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Towards the integration of dark and photo fermentative waste treatment. 1. Hydrogen photoproduction by purple bacterium *Rhodobacter capsulatus* using potential products of starch fermentation

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ABSTRACT

In this study, the H_2-photoproduction capacity of *Rhodobacter capsulatus* B10 was measured as a function of variations in the nature and concentration of volatile fatty acids (VFAs) and other products of dark fermentation. When an equimolar mixture of VFAs was provided, C4 substrates (butyrate and isobutyrate) were not consumed until the C2–C3 substrates (acetate, propionate, and lactate) became unavailable, but in order for the cells to produce H_2 at high rates they could not be exposed to severe growth substrate depletion. Among other possible fermentation products, the highest inhibition was observed by the addition of butanol (50% inhibition at 50 mM). The influence of high concentrations of VFAs, phosphate (used to stabilize the pH during dark fermentation) and some heavy metals (known inhibitors of methanogenesis) was also shown. Based on the results, the conditions of fermentation can be manipulated to avoid the inhibition of subsequent H_2 photoproduction by photosynthetic bacteria.

1. Introduction

Human society will soon be restricted by energy availability and by pollution that results from extensive fossil fuel use. Molecular hydrogen is a valuable alternative to fossil fuels since it can be generated from water, and water is the end product of its combustion. Several methods for H_2 production using alternative technologies including photobiological ones are under development. Purple photosynthetic bacteria can photoproduce H_2 using simple organic acids as electron donors in a nitrogenase-driven reaction, and they exhibit the highest production rates among photosynthetic microorganisms [1,2]. The immediate application of phototrophic bacteria for H_2 generation is not yet possible due to several scientific and technological challenges. One key issue is the lack of a cheap source of organic electron donors. The use of pure organic acids is not viable due to cost limitations, and the direct use of organic wastes in many cases may not be appropriate, because they usually contain a mixture of organic compounds that are not metabolized by purple

Abbreviations: Bchl, bacteriochlorophyll; VFA, volatile fatty acid.

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bacteria. One of the ways to address this problem is to integrate the process of waste treatment, which is dependent on anaerobic fermentation, with H_2 production by purple bacteria using the fermentative effluent as a substrate [3]. Dark fermentation produces H_2 and organic acids with traces of alcohols. Organic acids are easily assimilated by purple bacteria for growth and H_2 production, and individual organic acids (e.g. lactate, malate, and succinate) are common substrates used in the laboratory cultivation of purple bacteria. Moreover, the assimilation of acetate and butyrate by purple bacteria is well-documented [4–6]. Growth of phototrophic bacteria in a photobioreactor using direct feeding of the effluent from a dark, sugar-fermentation reactor has already been demonstrated [7,8]. Furthermore, continuous H_2 production in a photobioreactor, using either a mixture of acetate, propionate, and butyrate, or the effluent from an H_2-producing acidogenic reactor has been reported as well [9]. As long as the concentrations of volatile fatty acids (VFAs) in the effluent were not too high (8, 2, and 17 mM, respectively for the above-mentioned VFAs), wastes could be utilized without dilution after addition of 0.5 g/l glutamate as a nitrogen source. However, before the integration of these two H_2-producing reactors can be implemented routinely, additional questions must be answered. The direct pumping of the fermentation effluent into a photobioreactor requires that the components of the medium and the products of the fermentation from the first reactor do not affect the viability of the purple bacteria or the photoproduction of H_2 in the second reactor. Prior knowledge of potentially inhibiting factors present in fermentative effluents may allow for some modification of the fermentative process for optimization of H_2 photoproduction by the purple bacteria. Furthermore, this information would be helpful in interpreting results when real wastes are used. Finally, the strategy of using organisms that simultaneously or sequentially consume various organic acids out of a specific mixture of VFAs is of interest for the implementation of an optimal system as well.

The goal of this study was to determine the effect of potential fermentation products and medium components on the growth and H_2-photoproduction rates of the purple photosynthetic bacterium, *Rhodobacter capsulatus*, and to reveal the patterns of organic acid (as potential fermentation products) consumption from an equimolar mixture of VFAs.

2. Materials and methods

The strains of purple phototrophic bacteria used in this study were provided by the Department of Microbiology at Moscow State University (excepted as otherwise stated). Strains were pre-grown on Ormerod medium [10] with 1 mM $(NH_4)_2SO_4$ for experiments (nitrogenase-de-repressed conditions; 10 mM $(NH_4)_2SO_4$ commonly used for experiments will not allow the activation of nitrogenase during the time course of the following experiments). The medium normally contained lactate (16 or 20 mM as indicated in the text), but other substrates were also used as indicated in the text and Tables. Capped vials (15, 40 or 250 ml), filled to ~2/3 volume, were incubated at 30 °C at a light intensity of 15–20 W m^{-2} (incandescent lamps) for 3–7 days. When necessary, the H_2 content in the gas phase was assayed with a gas chromatograph (LkhM80, Moscow, Russia), equipped with a thermoconductivity detector and a 1 m × 3 mm with molecular sieve column (running temperature, 40 °C). Argon was used as gas carrier.

The observation of short-term H_2 production by *Rb. capsulatus* was performed in 12-ml vials, previously filled with argon. Active H_2-producing cultures were transferred anaerobically to the vials, repeatedly flushed with Ar, and incubated at 30 °C under saturating light (80 W m^{-2}) in a shaker bath. Substrates were added at the concentrations indicated in the table and figure legends. The duration of the experiments varied from 30 min at low substrate concentration (this included a 10-min, gas-sampling period) to 7 h (including a 1–2 h gas-sampling period), depending on the purpose of the experiment.

The acetylene-reduction test for nitrogenase activity was performed by gas chromatography in the same vials used for the short-term H_2-production experiments, except that 10% acetylene was introduced into the gas phase at the start of the analysis, and ethylene formation was determined as described previously [11].

Experiments with mixtures of VFAs (lactate, acetate, propionate, butyrate, and isobutyrate) were performed in 250-ml capped vials with 150 ml of culture. Samples were withdrawn twice a day from one vial for H_2 assays and from a second replicate vial for Bchl and VFA assays. The samples for VFA assays were centrifuged at 12,000 rpm for 15 min, acidified (to a pH below 3) by adding phosphoric acid, and assayed without additional pretreatment.

The concentrations of acetate, propionate, butyrate and isobutyrate were determined by gas chromatography (TCVET800, Dzerzhinsk, Russia). The gas chromatograph was equipped with a flame ionization detector and a 1 m × 2 mm glass column packed with Chromosorb W/AW-DMCS + 5% neopenthylglycolsuccinate in 100–200 mesh (Fluka). The injection port temperature was 145 °C, and the detector temperature was 185 °C. The column program temperature regime was as follows: 80 °C for 3 min; followed by a temperature increase to 175 °C at 6 °C/min; and finally 175 °C was maintained for 8 min. Carbon dioxide was used as the carrier gas at a flow rate 30 ml/min.

Lactate concentrations were determined by an enzymatic method and monitored as NAD reduction at 340 nm [12]. Bchl concentrations were measured spectrophotometrically at 772 nm following extraction in a 7:2 (v/v) acetone:methanol mixture [13]. Protein concentration was estimated according to the classical Lowry method after ultrasonic disruption of the cells.

3. Results and discussion

3.1. *Selection of a purple bacterium for H_2 production*

The optimal phototrophic bacterium for our proposed integrated system must possess high nitrogenase activity and be able to utilize a wide spectrum of organic acids. Purple non-sulfur photosynthetic bacteria belonging to the *Rhodobacter*, *Rhodopseudomonas*, and *Rhodocyclus* genera are known to meet

these requirements [14]. We tested six strains of purple bacteria for nitrogenase activity using two different assays: acetylene reduction and H_2 photoproduction (Table 1). No significant differences in nitrogenase activity were found among the tested strains. Since the metabolism of *Rb. capsulatus* B10 has been studied in more detail than other strains and the genome sequence is nearly finished, we selected it for further studies.

3.2. *Utilization of VFAs for growth and H_2 production by* Rb. capsulatus

We tested the effects of six VFAs at 20–80 mM concentrations on growth and H_2 production of *Rb. capsulatus* under nitrogenase-de-repressed conditions (medium containing 1 mM $(NH_4)_2SO_4$). Our data confirmed the known ability of this bacterium to utilize lactate, acetate, propionate, butyrate and, to a lesser extent, isobutyrate, for growth and concomitant H_2 production. Formate supported neither growth nor H_2 production. Growth was retarded, and H_2 production was inhibited at 80 mM butyrate or 40–60 mM isobutyrate. An equimolar mixture of the six VFAs did not affect growth nor H_2 production at 30 mM or even up to 60 mM total concentration, while inhibition occurred at 80 mM (data not shown). Growth inhibition by butyrate in batch cultures of *Rhodobacter sphaeroides* SCJ was previously reported to occur at >25 mM [6]. However, others reported high rates of H_2 photoproduction by phototrophic sludge predominantly containing *Rb. capsulatus* [15] or by a new strain ZX-5 [16] in the presence of as high as 50–60 mM butyrate. These differences might be explained by variability inherent in different bacterial strains or by the criterion used for estimating growth. For example, at high butyrate concentrations, a lag phase is observed, but the exponential growth rate and the final biomass seem to be the same as that seen with the lower butyrate concentration controls [6]. So, while in the presence of high butyrate concentrations, H_2 yields would be considered inhibited when compared at the earlier growth phase, it seemed unaffected if the comparison was made later in the growth phase.

The potential H_2-production rates by cell suspensions in the presence of different VFAs were determined by two types of experiments (Table 2). In both cases cells were sampled from the cultures and then studied for H_2 photoproduction in short-term experiments as described in Section 2. In the first type, cells were also pre-grown in the presence of a particular substrate (VFA) until they started to produce H_2, and were then tested for H_2 production immediately upon addition of the same substrate. The rate of the endogenous H_2 production was high throughout the experiment, and it was not stimulated by the further addition of 20 mM substrate. In the second case, cells pre-grown in the presence of lactate, were then tested for H_2 production upon addition of various other substrates. In the latter case, it was important to make the additions to cultures when they were almost depleted of lactate (at this point the rate of endogenous H_2 production was negligible; see Table 2).

Table 2 shows that four substrates out of five supported approximately equal H_2 production rates, if the cells were pre-grown in the presence of the same substrate that was added after growth. However, if the cells were pre-grown with lactate, the rates of H_2 production decreased ~50% upon the addition of acetate or propionate, ~75% with butyrate, and ~92% with isobutyrate. We concluded that more efficient utilization of butyrate and isobutyrate for H_2 production is dependent on the presence of the substrate during the pre-growth phase.

VFA concentrations in real wastes vary over a wide range. Very often acetate dominates in the mixture. For this reason, we tested H_2 production at both low and high acetate concentrations. Analysis of H_2 production in cell suspensions at low exogenous substrate concentrations is difficult due to the availability of endogenous (growth) substrates resulting in endogenous H_2 production. Two ways to exclude the growth substrate are (a) separating the cells from the medium or (b) determining the time when the cells have consumed the substrate naturally. Due to high sensitivity of nitrogenase to O_2, the second approach is preferable under the anaerobic conditions required to produce H_2. Consequently, we pre-grew *Rb. capsulatus* in medium with 1 mM of $(NH_4)_2SO_4$ and lowered (16 mM) acetate concentration. During cultivation, the cells continued to grew until the time that all the ammonium was consumed. After that point, nitrogenase was expressed and activated, and H_2 production commenced. In our experiments

Table 1 – Nitrogenase activity of various strains of purple bacteria.

Strain	Acetylene reduction (nmol min^{-1} mg^{-1} protein)	H_2 evolution (nmol min^{-1} mg^{-1} protein)
Rc. gelatinosus	42 ± 8	31 ± 6
Rps. palustris 1682	34 ± 7	39 ± 8
Rsp. fulvum K5	30 ± 6	42 ± 9
Rb. sphaeroides GL1[a]	23 ± 5	34 ± 7
Rb. capsulatus B10	32 ± 6	43 ± 9
Rhodopseudomonas sp. GI2[a]	47 ± 10	61 ± 1

a Strains that were isolated by the Institute of Basic Biological Problems, Pushchino, Russia.

Table 2 – Relative rates (%) of short-term H_2 production in the presence of different VFAs.

Substrate in the reaction mixture added at 20 mM	Cells pre-grown with the same substrate used in the H_2-production test experiments	Cells pre-grown with 20 mM lactate[a]
Lactate	100[b]	100[b]
Acetate	88 ± 18	48 ± 13
Propionate	103 ± 15	52 ± 14
Butyrate	103 ± 15	25 ± 15
Isobutyrate	39 ± 9	3 ± 7

a When lactate was depleted, the rate of endogenous H_2 production did not exceed 1.2 μl h^{-1} ml^{-1}. The data in the table have been corrected by subtracting the respective endogenous rates measured before the VFA was added.
b 100% Activity corresponds to ~35 μl h^{-1} ml^{-1}.

H_2 production was not observed after 40 h of pre-growth (evidently due to the continued presence of ammonium and lack of nitrogenase expression [data not shown]). However, after 64 h of cultivation, the cultures had accumulated H_2 in the gas phase indicating that ammonium was depleted and nitrogenase was expressed, and some acetate was still present during the intervening period. However, when the cells were assayed at this point (64 h), H_2-production activity (see Section 2, short-term experiments) was low or absent. On the other hand, H_2 photoproduction started immediately after acetate was added to the cells.

From this observation we concluded that after 64 h of cultivation, the cultures consumed the ammonium first and then the acetate. By 88 h of cultivation (no further additions of acetate were made at 64 h), the cultures contained H_2 in the gas phase; however, when the cells were assayed, they did not produce H_2 endogenously, and the addition of the acetate did not induce H_2 production. From this observation and from the difference between cells sampled after 64 and 72 h of cultivation (see below), we concluded that acetate depletion occurs at around 64 h, but prolonged acetate starvation (24 h after the point 64 h) destroys nitrogenase activity in the cultures.

To understand the difference between cells sampled immediately after acetate depletion and after prolonged acetate deprivation, we studied the influence of different acetate concentrations on the H_2-production rates of cells sampled after 64 h and 72 h of cultivation (Fig. 1). At the beginning of acetate depletion in the growth medium (64 h), endogenous H_2 production was low, but acetate-dependent H_2 production was still high. Fig. 1A (curve 1) shows that in this case the rate of H_2 production saturated at ~0.5–1.0 mM added acetate (the K_M was below 0.2 mM, although its precise determination was not possible using this method). The ratio of H_2-produced to acetate-consumed (mol/mol) was approx. 2.4 (Fig. 1B, curve 1).

However, after prolonged starvation (72 h), endogenous H_2 production disappeared, added-substrate-dependent H_2 production decreased, and a lag period appeared after the addition of the substrate but before the start of H_2 production (data not shown). In this case (Fig. 1A, curve 2), the K_M was ~0.5 mM, and the ratio of H_2-produced to acetate-consumed was approx. 1.0 (Fig. 1B, curve 2). In both cases the ratio of H_2/acetate was below the theoretical value of 4.0.

The change in K_M for glucose utilization as the result of physiological adaptation to the environment has been reported previously for *Escherichia coli* [17]. In that study, the increase in K_M was related to excess substrate conditions as compared to substrate-controlled, chemostat conditions. In our experiments, the increase in K_M occurred along with the simultaneous decrease in the rate of H_2 production and the efficiency of substrate utilization. This change in K_M probably reflects the redirection of substrate utilization, where the decreased ratio of acetate-consumed for H_2-produced suggests higher acetate utilization to support other processes such as cellular repair or maintenance.

It should be noted that the physiological state of the cells changed rapidly during the experiment shown in Fig. 1; hence, we focused our observations on the trends associated with these changes and not on the precise characteristics of each metabolic phase. From these results, we also concluded that in order to maintain high H_2-production activity, the cells must not be exposed to severe growth substrate depletion.

Acetate concentrations of 6–11 mM in growing batch cultures of *Rhodopseudomonas* sp. were reported to limit H_2 production [4]. Evidently, at low ammonium concentration, the fraction of acetate used for biomass production is proportionally lower, but that for H_2 production is higher compared to results with higher ammonium concentrations. In the absence of growth (due to the lack of nitrogen as with our experiments in Fig. 1), added acetate is used mostly for H_2 production, partly for the maintenance, but not for biomass accumulation. This is probably the reason why our data show much lower saturating concentrations of acetate for H_2 production than other authors [4]. Our short-term experiments with non-growing suspensions will be useful for determining whether the residual acetate concentration during continuous cultivation is saturating or not for H_2 production.

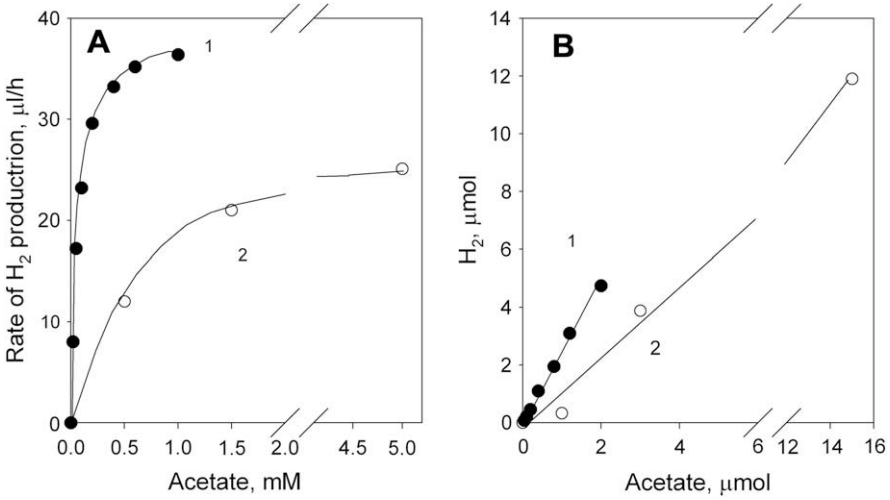

Fig. 1 – Rates (A) and stoichiometries (B) of acetate-dependent H_2 production in the early (1; 64 h) and the late (2; 72 h) phases of acetate depletion. The cultures were pre-grown in 1 mM of $(NH_4)_2SO_4$ (nitrogenase-de-repressed conditions) and 16 mM acetate, then assayed for H_2 photoproduction in short-term experiments as described in Section 2.

The effect of adding high concentrations of VFAs on H_2-production rates is shown in Table 3. For these experiments, the cultures were grown with 1 mM of $(NH_4)_2SO_4$ and 20 mM of lactate (or propionate where indicated in the Table 3). The cells were sampled (i.e. cells were withdrawn from the cultures) and assayed at the time when ammonium was depleted but the substrates were not (usually, not later than 10 h after the start of visible H_2 production by cultures).

When VFAs were added to sampled cells at a concentration of 40 mM, there were no visible changes in activity (data not shown). However, the addition of VFAs at 80 mM resulted in a 20–30% decrease in activity, including the experiment where four substrates were added simultaneously at 20 mM each. A 70% inhibition of H_2 production was observed with lactate-grown cells tested in the presence of 80 mM propionate. In contrast, propionate-grown cells showed only 30% inhibition upon further addition of 80 mM propionate, and 20% inhibition when 80 mM lactate was added. So, there is a much greater lactate to propionate affect than propionate to lactate.

3.3. *H_2 production and utilization of VFAs from an artificial mixed medium*

It should be noted that the rate of H_2 production by a cell suspension in the presence of a mixture of 4 VFAs (at 20 mM each) was inhibited 16% as compared with the experiment without any additions. This was about the same when compared with the addition of the individual VFAs at 80 mM (Table 3). At this point it was not clear what the microbial strategy was for substrate utilization out of this mixture for H_2 production: were various substrates utilized simultaneously or sequentially? Fig. 2 demonstrates the growth characteristics of *Rb. capsulatus* using Ormerod medium with 1 mM $(NH_4)_2SO_4$ and a mixture of acetate, lactate, propionate, butyrate, and isobutyrate (5 mM each). It is clear that the H_2-production kinetics followed the growth kinetics and that all VFAs (with exception of propionate, which was utilized before H_2 production started) were utilized for both growth and H_2 production. From Fig. 2B, it is evident that VFAs containing 2–3 C-atoms (propionate, acetate and lactate) were utilized first, while the alternate group of 4-C acids (butyrate and isobutyrate) were used later. When the acids in the first group were mostly depleted, the utilization of the second group started. Butyrate was consumed first and then isobutyrate. These results are consistent with Table 2, which indicates that cells pre-grown in the presence of lactate had a rather high potential for utilization of acetate and propionate but decreased capability for the consumption of butyrate (and especially isobutyrate). This could be interpreted in terms of the readiness of the cells to utilize a particular substrate, which would be dependent on the availability of that substrate during the pre-growth phase. We note that the inoculum for this experiment (Fig. 2) was pre-grown in presence of the same VFA mixture and, at the moment of inoculation, all organic acids had been consumed, as demonstrated by direct analysis of the medium (data not shown). This means that the experimental cells should be (and were) competent to use all VFAs, including butyrate and isobutyrate. The substrates were utilized in the order propionate, acetate, lactate, butyrate, isobutyrate, when we used inoculum from cell that were pre-grown one or more times with the VFA mixture (data not shown). So, independent of the culture's pre-exposure to all VFAs, C4 organic acids were metabolized

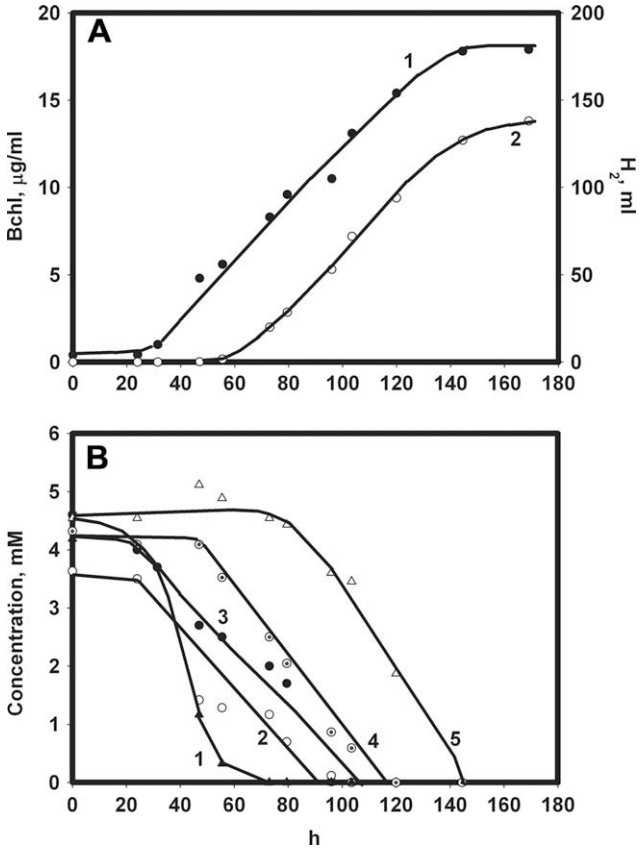

Fig. 2 – Growth (Bchl, A, 1), H_2 production (A, 2) and utilization of VFA out of an equimolar diluted mixture (B): propionate (1, closed triangles), acetate (2, open circles), lactate (3, closed circles), butyrate (4, open dotted circles), isobutyrate (5, open triangles). The cultures were pre-grown in 1 mM of $(NH_4)_2SO_4$ (nitrogenase-de-repressed conditions) and a mixture of acetate, lactate, propionate, butyrate, and isobutyrate (5 mM each).

Table 3 – The effect of high VFA concentrations (80 mM) on short-term H_2-production rates[a].

Substrate	Activity	Substrate	Activity
None added	100 ± 19[a]	Butyrate	69 ± 7
Lactate	87 ± 29	Isobutyrate	70 ± 5
	80 ± 5[b]		
Acetate	67 ± 11	Formate	82 ± 17
Propionate	30 ± 3	Lactate, acetate,	84 ± 16
	68 ± 3[b]	propionate, butyrate[c]	

a The cultures were pre-grown in 1 mM of $(NH_4)_2SO_4$ (nitrogenase-de-repressed conditions) and 20 mM lactate (unless stated otherwise). 100% activity corresponds to ~33 μl h^{-1} ml^{-1}.
b The cells were pre-grown in 1 mM of $(NH_4)_2SO_4$ and 20 mM propionate.
c The substrates were added simultaneously at 20 mM each.

only after the depletion of C2–3 organic acids from the medium.

The exact nature of the consumption of any individual substrate out of a mixture depends on the bacterial strain, the concentration of that particular VFA, and its ratio with other substrates in the mix. For example, during continuous cultivation of *Rb. capsulatus* (strain not indicated) with a mixture of acetate + propionate + butyrate (30, 2.7 and 11 mM, respectively), all substrates were consumed partially [9], suggesting simultaneous consumption of C2–C4 acids.

The sequential utilization of C2, C3 and C4 VFAs demonstrated above is of interest from both theoretical and practical points of view. The occurrence of this phenomenon among purple bacteria, its significance, and its biochemical mechanisms need to be studied. The ability to simultaneously utilize various substrates is considered to be very important at low substrate concentrations, provides higher growth rates, and results in a competitive advantage for a particular organism [17]. Nevertheless, the data obtained in this study suggest some application strategies. First, our bacterial strain would be highly successful in the selective utilization of propionate out of propionate-rich wastes. Furthermore, immobilized *Rb. capsulatus* could be successfully utilized in a photobioreactor under continuous flow of mixed-substrates medium (wastes). In this case, the consumption of different acids would be non-proportional to their concentrations, and the residual proportional concentration of C4 acids would be higher after treatment. This would give rise to substrate-specific zones, with C2–C3 VFA utilization near the input of the reactor and C4 VFA utilization near the output. A second photosynthetic bacterium could then be utilized to selectively metabolize the C4 VFAs in this output zone.

The data presented in Fig. 2 allow us to estimate the efficiency of VFA-mixture utilization for H_2 production. The stoichiometry of VFA utilization is known and is as follows:

$$C_2H_4O_2 + 2H_2O \rightarrow 4H_2 + 2CO_2$$

$$C_3H_6O_2 + 4H_2O \rightarrow 7H_2 + 3CO_2$$

$$C_3H_6O_3 + 3H_2O \rightarrow 6H_2 + 3CO_2$$

$$C_4H_8O_2 + 6H_2O \rightarrow 10H_2 + 4CO_2.$$

The total theoretical H_2 production yield in this experiment should be 622 ml but the actual volume of H_2 produced was only 176 ml (i.e. the substrate conversion efficiency was only 28%). The lower than expected value is evidently due to partial utilization of the VFAs for biomass production. Furthermore, one must take into account that non-saturating light intensity was used during the experiment.

3.4. *Effect of additional factors on growth and H_2 production on waste effluents*

Up to now we have been reporting results with VFAs added as pure chemicals; however, for energy applications we would like to use waste effluents. Assuming that a waste fermentation effluent contains other products such as alcohols, pH-regulators, and inhibitors of methanogenesis, we tested the effect of these chemicals on growth and H_2 production by *Rb. capsulatus*. We found that ethanol and methanol inhibited growth by 50% at ~0.65 M, and displayed 50% inhibition of short-term H_2 production at ~1.3–1.8 M. In contrast, butanol inhibited both growth and H_2 production by 50% at only ~0.05 M (Table 4). Acetone, up to 50 mM, affected neither growth nor short-term H_2 production (data not shown).

Ethanol does not support growth of *Rb. capsulatus* [18]. While it cannot derepress nitrogenase function, it can serve as an electron donor for derepressed nitrogenase [19]. In our experiments, ethanol significantly inhibited H_2 production by derepressed cells only at concentrations above 1.3 M.

We also tested the influence of CO_2 (one of the production of fermentation) on H_2 production. Increases in CO_2 content in the gas phase up to 20% (v/v) did not inhibit H_2 evolution (not shown).

An evaluation of the effect of high phosphate concentrations also seemed to be important, since phosphate buffer is often used to maintain a desirable pH (>4.9) during fermentation processes, particularly when large amounts of waste result in production of high organic acid content. A 50% inhibition of growth (as judged from Bchl content) of *Rb. capsulatus* was observed on the 3rd day of culture, in the presence of 120 mM phosphate, while growth retardation (after 1 day) was observed at 75 mM phosphate. In the short-term experiments, H_2 production was inhibited by 50% at 150 mM phosphate (Table 4). As reported earlier, *Rb. sphaeroides* is the most phosphate-tolerant strain among the purple bacteria tested, and in contrast to other species, concentrations of phosphate as high as 300 mM did not prevent the growth of *Rb. sphaeroides* [20]. It should be noted that phosphate addition causes an increase in the total salinity of the medium. For example, an increase in phosphate concentration from 11 (Ormerod medium) to 120 mM results in an increase in total salinity (measured as conductivity) from 4 to 13 mS (data not shown). According to our unpublished data, salinity level (provided by addition of KCl) that was most critical for growth was 23 mS. Consequently, the total salinity of wastes should be taken into account before feeding them to a photosynthetic bacterial treatment system.

Special attention should be paid to the problem of the possible shift in the fermentation process towards methane

Table 4 – The effect of added chemicals on the growth and short-term H_2-production rates[a].

Added chemical	Inhibition, %	Concentration of the added chemical, mM	
		Growth[b]	H_2 production[b]
Ethanol	50	650	1300
Methanol	50	940	1800
Butanol	50	50	50
Phosphates	50	120	150
Cu	100	0.16	0.16
Zn	0	0.31	0.31

a Cultures were pre-grown as described in Table 3.
b Concentration of the added chemical to give the percentage inhibition reported.

production, which would result in a significant decrease in H_2 production. This is especially important for long-term processes with an inadequate pH control. Different inhibitors of methanogenesis are known, namely bromosulfonates, anthraquinone dye, propionic acids, antibiotics, mevinoline, heavy metals and others [21–23]. Among them, metal ions are the simplest and cheapest employ. It is known that a number of methanogenic bacteria are very sensitive to ~0.1 mM copper and zinc at pH 6.5 [21]. In the current study, *Rb. capsulatus* showed extreme sensitivity to Cu^{2+} but a rather high tolerance to Zn^{2+} (up to 0.31 mM during both the growth and short-term H_2 production) as seen in Table 4. We conclude that the addition of zinc to prevent the shift of fermentation towards the methane production is not detrimental for phototrophic bacteria. Furthermore, it can be used in an integrated system where waste-water is initially fermented by a microbial community yielding H_2 and organic acids in the dark, which subsequently are utilized by photosynthetic bacteria to yield more H_2 and CO_2.

To summarize, in this study we estimated the effect of the addition of VFAs and other potential products from starch fermentation on growth and H_2-photoproduction rates of *Rb. capsulatus*. The purpose of these experiments was to understand how the addition of known amounts of define VFAs affect H_2 production, so that future experiments with mixed waste fermentation products can be better designed. The utilization of VFAs for H_2 production was characterized under a wide range of concentrations. Short-term H_2 production rates were rather stable upon addition of up to 80 mM concentrations of different VFAs. The saturating concentrations of added acetate were found to depend on the time after the culture had consumed it growth substrate, but in any case were below 2 mM. Among the other potential fermentation products, butanol caused the greatest inhibition of growth and H_2 production. The sequential order of VFA utilization out of an equimolar diluted mixture was also demonstrated and discussed. Finally, our data show that inhibition of methanogenesis during fermentation stage by the presence of Zn^{2+} in the fermentation effluent should not affect H_2 production by *Rb. capsulatus*.

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