Fermentative hydrogen production by *Clostridium butyricum CWBI1009* and *Citrobacter freundii CWBI 952* in pure and mixed cultures

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As a clean renewable energy resource H_2 is considered as the fuel of the future…

Two microbial pathways for biohydrogen production

Photo fermentation — Dark fermentation

Carbon source ($C_6H_{12}O_6$)
Anaerobiosis & nutrients

Phototrophic microorganisms
- Low production rate
- High yield
- Light energy consumption

Chemotrophic microorganisms
- High production rate
- Lower yield
- Low temperature (30 °C)

Light

Hydrogen production + CO_2

Alcohols, acids, ... in aqueous solution
... → $2CH_3COOH + 2CO_2 + 4H_2$

Valorization (methanisation)

Dark fermentation is more adapted for industrial H_2 production from wastewater and biomass (no light, higher production rate, effluent valorization, …). → Pollution reduction; Energy generation

Objectives

1. Compare the hydrogen production of two strains .recently isolated and characterized at the laboratory:
   - A facultative anaerobes *Citrobacter freundii* CWBI 952 (Hamilton et al. 2010)
     - Advantages: oxygen tolerant, high hydrogen production rate
     - Disadvantages: non sporulating strain, low yield
   - A strict anaerobes *Clostridium butyricum* CWBI 1009 (Masset et al. 2010)
     - Advantages: sporulating strain, high yield, high production rate, degrade many carbohydrate sources
     - Disadvantages: strongly inhibited with oxygen (need expensive reducing agents)

   Five substrates are investigated. The hydrogen production and the metabolites in the media are analyzed.
2. Investigate the co-culture of the two strains and its ability to grow on glucose and starch:
   - Advantages: *C. freundii* may consume the oxygen in the media and promote sustainable anaerobic conditions for *Cl. butyricum* to grow.
   - The high yields of *Cl. butyricum* may be lowered.
   - Analyze the ability to consume starch (a more complex substrate than glucose)
   - Observing the competition between the two strains.

Methods

BHP test (**B**iochemical **H**ydrogen **P**otential) (Lin et al. 2007)

Batch cultures in 270 ml serum bottles in MD media

	Pure *Citrobacter freundii* CWBI952 (Hamilton et al. 2010)	Pure Clostridium butyricum CWBI1009 (Masset et al. 2010)	Mixed culture of C. freundii and Cl. butyricum
Glucose	X	X	X
Maltose		X	
Sucrose	X	X	
Lactose	X	X	
Starch	X	X	X

- Biogas volume measured with a sterile syringe and needle trough a butyl septum
- Hydrogen content in biogas determined by GC-TCD and ABB EL 1020 gas analyzer
- Soluble metabolites concentration determined by HPLC-RID
- Aerobic contamination and purity check on PCA media in Petri dishes

Results 1 Pure cultures with several different substrates

Table 1: Final hydrogen production and hydrogen yield in 270 ml batch culture with five different substrates during 48 hours.

Table 1		Hydrogen production (ml)	Hydrogen yield ($mol_{H2} \cdot mol_{hexose}^{-1}$)
***Citrobacter freundii* CWBI952**	Glucose	46.1+-5.7	0.24+-0.03
	Maltose	N.D.	N.D.
	Sucrose	19+-2.8	0.10+-0.02
	Lactose	35.3+-7.2	0.18+-0.04
	Starch	0	N.D. (0)
***Clostridium butyricum* CWBI1009**	Glucose	95.9+-2.0	0.58+-0.01
	Maltose	100.8+-2.0	0.51+-0.01
	Sucrose	98.3+-0.5	0.52+-0.00
	Lactose	123.9+-2.0	0.69+-0.00
	Starch	79.1+-2.1	0.49+-0.02

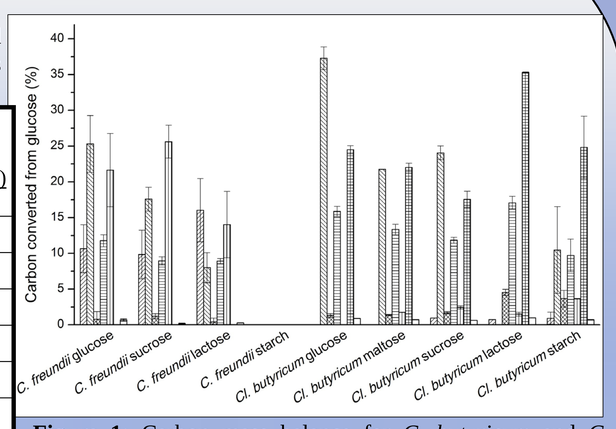

Figure 1: Carbon mass balance for *C. butyricum* and *C. freundii* in 270 ml batch fermentation with different carbon sources, expressed in percentage of carbon converted from the consumed carbon source (succinate; lactate; formate; acetate; ethanol; butyrate; carbon dioxide).

These results clearly show that better performance was obtained with *Cl. butyricum* compared to *C. freundii* which produced hydrogen less efficiently. With starch *C. freundii* produced no hydrogen. By contrast *Cl. butyricum* is able to produce hydrogen by degrading starch. For every substrate investigated, higher hydrogen yields were obtained with *Cl. butyricum*. The main soluble metabolites (ethanol, lactate, acetate, succinate and formate for *C. freundii* and butyrate, lactate, acetate, formate and ethanol for *Cl. butyricum*) were analyzed by HPLC at the end of the culture (48 hours). The carbon mass balance for these metabolites is indicated in the figure. Due to the difference in the metabolic pathways involved, butyrate is only produced by *Cl. butyricum* and succinate only by *C. freundii*. In addition more of the carbon source is converted to ethanol by *C. freundii* (ten times more than with *Cl. butyricum*).

Results 2 Mixed cultures with glucose and starch as substrate

Table 2: Hydrogen production and yields from glucose and starch fermentation in pure or mixed culture with *Cl. butyricum* and *C. freundii*. *C. freundii* is not tested in pure culture on starch because it doesn't degrade starch. The yields are calculated at the end of the fermentation.

Table 2		Cumulative hydrogen production (ml)		Hydrogen yield ($mol_{H2} \cdot mol_{hexose}^{-1}$)
		24 hours	End of fermentation	
Glucose	Pure *C. freundii*	38.4 +- 1.2	40.3 +- 5.4	0.25 +- 0.03
	Pure *Cl. butyricum*	0	99.6 +- 8.8	0.53 +- 0.04
	Mixed *C. freundii* and *Cl. butyricum*	54.4 +- 2	62.6 +- 2.7	0.38+- 0.02
Starch	Pure *C. freundii*	N.D.	N.D.	N.D.
	Pure *Cl. butyricum*	N.D.	92 +- 5.7	0.69 +- 0.04
	Mixed *C. freundii* and *Cl. butyricum*	44 +- 1.4	96.5 +- 0.7	0.73 +- 0.01

The sustainable influence of *C. freundii* in the mixed culture with glucose and starch allowed the production of hydrogen to begin earlier than with the pure *Cl. butyricum* culture. However, the use of a mixed culture in batch fermentation decreased the final hydrogen yield. Tests on PCA media, carried out 24 hours after inoculation, did not show any aerobic bacterial development indicating that *C. freundii* did not survive or that the cell concentration had dramatically decreased. This suggests that the strain enhanced the initiation of hydrogen production, but was then rapidly overgrown or strongly inhibited by *Cl. butyricum*.

Conclusions

Our work highlights the fact that mixed cultures of *Citrobacter freundii* CWBI952 and *Clostridium butyricum* CWBI1009 can efficiently maintain the production of hydrogen at acceptable yields compared to pure cultures of *Cl. butyrcium*. Moreover, it could consume efficiently many different carbon sources efficiently since *Cl. butyricum* was able to degrade simple carbohydrates or even starch. However the survival of *C. freundii* in competition with *Cl. butyricum* was compromised since it was overgrown especially with the glucose substrate. Further work will be done to find other facultative aerobic strains which are able to enhance anaerobic conditions in the culture media without being overgrown by *Cl. butyricum* and without decreasing the hydrogen yield.

Acknowledgements

This work is promoted by the FNRS (National Fund for Scientific Research, Belgium) for a grant of Research Fellow and is part of an ARC project called "MicroH2" supported by the "Communauté française de Belgique".
This poster is the result of a paper submitted in the journal "BASE" in February 2010

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