

# **HYDROGEN PRODUCTION FROM *Klebsiella oxytoca* AND MEDIUM INDUCED METABOLIC SWITCHES**

MARINELLA BROGLIA, FRANCESCO PETRAZZUOLO

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AGENZIA NAZIONALE PER LE NUOVE TECNOLOGIE,  
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# HYDROGEN PRODUCTION FROM *Klebsiella oxytoca* AND MEDIUM INDUCED METABOLIC SWITCHES

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## **Riassunto**

Un ceppo di *Klebsiella oxytoca* è stato isolato da un impianto per la depurazione delle acque municipali e le sue capacità di produzione di idrogeno sono state esplorate. La cinetica a breve termine del rateo di produzione di idrogeno è stata investigata in 'batch' e il rateo massimo di produzione è stato attivamente mantenuto in un reattore a flusso continuo pilotato dal pH. I primi tentativi di 'shift' metabolico, effettuati cambiando la composizione iniziale del mezzo di fermentazione, hanno spostato il metabolismo batterico su differenti percorsi di fermentazione.

**Parole chiave:** bioidrogeno, *Klebsiella*, fermentazione, digestione anaerobica, 'switch' metabolico

## **Abstract**

*A strain of Klebsiella oxytoca has been isolated from a municipal sewage sludge treatment plant and its performance in biohydrogen production has been explored. The short term kinetics of the hydrogen production rate has been investigated in batch and the maximum production rate has been actively maintained in a pH-driven continuous flow reactor. First attempts of metabolic shifting, made by changing the initial composition of the fermentation medium, switched the bacteria metabolism to different fermentation pathways.*

**Keywords:** biohydrogen, *Klebsiella*; fermentation, anaerobic digestion, metabolic switch



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## 1. Introduction

Hydrogen is generally considered the fuel of choice for the near future, water being the product of its combustion [1]. Indeed hydrogen, unlike oil or methane, cannot be found free in nature and, in this sense, it cannot be considered a real energy source: nevertheless it could be exploited as a carrier of otherwise produced energy, just like to electricity but with a different chance of storage [2].

Hydrogen production by fossil [3] or nuclear fuels [4] would not remove the environmental drawbacks of energy production, but would allow to confine them for a cleaner diffused energy utilization in fuel cell technology. A new perspective is opened by the use of renewable energy sources to produce hydrogen [5]: sun, wind, waves, biomass energy can be transformed in electricity or in hydrogen as well.

A quite different approach lies in microbial hydrogen production [6]. In several millions of years algae and bacteria developed their own “technology” for sun light capture and hydrogen production. Indeed, in anaerobic conditions they produce hydrogen in nature, but biological feedback regulation and association with hydrogen consuming bacteria likely prevent hydrogen from accumulation in wells or mines. Microbial hydrogen production offers some advantages: it works at ambient pressure and temperature and can reduce either water or many kind of organic wastes [7]. Moreover biological CO<sub>2</sub>, produced together with biohydrogen, does not increase total CO<sub>2</sub> in the atmosphere. Therefore, controlled reproduction of conditions that in nature induce bacteria to produce hydrogen, in a bioreactor where biohydrogen can be continuously removed, seems to be an interesting energy option.

Between all the bacterial processes for hydrogen production, anaerobic dark fermentation of organic wastes stands out on photosynthetic processes for less extensive installations required, the day-night working time and the higher production rates due to very efficient hydrogenases [8]. Nevertheless, the waste material being reduced to organic acids, yields are quite low and should be increased by a second bacterial stage, photosynthetic or methanogenic [9].

The anaerobic digestion stage of plants for municipal sewage sludge treatment can be a good source of dark fermenting hydrogen producing bacteria. Not all of them are strict anaerobes. Facultative anaerobic bacteria are often preferred for their high growth rates and their tolerance to high hydrogen concentrations, in spite of a lower hydrogen yield due to a more branched metabolism [10].

## 2. Materials and Methods

### 2.1 Bacterial strain isolation

Our hydrogen producing strain has been isolated from the output sludge of the municipal sewage treatment plant in Cremona (Italy). The sludge has been diluted then plated on Luria Broth agar medium (for 1 liter: 10 gr Tryptone, 5 gr Yeast Extracts, 10 gr NaCl, 0.32 ml NaOH 10 N, 15 gr Agar) and aerobically grown overnight at 30°C. Single colonies have been subcultured in 3 ml and then in 100 ml 8 gr/l Nutrient Broth (DIFCO) liquid medium (NB); these cultures have been transferred with 150 ml of a buffered minimal medium (48 mM Na<sub>2</sub>HPO<sub>4</sub>, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 8.5 mM NaCl, pH=6) (MM) and 1% sucrose in 250 ml plastic bottles (head space about 20 ml) and left

sealed and still on the bench at room temperature. About 72 hours later bottles have been gently shaken: bottles that swelled due to some gas released from the liquid phase into the head space were set out for gas phase analysis. The main hydrogen producing strain has been identified by 16S rDNA amplification, sequencing and comparison to genetic data banks as *Klebsiella oxytoca*.

Briefly, 1 ml of saturated culture were centrifuged (15000 g x 5 min); pellet was washed and resuspended in 300 µl of sterilized distilled water; then it was incubated 15 min at 95°C. After centrifugation, 5 µl of a 100 dilution of the supernatant were used for 16S rDNA amplification by polymerase chain reaction (PCR) in a 50 µl total reaction volume containing 1.5 mM MgCl<sub>2</sub>, 150 ng (each) of primers (bacfor: 5'-CAGACTCCTACGGGAGG-3', bacrev: 5'-CATTGTAGCACGTGTGTAG-3'), 200 µM (each) deoxynucleoside triphosphates, 1.25 units of Gold *Taq* DNA polymerase (Applied Biosystem) in Gold *Taq* buffer. The reaction mixtures have been incubated in a thermocycler (MJ Research) at 95°C for 10 min and then cycled 30 times through the following temperature profile: 95°C for 30 s, 55°C for 30 sec, 72°C for 30 s. Finally the mixtures were incubated at 72°C for 10 min. Ten microliters of the amplified DNA was verified by gel electrophoresis (1% wt/vol agarose, 200 ng/ml of ethidium bromide in TBE buffer (0.045 M Tris-borate, 1 mM EDTA)). Sequencing analysis was performed by the ENEA GENELAB.

## 2.2 Bioreactor and analysis

Fermentation has been investigated in a plexiglass home-made bioreactor (Fig.1). A total volume of 1.2 l is water jacketed for thermal conditioning and continuously magnetically stirred. The reactor is equipped with a glass electrode for pH control (Metrohm Herisau); NaOH 1N or a phosphate buffer can be automatically added when stable pH operation is required. A membrane micropump (KNF) is used to remove the gas from the bioreactor head-space, skimming the liquid level at 1 l volume, through a foam-liquid collector bottle, to a water displacing cylindrical system; here the gas phase is looped with the gaschromatograph sampling valve (sampling volume 125 µl). The residual pressure in the reactor is about 380 mbar. All connections are made by teflon tubes.

Both H<sub>2</sub> and CO<sub>2</sub> are detected with a GC6000 gaschromatograph (Carlo Erba Instruments) equipped with a silica gel (60/80 mesh) packed column (length 6 m, internal diameter 2 mm, carrier gas Ar, temperature program: 50°C 10 min, steep slope 35°C/min, 150°C 12 min) and a thermal conductivity detector. The system response curve has been determined with a set of standard mixtures of H<sub>2</sub> and CO<sub>2</sub>. During fermentation H<sub>2</sub> and CO<sub>2</sub> volumes and the production rates have been calculated from the gas analysis.

Simultaneously with gas analysis the fermentation broth is sampled for biomass, sugars, alcohols and volatile fatty acids (VFA) determination. Microbial biomass is determined by measuring the optical density at 600 nm (Optima 3000 UV/VIS Spectrophotometer); correspondence between optical density and microbial dry weight biomass has been determined by centrifugating (8000g x 20 min) 500 ml of a saturated culture of *K. oxytoca* in NB medium, quickly washing pellet with sterile water, and drying it at 90°C until stable weight was attained. Cell viability has been assessed by plating on agar solidified NB medium 100 µl of suitable dilutions of the working bacterial suspension and counting visible colonies after overnight incubation at 37°C. When required, a real time measure of glucose is obtained by glycaemia test strips and nitrogen is checked by the Nessler method. Concentration of sugars (glucose, sucrose or lactose) during fermentation is measured by liquid chromatography (column Alltech IOA-1000, temperature 70°C, mobile phase 0.01 N H<sub>2</sub>SO<sub>4</sub>, flow rate 0.5 ml/min, refractive index detector). Alcohols and volatile fatty acids in the fermentation

broth are detected both by liquid chromatography and by a 3600 Varian gaschromatograph equipped with a glass packed column with 80/120 Carbopack B-DA/4% Carbowax 20M (carrier gas N<sub>2</sub>, temperature program: 100°C 3 min, steep slope 50°C/min, 175°C 36 min) and a flame ionization detector. Gas and liquid chromatograph systems are connected to a personal computer where the CSW32 Software from Data Apex is used for data collection and processing.

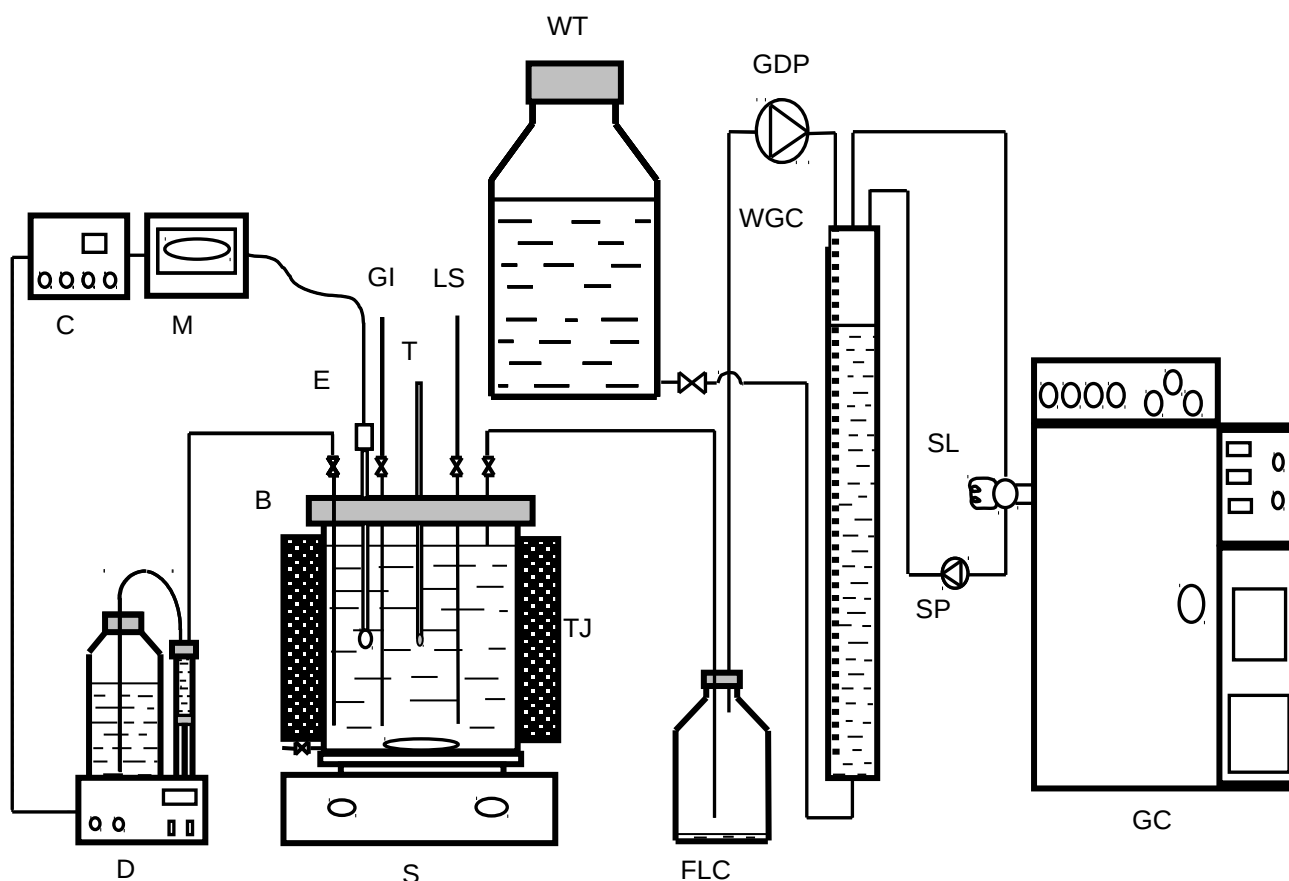


Figure 1 – Experimental setup

B bioreactor, E pH electrode, M pH measurement, C controller, D dispenser for pH stabilization, T termometer, S magnetical stirrer, TJ termal jacket, GI gas input for inertization, LS liquid sampling, FLC foam-liquid collector, WT water tank, GDP gas displacing pump, WGC water-gas cylinder, GC gaschromatograph, SL sampling loop, SP sampling pump.

### 3. Results and Discussion

#### 3.1 Microbial growth and fermentation

Several growing media have been explored to obtain high cell density starting conditions. Generally, when sugars were added to the growing medium as carbon source, glycolysis was the preferred metabolic way, then volatile fatty acids were produced and stopped the growth unless high phosphate concentrations had been used to buffer the medium. This is the reason why we preferred protein media, like NB, that enter the Krebs Cycle allowing to grow bacteria at higher microbial density without any buffering. The addition of 5gr/l yeast extracts (YE) improved the growth and with a double strength 2x(NB+YE) medium we generally obtain about  $8 \times 10^9$  viable cells/ml (corresponding to 2.5-3 gr dry weight/l) in 16 hours (Fig.2).

Glucose fermentation has been investigated in 1 liter batch reactor. A 500 ml saturated culture of *K. oxytoca* in NB medium has been diluted with sterile MM pH 5.5 and sparged with argon to attain anaerobic condition. A 0.2  $\mu\text{m}$  filtered glucose stock solution has been added for fermentation: the glucose concentration has been periodically checked and manually restored to the starting value.

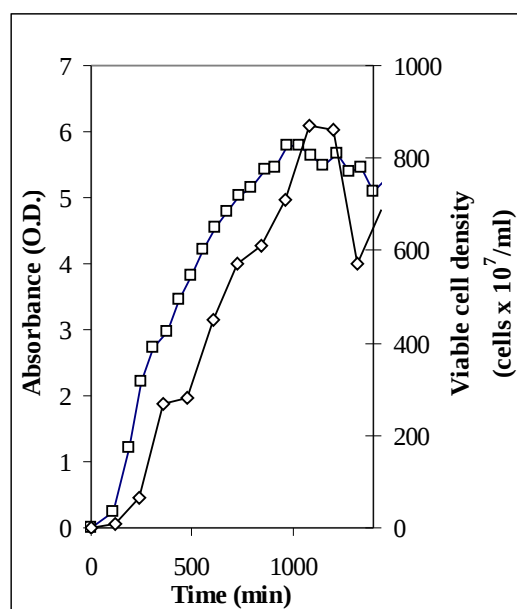


Figure 2 – Growth curve of *Klebsiella oxytoca* in 2x(NB+YE).  $\square$  Absorbance at 600 nm,  $\diamond$  Viable cell density assessed as colony forming units.

The pH initial value was about 6 with a decreasing trend due to volatile fatty acids production. A first set of batch fermentations has been performed at pH 6 and different temperatures (30°, 34°, 37°, 40°C): the optimal temperature of 34°C has been obtained as the one with the higher value of the maximum production rate ( $\text{mmol H}_2/\text{gr}_{\text{DW}}/\text{h}$ ). In a second set of batch fermentations the pH has been hooked and maintained at different values between 5 and 6 (5.0, 5.25, 5.5, 5.75, 6.0), at 34°C: the pH 5.5 corresponding to the maximum value of hydrogen production rate has been adopted for further investigation. A starting glucose concentration between 0.5 and 2% resulted not to be critical and 1% has been generally used as starting glucose concentration.

As facultative anaerobe, *K. oxytoca* also grows during fermentation even if at slower rate. Metabolism during fermentation has been investigated by the analysis of the fermentation products. For carbon balance purpose the following medium has been used as fermentation medium: Na<sub>2</sub>HPO<sub>4</sub> 48 mM, KH<sub>2</sub>PO<sub>4</sub> 22 mM, NaCl 8.6 mM, MgSO<sub>4</sub> 1 mM, CaCl<sub>2</sub> 0.1 mM, MnSO<sub>4</sub> 0.7 mM, FeCl<sub>2</sub> 40 μM, ZnCl<sub>2</sub> 40 μM, CoCl<sub>2</sub> 40 μM, Na<sub>2</sub>MoO<sub>4</sub> 40 μM, H<sub>3</sub>BO<sub>3</sub> 20 μM, pH 5.5 by HCl (after autoclaving and cooling, this medium presented a slight precipitate that had to be filtered by a sterile 0.45 μm filter) [11]. Bacteria grown in 2x(NB+YE) were centrifuged (6000g x 20 min) and resuspended in the above-mentioned minimal medium. Glucose 0.5-1% and NH<sub>4</sub>Cl 10-20 mM were maintained in the fermentation medium by frequently checking and restoring starting concentration when necessary. In Fig. 3 we report the microbial growth during fermentation, total gas volumes, gas production rates and the fermentation products accumulation. Carbon balance as calculated at the end of fermentation is reported in Table 1.

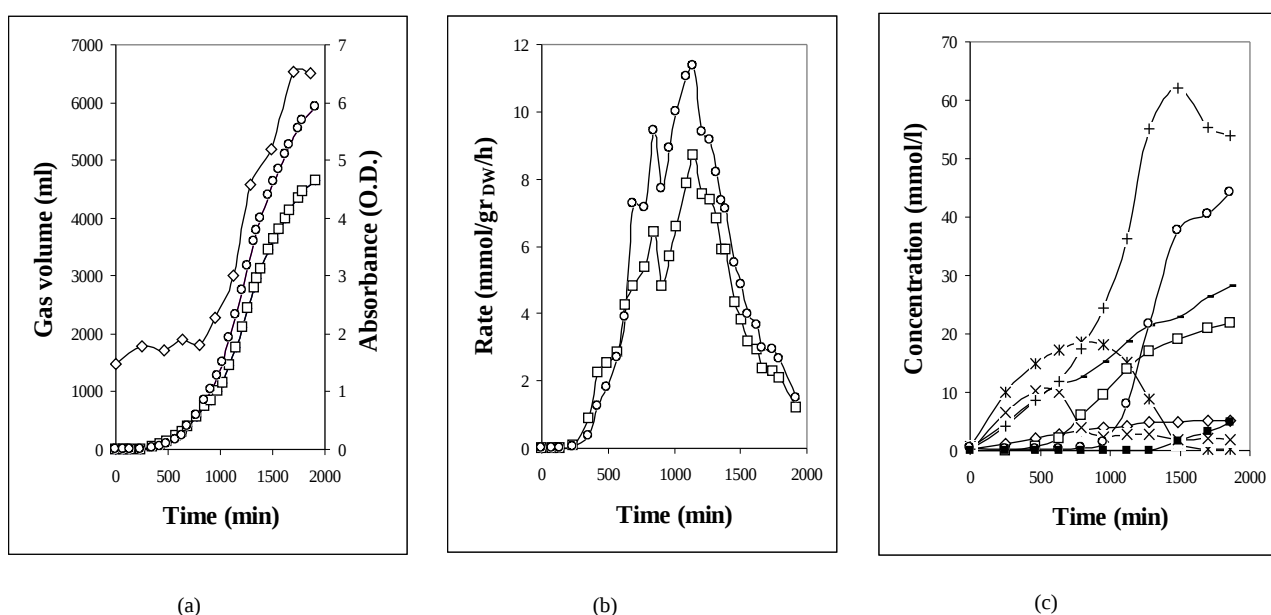


Figure 3 – Batch fermentation in minimal medium for carbon balance assessment. (a) Microbial growth and total gas production:  $\diamond$  absorbance at 600 nm,  $\square$  H<sub>2</sub> volume,  $\circ$  CO<sub>2</sub> volume. (b) Production rates:  $\square$  H<sub>2</sub>,  $\circ$  CO<sub>2</sub>. (c). Total products: + ethanol,  $\circ$  butyrate, - acetate, \* lactate,  $\square$  2,3-butanediol,  $\diamond$  succinate, x formate,  $\blacksquare$  butanol.

<b>Converted Glucose</b>	<b>1009.9</b>				
Biomass	145.2				
Carbon dioxide	264.3				
Alcohols	ethanol	butanol	2,3-butanediol		
	147.2	21.6	110.9		
Volatile fatty acids	formate	acetate	butyrate	succinate	lactate
	2.1	71.8	216.9	27.0	3.2
<b>Total products</b>	<b>1010.2 (+0.03%)</b>				

Table 1 – Carbon balance for the fermentation of Figure 3. All values are given in C-mmol. The average dry biomass composition CH<sub>1.74</sub>O<sub>0.43</sub>N<sub>0.23</sub> reported by Roels (1983) has been considered [15].

Kinetics of the hydrogen production rate in batch stimulated further investigation. Generally, in the complex fermentation medium, the maximum value of the hydrogen production rate is attained in 200-300 min, after that an abrupt drop in the hydrogen production rate is always detected, irrespective of free or controlled pH mode (see Fig. 4 (a), (b)). Quite differently and depending on the fermentation conditions the CO<sub>2</sub> production rate (not shown in the figure) can stay longer high, meaning that different metabolic ways are producing H<sub>2</sub> and CO<sub>2</sub>: this seems to indicate that hydrogen production is a prompt electron capture mechanism of the bacterial cell in anaerobic conditions, but it is dropped when other methabolic ways become practicable. If so, in spite of the substrate economy the microbial growth should be substained during fermentation to maintain a high hydrogen production rate. Indeed, switching unsaturated suspensions to fermentation we noted that the bacterial growth in anaerobic conditions sustain the hydrogen production rate (e.g. see Fig. 5). Alternatively, taking advantage from using facultative anaerobic bacteria, we could maintain the maximum production rate by growing bacteria aerobically and continously introducing them into an anaerobic flow reactor (without any change in microbial density). Exploring this possibility in our experimental apparatus, in order to simultaneously stabilize pH, dilute the products of fermentation and refresh the microbial population, fresh cultured bacteria have been suspended in a phosphate solution MM pH 7 and this suspension was used for pH stabilization. The foam-liquid collector bottle has been removed and two synchronous pumps, driven by the pH stabilization system, have been used to maintain constant the fermentation volume. This operation mode can be considered a pH-driven continuous flow reactor and indeed enabled us to maintain the maximum production rate (Fig. 4d). In our opinion this separation between growth and fermentation could be of interest to obtain biohydrogen from a conventional plant for sewage sludge treatment, where the facultative anaerobic microflora can vigorously be grown in the aerobic stages.

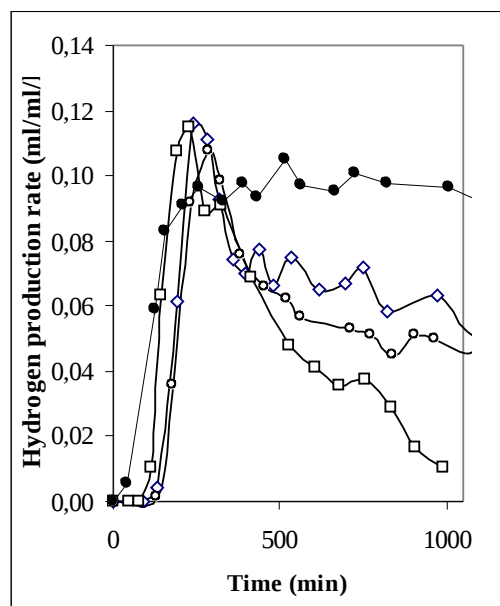


Figure 4 – Hydrogen production rates for different fermentation modes: □ batch at free pH, ◇ batch at fixed pH=5.5, ○ pH-driven continuous flow (neutralization buffer phosphate pH=7) ● pH-driven continuous flow (neutralization buffer phosphate PH=7 + aerobically grown bacteria).

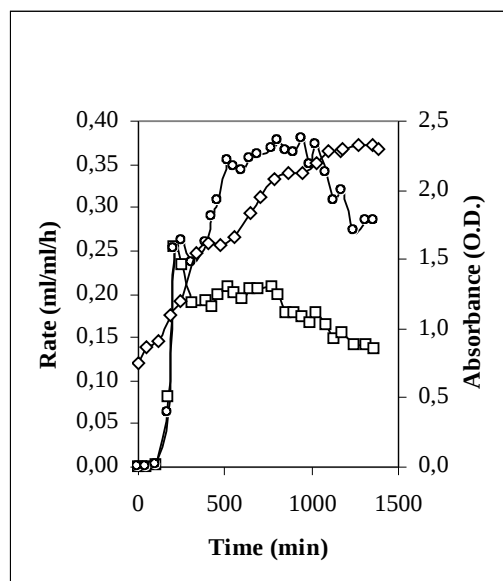


Figure 5 – Batch fermentation in growth sustaining conditions. 500 ml of NB 3x have been inoculated and bacteria have been grown aerobically until the exponential phase. Then the suspension has been diluted by 500 ml of MM pH 5.5 + 1% glucose and switched to anaerobic condition. □ H<sub>2</sub> production rate, ○ CO<sub>2</sub> production rate, ◇ absorbance at 600 nm.

### 3.2 Medium induced metabolic switches

The low yield of hydrogen production from facultative anaerobic bacteria, due to their metabolism more branched with respect to strict anaerobic and thermophilic bacteria, is considered the main hindrance to their application on a large scale [9]. Mutagenesis and genetic engineering have been used to obtain strains of facultative anaerobes with less branched metabolisms and higher hydrogen yields [12].

Indeed the medium initial composition seems to have an important role in addressing bacterial metabolism. *Klebsiella oxytoca* has been diffusely investigated in the twentieth century as 2,3-butandiol producer [13]. In our hands 2,3-butandiol and ethanol are the main products when a glucose containing complex medium is used for fermentation (see Fig. 6a), but we did not observe a significant production of 2,3-butandiol in the minimal medium we used for carbon balance (cfr. Fig. 3). In the complex fermentation medium bacterial growth was prompt but a low ratio H<sub>2</sub>/CO<sub>2</sub> was attained due to a dominant alcoholic fermentation and the yield was about 0.8 mol H<sub>2</sub>/mol glucose. In the minimal medium, bacteria started to grow after a lag phase of about 17 hours and production of ethanol and butyrate were the preferred metabolic ways, with a H<sub>2</sub>/CO<sub>2</sub> ratio near to 1 and a higher yield of about 1.2.

As a matter of speculation we wondered if the addition of small quantities of alcohol and volatile fatty acids in the initial composition of the fermentation medium would have been able to induce bacteria to not produce them further. In Fig. 6b the initial fermentation medium was exactly the same as in Fig. 6a, but before bacteria resuspension, alcohols and volatile fatty acids had been

added as indicated in the figure caption and the pH has been re-adjusted to 5.5 by NaOH. Actually production of 2,3-butanediol and ethanol was almost stopped, favouring a moderate mixed acid fermentation. In this case the H<sub>2</sub>/CO<sub>2</sub> ratio comes closer to 1. Referring to the scheme in Fig. 7 [14], we noted that all the metabolic ways can be explored by *Klebsiella oxytoca* depending on the initial conditions and on the time. Synchronous lactate decrease and butyrate increase could indicate the additional occurrence of a lactate to butyrate fermentation with H<sub>2</sub> and CO<sub>2</sub> production (2 lactate => 1 butyrate + 2 H<sub>2</sub> + 2 CO<sub>2</sub>). In such a case we should subtract from the total produced hydrogen the share of the initial lactate, to obtain the yield on glucose that approaches 1.2. Butanol and butyrate addition to the initial composition avoided their production by bacteria (Fig. 6c), but in this case a pure lactic fermentation took place, almost without any gas production.

Closing the other main metabolic ways but the butyrate one, we increased the initial lactate in order to inhibit lactate production. Addition of even 60 mmol/l of lactate to the initial composition of the fermentation medium was not sufficient to stop its production by bacteria. Indeed, lactate accumulation resulted to be the last metabolic way experienced in fermentation by a degenerating microbial culture (Fig. 8), generally avoided only in continuous flow regime.

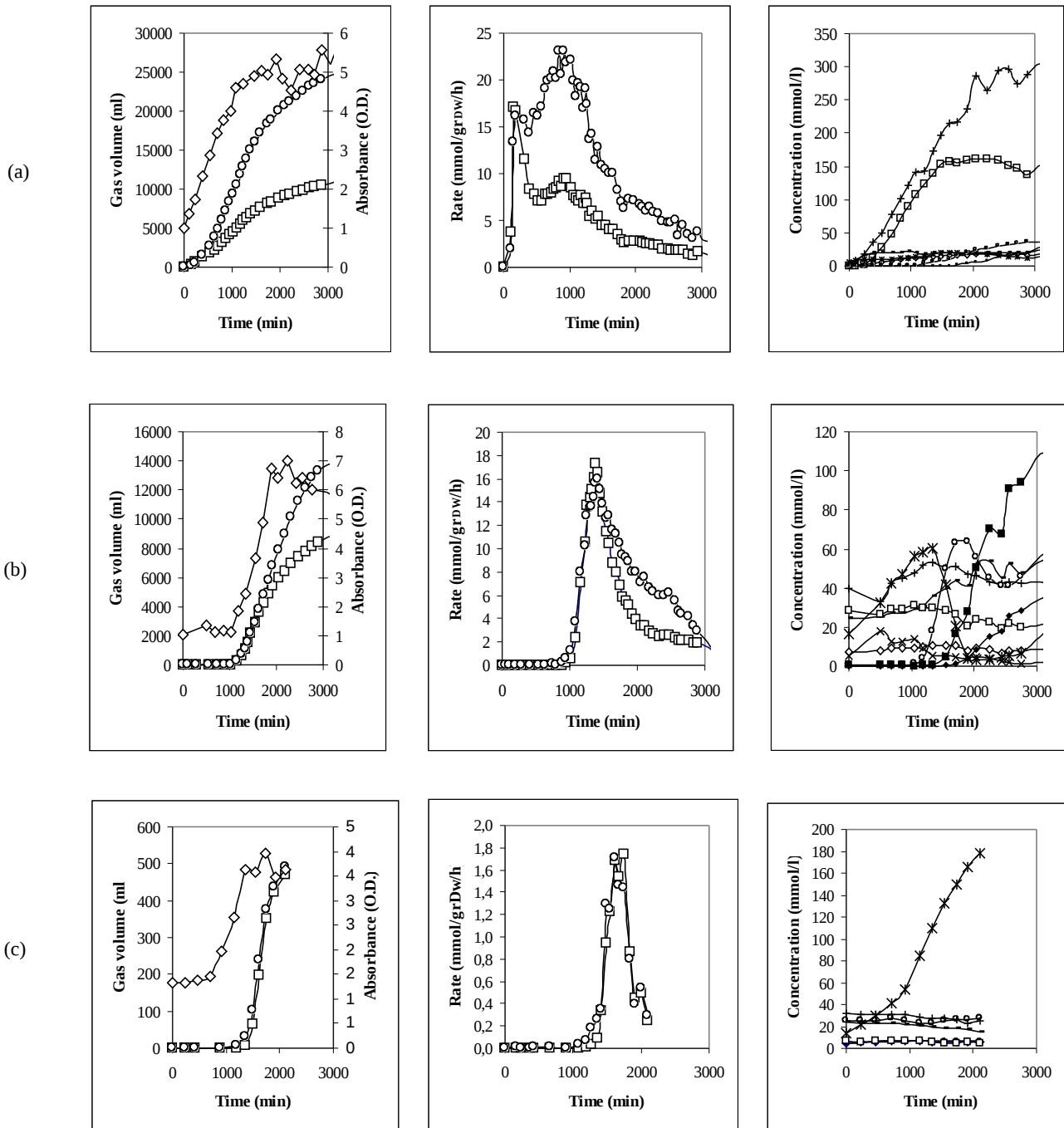


Figure 6 – Batch fermentations at different initial conditions. 500 ml of a saturated culture of *K. oxytoca* in 2x(NB+YE) have been centrifuged and resuspended in 500 ml 2x(NB+YE) + 500 ml MM pH 5.5 and 1% of glucose has been added and maintained during fermentation. (a) Referring basic composition. (b) Same as (a) but ethanol 40 mM, 2,3-butandiol 28 mM, acetate 24 mM, lactate 16 mM, succinate 7 mM, formate 5 mM, acetoin 1 mM, butyrate 1 mM. (c) Same as (a) but ethanol 32 mM, 2,3-butandiol 7 mM, acetate 24 mM, lactate 14 mM, succinate 5 mM, butyrate 25 mM, butanol 1 mM. For each composition total gas production, production rates and total products are given. Gas symbols:  $\square$   $H_2$ ,  $\circ$   $CO_2$ . Liquid products symbols: + ethanol,  $\diamond$  succinate, x formate,  $\blacksquare$  butanol.

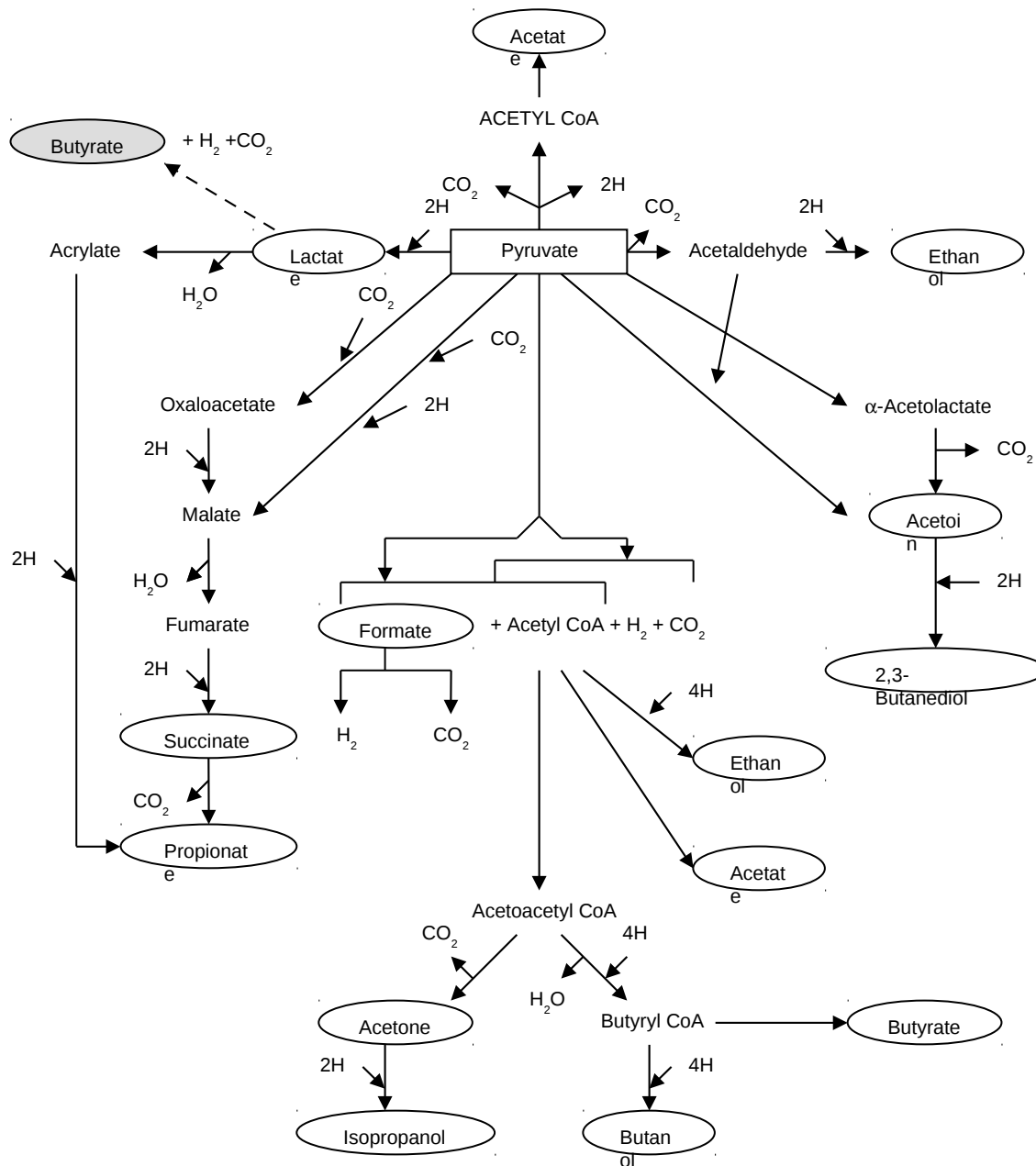


Figure 7 – Metabolic pathways of the microbial fermentations from pyruvate, as reported in Ref. 14. We inserted the lactate to butyrate fermentation way as it comes from our experimental results.

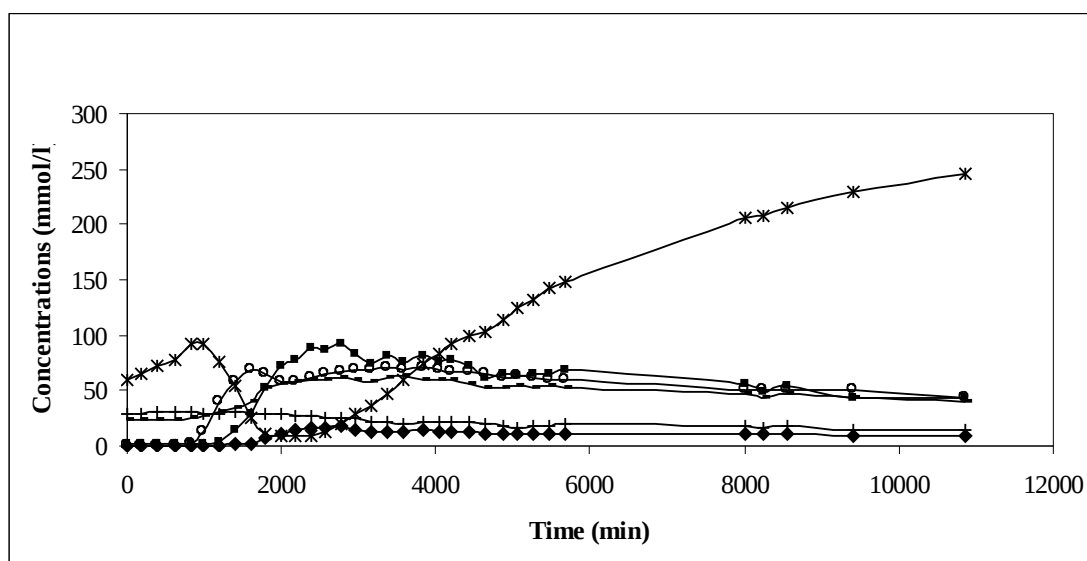


Figure 8 – Batch fermentation in the following initial medium composition: same as Fig. 6 (a) but lactate 60 mM, ethanol 30 mM, acetate 24 mM, 2,3-butanediol 7 mM, succinate 5 mM, butanol 1 mM. Symbols: + ethanol, o butyrate, - acetate, \* lactate, □ 2,3-butanediol, ◇ succinate, x formate, ■ butanol, ◆ isopropanol.

#### 4. Conclusions

*Klebsiella oxytoca* isolated from a municipal sewage sludge has been characterized and resulted an excellent microbial tool for biohydrogen investigation. The short-term kinetics of the hydrogen production rate has been modified refreshing the microbial population by introducing aerobically grown bacteria. Switches in metabolic pathways, induced by changing the initial composition of the fermentation medium, resulted in a 50% increase of the hydrogen yield on glucose.

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