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In vitro hydrogen photoproduction using *Pyrococcus furiosus* sulfhydrogenase and TiO_2

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Abstract

The [NiFe] sulfhydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus* has been tested as a biological catalyst in a light-driven in vitro hydrogen evolution system, using a solar simulator apparatus as a source of light. The enzyme is active when coupled to photo-induced titanium dioxide (TiO_2) dispersed as submicronic powder in a buffer solution and in the presence of the artificial redox mediator methylviologen (MV), carrying electrons from the TiO_2 particle to the enzyme. To optimize the system towards practical applications, a simplified version which works in the absence of MV was set up and its performance was assayed with different reductants, as a function of time and over a wide range of temperatures. The comparison of MV-mediated and not-mediated conditions in terms of rate and amount of H_2 photoproduced, highlights a lower efficiency, but an improved overall stability of the system when a direct electron transfer from the TiO_2 to the enzyme takes place. As a consequence, the reaction lifetime is productively extended counteracting the effect of the low H_2 evolution rate with regard to the total amount of H_2 photoproduced. In particular, at 60°C the reaction lifetime is prolonged to 8 h, thus resulting in significantly higher amount of H_2 evolved. The flexibility of the *P. furiosus* sulfhydrogenase/TiO_2 in vitro system with respect to the redox mediator MV and the evidence that the enzyme performance can be maintained over a wide range of temperatures (60–80°C) are promising advances in the identification of the optimal conditions for the development of an economically feasible hydrogen production process exploiting solar light as a source of energy and waste compounds as electron donors. © 1999 Elsevier Science S.A. All rights reserved.

Keywords: Hydrogen photoproduction; *P. furiosus* sulfhydrogenase; Titanium dioxide; Methylviologen

1. Introduction

The basic requirement for hydrogen to be considered as an alternative energy source of the future is the development of economically feasible production systems, since its advantages as a fuel are generally acknowledged. It is, in fact, efficient, renewable and environmentally friendly, since its combustion does not generate toxic byproducts.

Among hydrogen production technologies, those based on biological systems represent an attractive possibility. Since the early 1970s, research efforts in the field of biohydrogen have been particularly addressed to biophotolysis and photofermentations, the latter combining hydrogen production to the disposal of waste compounds used as growth substrates [1]. Both strategies exploit sunlight as an energy source for microorganisms and are promising to make the bioproduction of hydrogen competitive with conventional systems, such as chemical and electrochemical methods [1].

Unlike in vivo technologies, light-driven in vitro enzymatic systems have been less considered as potential methods for biohydrogen production. Nevertheless, since the early 1980s different hydrogenases, the enzymes catalyzing the reaction $2H^+ + 2e^- \Leftrightarrow H_2$, have been successfully employed in photoevolution processes coupled with semiconductors and sensitizers in the presence of artificial or natural electron carriers [2–5]. Likewise, the principle of the photoreactivity of finely dispersed TiO_2 powder in aqueous solution is a well studied phenomenon and many applications, mostly associated with polluted water treatment, are described in the literature [6]. TiO_2 has also been employed in photoevolution experiments coupled to redox enzymes, such as hydrogenases, able to successfully replace inorganic catalysts [7,8] in the generation of molecular hydrogen [3–5]. Interestingly, the ability of the hydrogenases tested, all of them isolated from mesophilic organisms, to directly accept electrons from the light-excited semiconductor without the requirement of any redox mediator was also reported [9,10]. These early experiments pointed out some relevant problems

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of these in vitro systems that remain to be solved in view of possible practical applications, namely the cost of the catalyst, its instability and the limited lifetime of the reaction.

With regard to the instability of the enzymatic component, the use of hydrogenases from thermophilic or hyperthermophilic organisms might represent an advantage, since these enzymes exhibit biochemical and structural features particularly attractive for applicative purposes [11,12]. In fact, in addition to thermostability, they are endowed with high catalytic efficiency, oxygen tolerance and resistance to chemical and denaturing agents.

We previously reported on the performance of two thermotolerant enzymes, the hydrogenase from the eubacterium *Acetomicrobium flavidum* and the bifunctional sulfhydrogenase from the archaeon *Pyrococcus furiosus*, in an in vitro heterogeneous system in which H_2 is photocatalytically produced by coupling the biocatalysts to the light-induced finely dispersed semiconductor TiO_2 [13,14]. This early set up also included the presence of the artificial redox mediator methylviologen (MV) and was assayed at temperatures close to the optimum for each enzyme. In the experimental conditions used, the overall performance of the *P. furiosus* sulfhydrogenase proved to be superior to that of the *A. flavidum* enzyme and the direct utilization of electrons, available from the irradiated semiconductor, could be demonstrated only in the case of the archaeal protein [14]. *P. furiosus* sulfhydrogenase was also successfully employed by others in a different in vitro system for the generation of molecular H_2 based on the oxidation of glucose and in combination with the glucose dehydrogenase from *Thermoplasma acidophilum* [15].

These experimental data brought us to the conclusion that the *P. furiosus* enzyme is of particular interest for further investigations aimed at establishing the optimal conditions for practical applications of the light-driven in vitro system coupled to TiO_2. *P. furiosus* sulfhydrogenase is a bifunctional enzyme the physiological role of which, depending on growth conditions is either proton reduction to H_2 or sulphur species reduction to H_2S [16]. Its bifunctionality is probably based on the presence of two structurally different components which are assembled as a tetramer ($\alpha\beta\gamma\delta$) in the native conformation [17]. Its physiological electron donor is NADPH [18] and this is very unusual, since only a few hydrogenases are able to utilize nicotinamide nucleotides as electron carriers.

Three critical points of the MV-mediated *P. furiosus* sulfhydrogenase/TiO_2 system, as far as the reaction kinetics is concerned, were identified [13,14]: (i) H_2 photoproduction is a high-rate but a short-term reaction; (ii) the presence of MV, carrying electrons from the irradiated TiO_2 particle to the biocatalyst, enhances the H_2 evolution rate but strongly affects the lifetime of the reaction, since it undergoes irreversible degradation; (iii) Tris, used as a buffer, proved to be an efficient electron donor for TiO_2, but its oxidation affects the stability of the system since under these conditions the pH changes in the course of the reaction. Alternative and possibly renewable compounds would be preferable as a source of electrons, particularly in view of a scaling-up.

These results prompted us to further investigate the performance of the *P. furiosus* sulfhydrogenase/TiO_2 system in the absence of MV as a first optimization step towards its practical application for biohydrogen production.

The present report deals with the setting up of a simplified system which includes only three reaction components: the inorganic semiconductor, the biological catalyst and the buffer. In particular, we describe the functional scheme of the not-mediated *P. furiosus* sulfhydrogenase/TiO_2 in vitro system and a comparative evaluation of its performance, in terms of rate and amount of H_2 produced, with respect to the mediated system in different experimental conditions.

2. Experimental

2.1. Chemicals

TiO_2 powder (50 m^2/g) was purchased from Degussa. Sodium dithionite, methyl viologen dichloride and Tris (tris(hydroxymethyl)aminomethane) were purchased from Fluka. Chromatographic media and columns were from Pharmacia. The other chemicals used were of the purest grade available commercially.

2.2. *P. furiosus* growth and sulfhydrogenase purification

P. furiosus (DSM3638) was cultured and its sulfhydrogenase was partially purified according to the procedures previously reported [19,20]. Hydrogenase activity was colorimetrically visualized after each chromatographic step by SDS-PAGE analysis [19]. Finally, the hydrogenase-containing fractions were diluted in 50 mM Tris-HCl pH 8 buffer at a protein concentration of about 3 mg/ml, as estimated by the Bio-Rad micromethod based on the Bradford assay [21]. The purity degree of the enzyme, as judged by SDS-PAGE analysis, was about 10% with a specific activity of 10.8 U/mg.

2.3. Hydrogenase activity assay

Hydrogenase activity was measured anaerobically at 80°C in 12 ml deaerated vials containing 50 mM Tris-HCl at pH 8, 1 mM methylviologen and 25 mM sodium dithionite ($Na_2S_2O_4$) as an electron donor. Reaction mixtures contained approximately 0.6 mg of sulfhydrogenase in 5 ml buffered solution. One unit is defined as the amount of enzyme which produces 1 μM of hydrogen per minute.

Hydrogen production was determined by gas chromatography on a Varian 3400 instrument equipped with a 5 Å MolSieve column ($6' \times 1/8''$) and Thermoconductivity detector. Carrier gas was Ar at a flow rate of 30 ml/min.

Usually, 0.2 ml of the gas phase present in the headspace of the reaction vial were injected at regular intervals.

2.4. Solar box experiments

Hydrogen photoproduction experiments were performed in an AM1 solar simulator box equipped with a Xenon lamp (1500 W/m^2) produced by CO.FO.ME.GRA, Italy. The photometric measurement of the irradiation inside the box (15 cm from the light source) was approximately 120 klux. Kinetic experiments with the *P. furiosus* sulfhydrogenase were performed at different temperatures in jacketed pyrex glass vials (14 ml, total volume) closed by a tight-fitting rubber stopper and under magnetic stirring to homogenize the photoinduction of the white colloidal suspension.

Typically, in not-mediated experiments, the reaction mixture (5 ml) contained: 50 mM Tris-HCl buffer solution at pH 8, 20 mg TiO_2, 0.2 ml of *P. furiosus* sulfhydrogenase (6.6 U). In mediated conditions, 1 mM MV was also added.

3. Results and discussion

The reaction scheme of the system set up for initial evaluation tests on *P. furiosus* sulfhydrogenase included MV as an artificial redox mediator and Tris as an electron donor (sacrificial) [13,14]. The electron transfer pathway among the different components in the mediated condition (A) is shown in Fig. 1.

Provided that the biological catalyst is in the redox active state, hydrogen evolution occurs, under light irradiation, as a continuous electron flow from the donor Tris to protons, with MV carrying electrons accumulated on the TiO_2 particle to the enzyme.

The electron transfer pathway in not-mediated conditions (B) is also illustrated in Fig. 1. Without the mediation of the electron carrier, the enzyme is directly reduced at the TiO_2 surface and electrons are transferred from the light-excited semiconductor to the biological catalyst. On the basis of previous results, a decrease in the rate of H_2 evolution in not-mediated conditions can be predicted as a result of the MV exclusion [13]. However, from an applicative point of view, the negative effect in terms of efficiency is balanced by a higher overall stability of the system components, which maximize the lifetime of the reaction and by the economical benefit related to the cost of MV.

3.1. H_2 evolution by *P. furiosus* sulfhydrogenase in mediator-independent conditions

The effect of the absence of the redox mediator MV on the kinetics of in vitro H_2 evolution by *P. furiosus* sulfhydrogenase was first evaluated by comparing the performance of the enzyme at 80°C using either sodium dithionite ($Na_2S_2O_4$) or photoinduced TiO_2 as a reductant in mediated and not-mediated conditions. Sodium dithionite is typically used as an electron donor in standard evolution assays of hydrogenase enzymes.

Table 1 shows the results obtained, as far as initial rates and amounts of H_2 evolved after 1 hour of reaction are concerned. As expected, in mediated conditions *P. furiosus* sulfhydrogenase is more efficient when coupled to $Na_2S_2O_4$ than when combined to the light-excited semiconductor TiO_2: both the initial rate and the total amount of hydrogen evolved are about 3.5 times higher in the former than in the latter system. In fact, the formation of reduced MV by $Na_2S_2O_4$ does not represent a rate limiting step and therefore hydrogen evolution is mainly dependent on the enzyme activity. On the other hand, using photoinduced TiO_2, the reaction kinetics is mostly dependent on the quantum efficiency of the photocatalytic process and on the fraction of enzyme productively adsorbed on the TiO_2 surface.

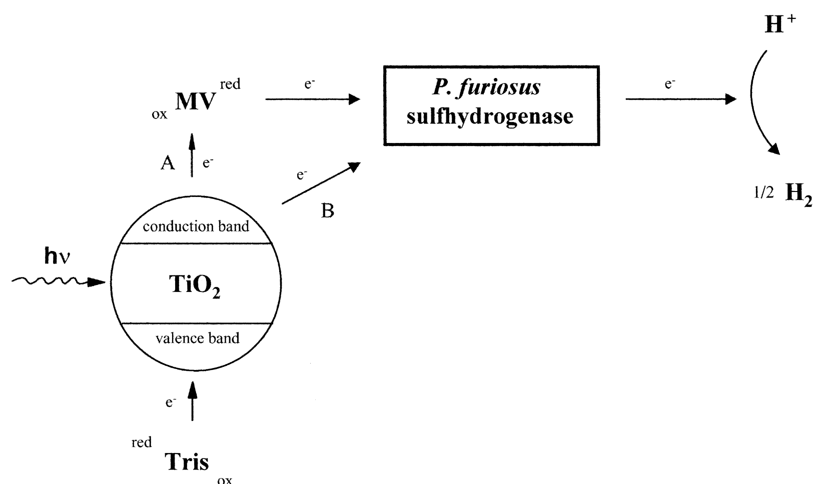

Fig. 1. In vitro H_2 photoproduction system based on *P. furiosus* sulfhydrogenase coupled to the semiconductor TiO_2: scheme of the electron transfer pathway in the presence (mediated, A) and in the absence (not-mediated, B) of the artificial redox mediator MV. Tris is the electron donor (sacrificial).

Table 1
Performance of *P. furiosus* sulfhydrogenase coupled to $Na_2S_2O_4$ or photoinduced TiO_2 in the presence or in the absence of the redox mediator MV[a]

Experimental condition	Initial rate of H_2 evolution (μmoles/min/mg)	H_2 amount after 1 h of reaction (μmoles)
$Na_2S_2O_4$/MV (mediated)	11	145
$Na_2S_2O_4$ (not-mediated)	1.7	48
hν/TiO_2/MV (mediated)	3.1	41
hν/TiO_2 (not-mediated)	1.3	29

Experiments were carried out at 80°C using 0.12 mg/ml of semipurified *P. furiosus* sulfhydrogenase. Hydrogen evolution was measured gaschromatographically. Concentrations of other reagents were: 25 mM $Na_2S_2O_4$, 4 mg/ml TiO_2, 50 mM Tris-HCl at pH 8 and 1 mM MV in the case of mediated conditions.

Without the mediation of MV, H_2 evolution was measured in both in vitro systems. This might suggest that the ability of directly accepting electrons from either $Na_2S_2O_4$ and TiO_2 is an intrinsic property of the *P. furiosus* sulfhydrogenase, probably based on the presence of the two additional subunits which are also likely to be involved in electron transfer.

As far as initial rates are concerned, the effect of the absence of the redox mediator MV is more significant using $Na_2S_2O_4$ as a reductant, being the value about 6.5 times lower than in mediated condition, whereas only a 2.4 fold decrease is measured using photoinduced TiO_2.

With regard to the amounts of gas obtained after 1 h of reaction, again the performance of the light-driven TiO_2 system is less dependent on the presence of MV, since the micromoles of hydrogen evolved in not-mediated condition are only 1.4 times less than in mediated condition, while a 3-fold decrease is measured using $Na_2S_2O_4$ as a reductant.

Therefore, the experimental data point out that the in vitro system combining *P. furiosus* sulfhydrogenase to photoinduced TiO_2 is quite flexible with respect to the presence or absence of the artificial redox mediator MV, allowing its exclusion from the reaction without a dramatic loss in terms of rate and amount of hydrogen production.

It is worth noting that with both reductants, the total amount of hydrogen evolved is less affected than the initial rate by the absence of MV. This is particularly true in the case of TiO_2-dependent heterogeneous photocatalysis, suggesting a different time-course of the reaction in mediated and not-mediated conditions.

3.2. *Time-course of H_2 photoproduction in mediated and not-mediated conditions*

The performance of the light-driven *P. furiosus* sulfhydrogenase/TiO_2 system in mediated and not-mediated conditions was then evaluated as a function of the reaction time. The time-course patterns of the rate and of the amount of hydrogen photoproduced at 80°C in mediated and not-mediated conditions are shown in Fig. 2(A) and (B), respectively. In the presence of MV, the rate of hydrogen evolution is high during the first 30 min and, as already pointed out, the initial value is about 2.4 times higher than in not-mediated condition (Fig. 2(A)). Afterwards, it rapidly decreases and within 1 h of reaction time it is close to zero. Values of hydrogen evolution rate remain low during the two additional hours of reaction, indicating that, under these experi-

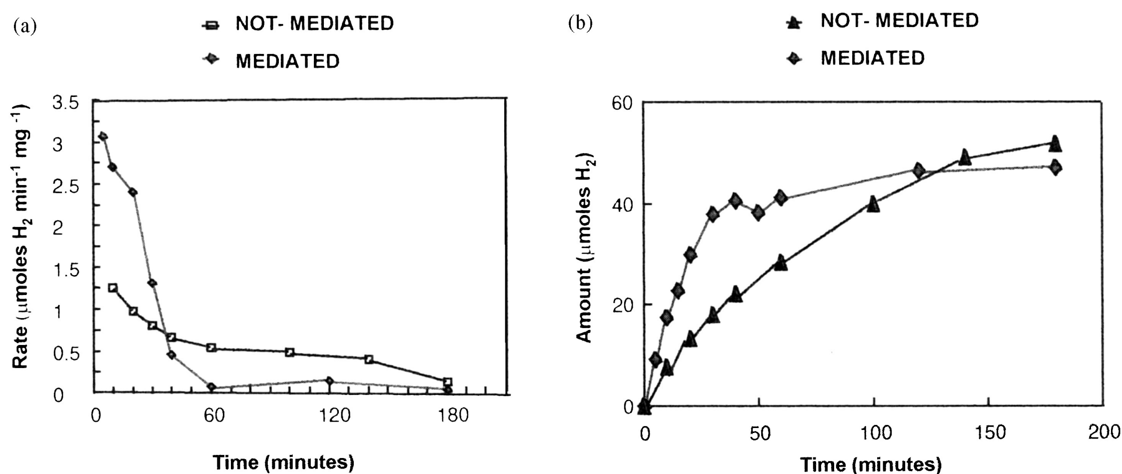

Fig. 2. Time-course of the rate (A) and the amount (B) of hydrogen photoproduced by *P. furiosus* sulfhydrogenase coupled to TiO_2 at 80°C in mediated and in not-mediated conditions.

mental conditions, the *P. furiosus* sulfhydrogenase/TiO_2 system is no longer active. After 3 h of reaction, the total amount of hydrogen evolved in mediated condition is about 47 mM, mostly as a result of the first hour of photoproduction (Fig. 2(B)). After that time, in fact, the hydrogen evolution reaches the plateau.

On the other hand, in the absence of MV the initial rate is rather low, but more constant and values close to zero are measured only after 3 h of reaction. As a consequence, the hydrogen evolution is productively extended and the plateau is reached later than in the presence of MV, thus giving rise to a similar amount of gas (about 52 mM).

In conclusion, the evaluation of the time-dependent performance of the light-driven *P. furiosus* sulfhydrogenase/ TiO_2 system in mediated and not-mediated conditions indicates that when a direct electron transfer takes place the system is less efficient as a lower H_2 evolution rate is obtained, but since the system gains in stability, the reaction lifetime is prolonged and the total amount of gas produced is similar to that obtained in MV-mediated condition. Therefore, for its overall performance and for its improved economics, the not-mediated *P. furiosus* sulfhydrogenase/TiO_2 system can successfully replace the early set up in view of practical applications in H_2 photoproduction.

3.3. Temperature-dependent H_2 photoproduction in mediated and not-mediated conditions

Another fundamental aspect to be considered for applicative purposes is the performance of the in vitro system as a function of temperature. In fact, besides economical benefits, temperatures lower than 80°C could be helpful to improve the thermochemical stability of the reaction components and therefore might have a further positive effect on the lifetime of the reaction. The flexibility of the system as a function of temperature will depend on the thermophilicity of the biological catalyst. This determines in fact, the range of temperatures within which the *P. furiosus* sulfhydrogenase/TiO_2 couple is functionally active in H_2 photoproduction.

The performance of mediated and not-mediated systems at different temperatures was evaluated in terms of total amount of H_2 evolved and duration of the reaction. In Fig. 3, the results obtained are reported.

As a general effect of lowering the reaction temperature below 80°C, a significative extension of the H_2 photoevolution lifetime is obtained both in mediated and not-mediated condition. On the other hand, at 90°C the performance of the in vitro system is clearly compromised, especially in terms of gas production, independently from the presence or the absence of the redox mediator MV.

At temperatures lower than 80°C in mediated condition, the prolonged lifetime of the reaction does not correspond to a remarkable increase in H_2 evolution due to the irreversible degradation of the redox mediator MV from the first hour reaction on. On the other hand, with the not-mediated system run in the same temperature conditions, the extension of the reaction lifetime is coupled to a significantly higher amount of gas evolved down to 50°C, which therefore represents the lower temperature limit for the thermophilic sulfhydrogenase to be active. The temperature range included between 60° and 70°C thus represents the best compromise between the thermophilicity of the enzyme and the thermochemical stability of the reaction components.

Particularly promising are the results obtained in not-mediated condition at 60°C, the temperature which gives rise to an extension of the reaction lifetime from 5 to 8 h and to the highest amount of H_2 evolved (Fig. 3(A)). Although 60°C does not represent the optimum temperature as far as the initial rate is concerned, being about 2.6 times lower than at 80°C, the values remain stable over a longer period of time, leading to a 1.8 fold increase of H_2 photoproduced with respect to the same condition at 80°C and to a 1.6 fold

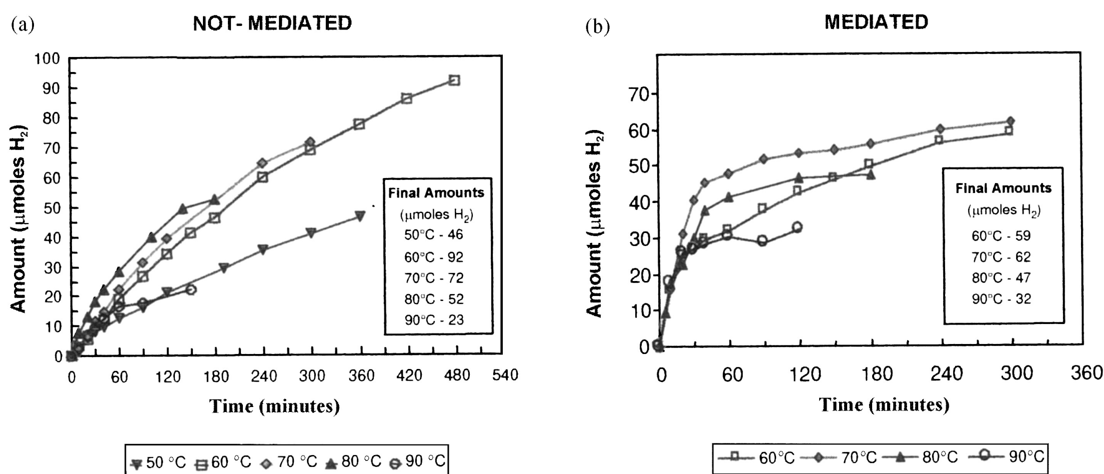

Fig. 3. Time-course of the amount of hydrogen photoproduced by *P. furiosus* sulfhydrogenase coupled to TiO_2 at different temperatures in not-mediated (A) and in mediated conditions (B). Reaction lifetimes correspond to the period of active H_2 photoproduction.

increase when compared to the mediated system run at the same temperature.

In conclusion, the not-mediated *P. furiosus* sulfhydrogenase/TiO_2 system represents a first optimization step towards the development of an economically feasible in vitro hydrogen production process which should be driven by solar light and should utilize waste compounds as source of electrons. The present version, in fact, shows the following advantages compared to the mediator-dependent approach: (i) a simpler and more inexpensive experimental set up, including only three components (the inorganic semiconductor, the biological catalyst and the buffer) instead of four; (ii) an improved overall stability, which allows the lifetime of H_2 evolution to be productively extended and (iii) a better performance at lower temperatures, since the extension of the reaction lifetime is coupled to a significantly higher amount of H_2 photoproduced.

It is also worth mentioning some advantages related to the use of the *P. furiosus* sulfhydrogenase as a biological catalyst. The evidence that a minimal degree of purification is required for the enzyme to be employed in the in vitro system represents another advantage from an economical point of view, since its purification procedure is a particularly complex and expensive multi-step operation. In addition, its stability as a biological catalyst and its versatility in terms of reaction temperatures (60–80°C) represents a further evidence of the biotechnological potential of this enzyme.

Future investigations will be addressed to optimize another critical point of the system which concerns the use of Tris and its dual role as a buffer and as an electron donor for TiO_2. Alternative and renewable electron donors will be tested in mediator-independent condition. The screening of sacrificials different from Tris will be also performed with the aim of improving the oxidative transfer rate at TiO_2 surface. The photo-oxidation process, in fact, represents a fundamental issue as far as the efficiency of the system is concerned. As already pointed out, the use of waste products as source of electrons will be considered mainly because their disposal, coupled to H_2 photoproduction, would allow lower costs of process realization and management, besides making the system environmentally attractive.

The replacement of Tris with waste products will cause the absence of a buffering system to become the critical factor and the possibility of developing an on-line pH control system will be considered.

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