, as seen for the cell immobilization mechanism. In the later stages of the growth, amount of volatile fatty acid production had been increased gradually that had caused the dissolution of the iron flocculants and releasing of flocculated iron.

In a similar manner, the pronounced lag (first 8th h) observed in the pH value of our iron supplemented media (Fig. 3, +Fe), in addition to its overall lower pH values compared to the control and Mo-added cultures, might have been caused by the possible formation and precipitation of iron-polyphenol chelates in the media for the first stage of the experiment (from the beginning until the time interval of around 42–59 h). After the 59th hour, possible accumulation of volatile organic acids as well as the H^+ and CO_3^{-2} ions in the liquid media might have triggered the dissolution of some iron-polyphenol chelates and granted an extension of the biomass and hydrogen gas accumulation (mentioned as secondary stages in Fig. 1, +Fe and Fig. 2, +Fe). As an overall, such a two-stage iron mechanism yielded faster biomass accumulation (Fig. 1, +Fe) and higher H_2 production (Fig. 2, +Fe) for the iron supplemented media.

These results bear similarity with the findings about the possible effects of the iron-metal on the phenolic content of some food. The chemical affinity of phenolic compounds for iron to form iron-polyphenol chelates has been observed in other biological systems [28,29]. In the study of Kapsokefalou et al. (2006) iron ingestion with green tea is shown to diminish the absorption of the polyphenolic compounds present in

green-tea [28]. In this context, better growth and hydrogen production observed for the current study by iron addition can be also beneficiated by the degradation of some aromatic compounds that can be harmful for the cultures, by being trapped through chelating with iron.

Overall results for the H_2 production parameters are shown in Table 1. It is also necessary to mention that the present experiments for the control culture have lower hydrogen production rates (based on the amount of concentrated OMW in the culture media) in comparison with the results that have been reported for the same strain in our previous paper [6], although they were both investigated within the same type of photobioreactors. The main reason for that is based on the variations in the compositions of OMW samples. It can be concluded from Table 1 that the supplementation of iron significantly enhanced the hydrogen production yield ($17.4\ L_{H_2}\ L_{OMW}^{-1}$) of the control reactor ($5.6\ L_{H_2}\ L_{OMW}^{-1}$), approximately by the factor of 3. Mo addition also achieved a higher H_2 production yield ($8.6\ L_{H_2}\ L_{OMW}^{-1}$) compared to the control reactor.

Maximum H_2 productivity (r_{H_2}) has been calculated by dividing the total volume of hydrogen gas produced by the volume of the culture and by the duration of H_2 production, yielding a unit of $mL_{H_2}\ L_{culture}^{-1}\ h^{-1}$. Due to the fact that r_{H_2} has an inverse proportion with the duration of hydrogen production (Δt_{H_2}), this time Mo supplemented reactor has the highest productivity ($6.4\ mL_{H_2}\ L_{culture}^{-1}\ h^{-1}$) compared to the +Fe($4\ mL_{H_2}\ L_{culture}^{-1}\ h^{-1}$) and the control reactors ($2.1\ mL_{H_2}\ L_{culture}^{-1}\ h^{-1}$), in coordination with its shortest Δt_{H_2} (27 h) (Table 1).

Another important parameter for such biohydrogen production studies is the light conversion efficiency (η). It is the ratio of the total energy value of the obtained hydrogen (heat of combustion) to the total energy input of the photobioreactor by solar radiation, and depends on the light intensity, irradiated area, duration time of H_2 production and total amount of hydrogen accumulation [30,31]. Light conversion efficiency of +Mo reactor (0.51%) was much higher than the ones obtained for the +Fe added (0.32%) and the control (0.16%) cultures (Table 1). Higher light conversion efficiency of Mo-added culture is also in parallel to its considerably shortest Δt_{H_2} (27 h) (Table 1).

In the present study, main organic compounds that can be metabolized into hydrogen gas are listed in Table 2, according to their initial concentrations in the OMW sample. The theoretical yield of hydrogen that could be produced from the complete conversion of these organic compounds to H_2 and CO_2 is given in Table 2. Theoretical amounts were obtained by taking into consideration that all of the substrate was converted into hydrogen and carbon dioxide production according to the following theoretical reaction [32,33]:

$$C_xH_yO_z + (2x - z)H_2O \rightarrow (y/2 + 2x - z)H_2 + xCO_2 \quad (1)$$

As given in Table 2, the total theoretical hydrogen yield was calculated to be 1.04 mol/L_{OMW}. On the other hand, the experimentally produced hydrogen gas was obtained as 0.20 mol/L_{OMW} for Control, 0.31 mol/L_{OMW} for +Mo and 0.63 mol/L_{OMW} for +Fe (Table 1). Likewise, these results illustrate highly enhanced organic conversion efficiency for Fe supplemented conditions into hydrogen (~60%), compared to the control reactor (~19%). Mo addition resulted in ~30% of organic conversion efficiency. For all conditions, the remaining portion of the organic substrates might have been utilized for the probable shift of the metabolism into bacterial growth and/or other kinds of bio-product synthesis instead of hydrogen evolution.

As the composition of the gas produced in all three reactors contains approximately 98% of H_2 and 2% of CO_2; CO_2 gas production yield has been obtained as 4 mmol CO_2 per liter of OMW for Control, 8 mmol CO_2 per liter of OMW for +Mo, and 15 mmol CO_2 per liter of OMW for +Fe (Table 1). Due to the high final pH values of the systems (between 7.6 and 8.0), the main portion of CO_2 remained in the liquid culture in the form of bicarbonate and carbonate (316 mmol for Control, 383 mmol for + Mo, and 478 mmol for + Fe per liter of OMW) as given in Table 1. After the addition of these two numbers to

Table 1 – Comparison of Fe and Mo-added cultures with the control reactor (2% OMW) on the base of medium composition, hydrogen production parameters, initial and final colors of the medium, and amount of CO_2 present in the form of liquid and gaseous phase.

Property	2% OMW + Mo	2% OMW + Fe	2% OMW (Control)
Total [Fe] (mM)	0.01	0.1	0.01
Total [Mo] (μM)	16.5	0.07	0.07
C/N molar ratio	42	43	42
Max. cell conc. (g/L_c)	0.425 ± 0.018	0.563 ± 0.015	0.385 ± 0.020
Total H_2 (mL)	62 ± 4	125 ± 5	40 ± 2
Lag time for H_2 production (h)	15 ± 2	24 ± 3	15 ± 3
Duration of H_2 production (h)	27 ± 3	87 ± 5	54 ± 3
H_2 production rate (mL H_2/L_c/h)	6.4 ± 1.0	4.0 ± 0.4	2.1 ± 0.2
Yield (L_{H_2}/L_{OMW})	8.6 ± 0.6	17.4 ± 0.7	5.6 ± 0.3
Light conversion efficiency (η%)	0.51 ± 0.08	0.32 ± 0.01	0.16 ± 0.02
$Color_i$ (PtCo APHA)	2325 ± 80	2475 ± 25	1260 ± 40
$Color_f$ (PtCo APHA)	2700 ± 50	2875 ± 45	2100 ± 30
CO_2 in liquid phase, in the form of total carbonate and bicarbonate (mmol/L)	383 ± 2	478 ± 5	316 ± 2
CO_2 in gas phase (mmol/L_{OMW})	8 ± 0.5	15 ± 0.6	4 ± 0.2
Total CO_2 (mmol/L_{OMW})	391 ± 2.5	493 ± 5.6	320 ± 2.2

Table 2 – Concentrations of organic compounds present in raw OMW sample (calculated from Eroglu et al., 2009 [22]) and their theoretical yields for H_2 and CO_2 production.

Organic compound	Amount (mmol/L_{OMW})	Theoretical H_2 yield (mmol H_2/L_{OMW})	Theoretical CO_2 yield (mmol CO_2/L_{OMW})
Acetic acid	66	264	132
Propionic acid	9	63	27
Butyric acid	9.5	95	38
Lactic acid	6	36	18
Glucose	2.5	30	15
Xylose	1.5	15	7.5
Arabinose	0.5	5	2.5
Methanol	3.5	9.5	3.5
Ethanol	20	120	40
Phenol	8.5	119	51
p-Cresol	5	85	35
m-Cresol	9.6	163	67.2
o-Cresol	2.0	34	14
Total theoretical yields		*1038.5 mM*	*437.7 mM*

the both phases, the total CO_2 production was calculated as 320 mmol CO_2 for Control, 391 mmol CO_2 for +Mo, and 493 mmol CO_2 for +Fe per 1 L of OMW.

As listed in Table 2, only the consumption of organic compounds would have caused the production of 438 mmol CO_2 per L_{OMW} on theoretical basis, which is higher than the experimentally produced amount of CO_2 gas for the Control (320 mmol/L_{OMW}) and the Mo-added cultures (391 mmol/L_{OMW}). However, iron supplementation yielded higher CO_2 production on the experimental basis (493 mmol/L_{OMW}). That can be explained by the consumption of additional organic materials extra for the ones given in Table 2, which have not been analyzed but might be present in this OMW sample.

Since Fe was added in the form of iron-citrate; small amount of carbon has been added externally. In order to see the probable effect of this artificial carbon; molar ratios of carbon to nitrogen was calculated and found to be 43 (Table 1). However, the difference between this value and the C/N molar ratio of the control medium (42) can be accounted as insignificant.

Initial color of the control media (1260 PtCo APHA) gets darker after the addition of +Fe (2475 PtCo APHA) or +Mo (2325 PtCo APHA) (Table 1). Besides the dark nature of these metals, extra color might have also been caused by the metallo-pigment complex formation between the aromatic components of OMW, which would assist metal-supplemented cultures to trap some toxic phenolic compounds as evidenced by achieved better productivities. After all H_2 production processes, the color of the media increased for all runs including the control reactor (Table 1). The increase in the initial color of the control reactor is consistent with the findings reported in one of our previous manuscript [4]. As shown in Fig. 3, the pH of all three media was increased from ~7 to around 8. It has been known that the solubility of phenolic or other types of hardly biodegradable components increase at basic conditions [34]. Therefore, during the later stages, these color-giving components are probably dissolved more in the effluent media and resulted darker color.

In the literature, there have been several studies about the effect of external metal ion addition on the biological hydrogen production by a single strain or mixed cultures via dark or photofermentation. In a study given by Lee et al. (2001), the effect of Fe^{+2} concentrations on the fermentative hydrogen production from sucrose has been investigated for the iron concentration in between 0 and 4000 mg/L $FeCl_2$ (31.6 mM Fe^{+2}) [20]. They obtained a maximum hydrogen yield (131.9 mL/g sucrose) at the Fe^{+2} concentration of 800 mg/L $FeCl_2$ (6.3 mM Fe^{+2}). Likewise, Wang and Wan (2008) reported that the H_2 production from the glucose media was increased from 194 to 302 mL after increasing the concentration of Fe^{+2} concentration from 0 to 300 mg/L (5.4 mM Fe^{+2}) [21]. They also observed a decrease in the amount of hydrogen production once iron concentration is higher than 300 mg/L (5.4 mM Fe^{+2}), which has been confirmed as the threshold iron concentration. Zhang and colleagues (2005) investigated the effect of additional iron concentration on the hydrogen yield of heat-shocked mixed cultures, while growing on a sucrose media [18]. They observed maximum amount of hydrogen production (2.73 mol/mol sucrose) at 1600 mg/L $FeSO_4$ (10.5 mM Fe^{+2}) concentration.

Apart from the previously mentioned dark-fermentative hydrogen production processes, other groups of researchers investigated the effect of metals on the photofermentative hydrogen production by single photosynthetic bacteria. Zhu et al. (2007) showed that hydrogen production from sodium lactate by *R. sphaeroides* increased linearly with an increase in Fe^{+2} concentration in the range of 0 to 1.6 mg/L (0.03 mM Fe^{+2}), while reaching a maxima at 2.4 mg/L Fe^{+2} (0.04 mM Fe^{+2}) [16]. This concentration (2.4 mg/L) was reported to be identical with the Fe^{+2} concentration contained in the Ormerod medium [35]. In their study, Zhu et al. (2007) did not observe any increase on the amount of hydrogen production when iron concentration was higher than 2.4 mg/L (0.04 mM Fe^{+2}) [16]. They correlated this observation with the coagulation of the $FeSO_4 \cdot 7H_2O$ at higher iron concentrations.

In a recent study by Afsar et al. (2009), the effect of iron and molybdenum addition on the H_2 production performance of *Rhodobacter capsulatus* has been investigated when the dark fermenter effluent of potato steam peel hydrolyzate was used as the substrate source [15]. They observed an improvement on the hydrogen productivities of Mo (40 μg/l $Na_2MoO_4 \cdot 2H_2O$ = 0.165 μM Mo) and Fe (5 mg/L Fe(III)-citrate = 0.02 mM Fe^{+3}) co-supplemented cultures from 0.08 to

0.5 mg L^{-1} h^{-1}. Uyar et al. (2009) also observed about 3-fold increase on the amount of hydrogen production from *R. capsulatus* (DSM 155) (from 0.3 $L_{H_2}/L_{culture}$ to 1.0 $L_{H_2}/L_{culture}$) after the supplementation of the iron (5 mg/L Fe(III)citrate = 0.02 mM Fe^{+3}) into the dark fermentation effluent of the Miscanthus hydrolyzate that was fermented by a thermophile (*Thermotoga neapolitana*) [13]. In a recent study by Ozgur et al. (2010), the hydrogen production by wild type *R. capsulatus* significantly improved after the addition of Fe^{+3} (0.1 mM) into 3 times diluted dark fermenter effluent of sugar beet molasses, which was fermented by *Caldicellulosiruptor saccharolyticus* [14]. They observed an enhancement on the H_2 productivity from 0.73 mmol H_2/L_c h to 1.10 mmol H_2/L_c h. On the other hand, the effect of Mo addition has some alterations for various organisms. For example, supplementary Mo (0.16 μM) significantly improved the hydrogen production for the *hup*$^-$ strain of *R. capsulatus* (from 0.72 mmol H_2/L_c h to 1.37 mmol H_2/L_c h) and for *Rhodopseudomonas palustris* (from 0.89 mmol H_2/L_c h to 1.16 mmol H_2/L_c h). However, addition of Mo did not enhance the H_2 production for the wild type *R. capsulatus*.

In a study done by Kars et al. (2006), *R. sphaeroides* O.U.001 was grown in the defined media with different concentrations of molybdenum and iron, in order to study the expression level of *nif*D and *nif*K genes that are known to code for the large subunit of Mo-nitrogenase and *hup*S gene coding for the small subunit of uptake hydrogenase [11]. They observed an enhancement on the amount of hydrogen production once the concentrations of the molybdenum and iron have been increased independently, and the highest total hydrogen accumulation was achieved for the culture media, individually containing 16.5 μM Mo (0.84 L H_2 per L culture) and 0.1 mM Fe^{+3} (1.14 L H_2 per L culture). The gene expression analysis showed that *nif*K expression in Mo deficient medium was significantly reduced while it was considerably expressed for the Mo supplemented media. They also showed that iron deficiency led to significant reduction in the expression levels of *nif*D and *hup*S.

3.2. COD removal efficiencies under iron and molybdenum supplementation

On the aspects of environment, chemical oxygen demand (COD) removal efficiencies of each culture condition had been investigated and observed to reach a significant improvement after the supplementation of Fe (48.1%) compared to the Mo supplemented (35.7%) cultures and the control reactors (30.2%), respectively (Fig. 4). COD removal efficiency for the control reactor is close to the previous findings with OMW samples [4,6].

Despite degradation of the COD contents for all reactors, it can be observed from Table 1 that the final solutions were not decolorized. These results underline the fact that degradation products of photofermentative hydrogen production are recalcitrant. This is mainly related with the aromatic contents, and metal-phenol chelations for the raw and metal supplemented media, respectively. For this reason, several pre-treatment techniques can be applied to the raw OMW samples prior to photofermentation process, such as clay treatment [24] that would also overcome high dilution-rate requirements.

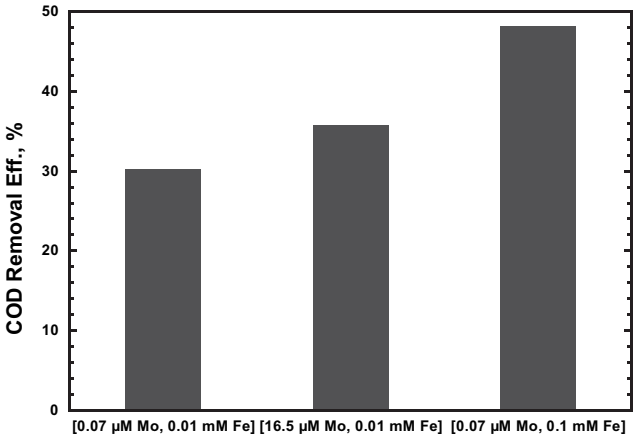

Fig. 4 – COD removal efficiencies of photofermentative H_2 production processes occurred under control (0.07 μM Mo, 0.01 mM Fe), molybdenum supplemented (16.5 μM Mo, 0.01 mM Fe) or iron supplemented (0.07 μM Mo, 0.1 mM Fe) reactors.

In wastewater treatment, iron(III) has often been used as an electron acceptor for biodegradation purposes, such as phenol degradation [27]. Several studies used iron (i.e, iron(III) chloride) as a precipitating reagent for wastewater treatment [36]. Reaching a higher COD removal efficiency as a result of Fe addition is mainly achieved by (i) increased organic matter consumption in parallel to the culture's enhanced hydrogen production activity via triggered nitrogenase-enzyme and ETS systems, and (ii) possible removal of several organic materials after forming a chelation between the iron in the media (such as iron-polyphenol chelates).

4. Conclusions

Once a waste material has been used as the substrate source, the knowledge on the effect of metal ions on the photofermentative hydrogen production becomes essential from the manufacturing point of view. Since these kinds of substrate sources are diverse in their metal concentration, their external supplementation can be of critical importance for an effective hydrogen production. In this research, the effect of molybdenum and iron addition to olive mill wastewater was analytically studied toward the outcomes on the photofermentative hydrogen production by *R. sphaeroides*.

Our results demonstrated that the metal addition to OMW can be a simple and convenient way to enhance the hydrogen production, biomass accumulation, and COD removal. The amount of hydrogen production observed for the control reactor has been enhanced by 3 times for the iron-added; and 1.5 times for the molybdenum-added cultures. Although biomass accumulation is also higher for the iron-added conditions, maximum hydrogen productivity and light conversion efficiencies are the highest for Mo enriched conditions, as it has the shortest duration time for hydrogen production.

Obtaining a prolonged photofermentative process, yielding a higher biomass and hydrogen accumulation, and higher COD removal by the iron supplementation could be connected to a number of reasons, while still in need of support by the further measurements of nitrogenase-enzyme and chelated-polyphenols. These can be listed as: (i) enhanced photosynthesis via improved nitrogenase-enzyme and ETS system; (ii) reduction on the amount of some toxic aromatic compounds by being trapped via making chelations between the iron; (iii) efficient utilization of the iron in the media toward the possible formation/precipitation of iron-polyphenol chelates during the first stage of the experiment, and (iv) the complementary dissolution of some of these iron-polyphenol chelates during the second stage that would be benefited toward the extension of the biomass and hydrogen gas accumulation.

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