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La Fabbrica della Bioenergia

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CNR EXPO Lombardy Dialogues

Innovazioni tecnologiche
per il recupero di energia e risorse da reflui dell'agroindustria

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Dark fermentation: aspetti microbiologici

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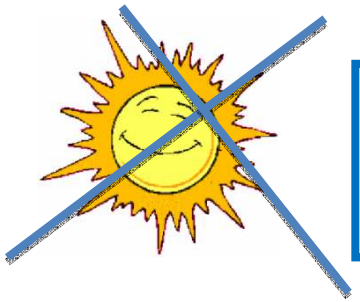
Bio - Hydrogen Production

- Direct biophotolysis
- Photofermentation

→ **Bio hydrogen**



Organic rich substrates

→ **Dark fermentation** → **Bio hydrogen**

- Microbial electrolysis cell

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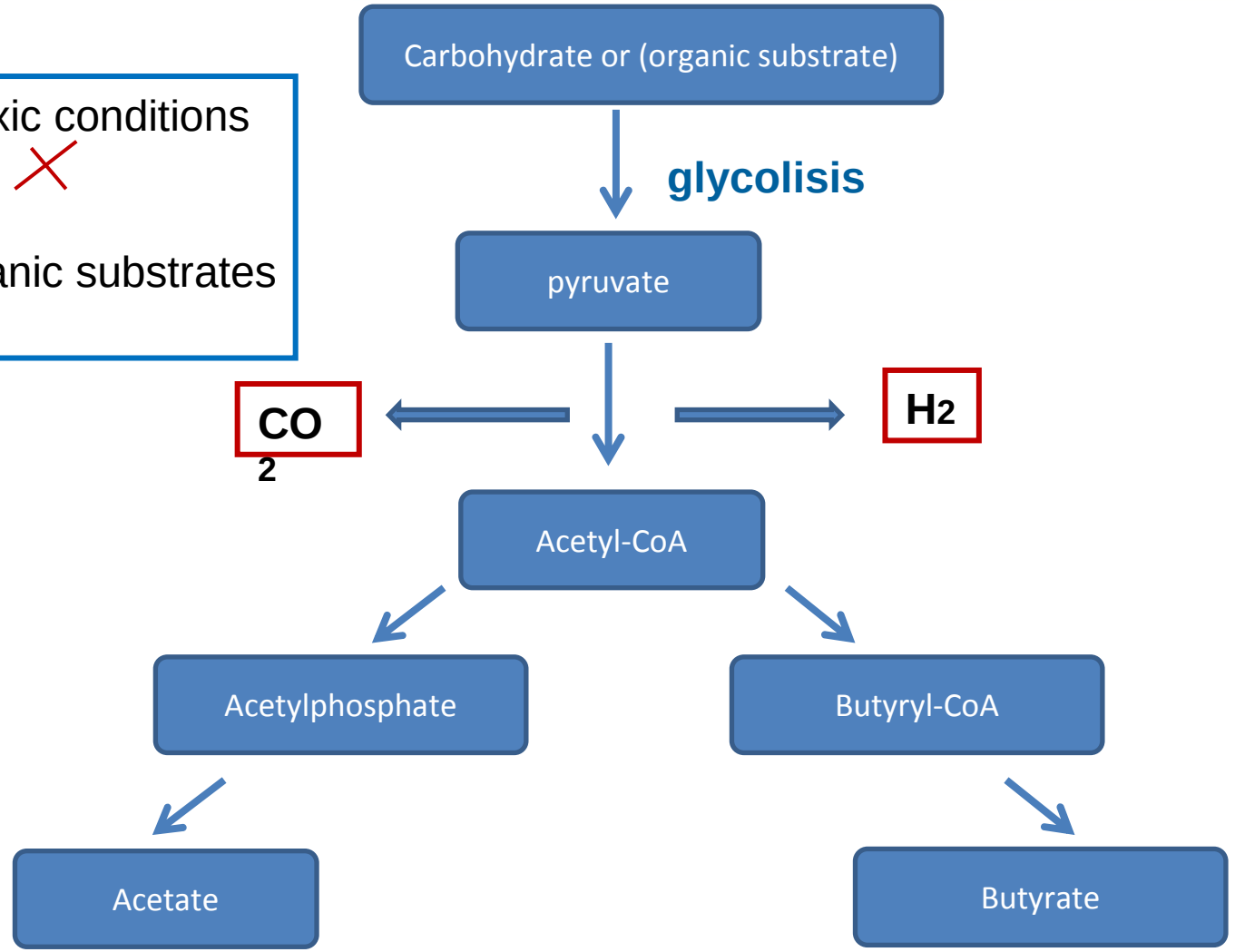
Dark fermentation

Anaerobic bacteria

- Anoxic conditions
  O_2 ✗
- Organic substrates



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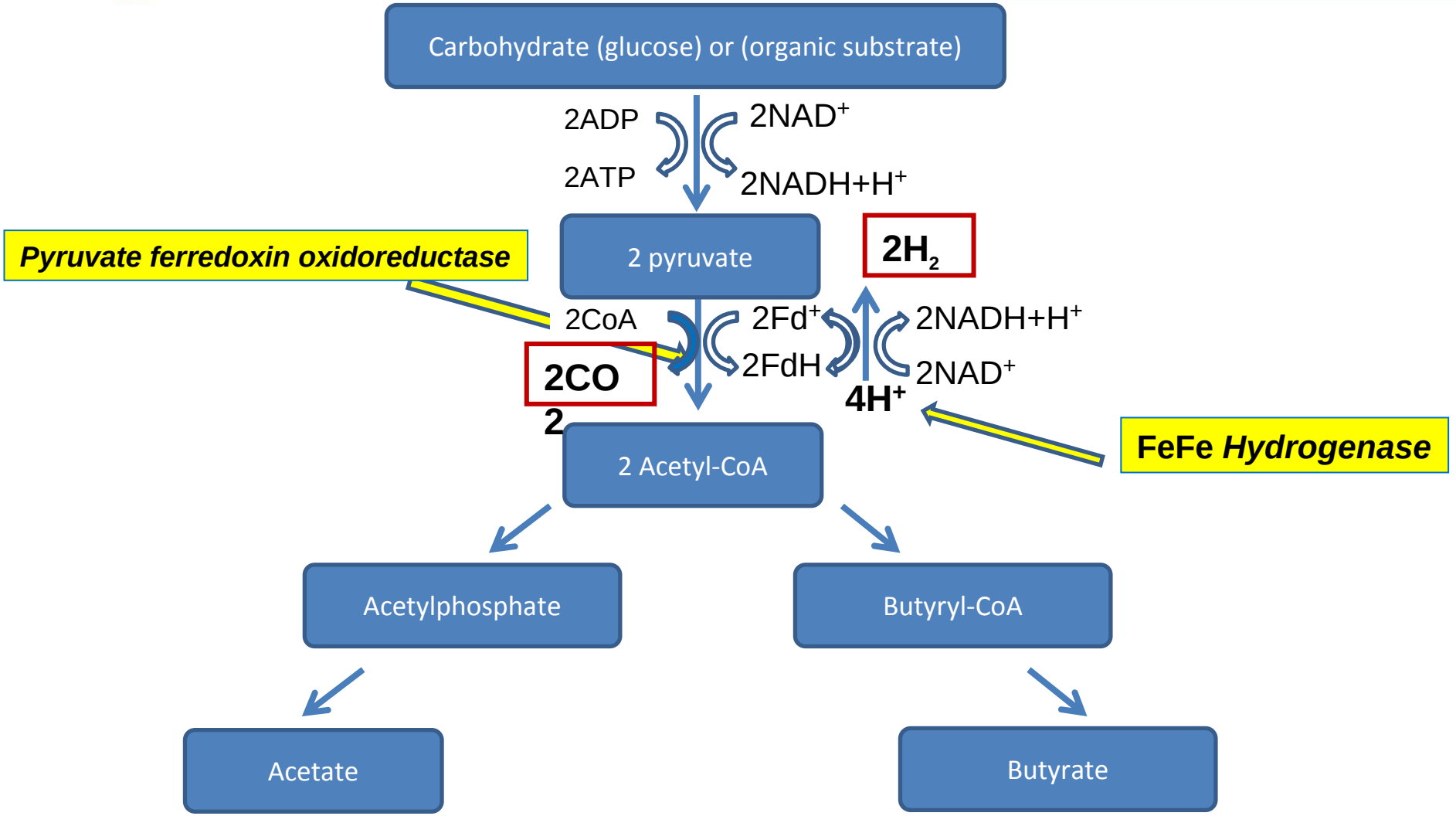
Dark fermentation

Anaerobic bacteria



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Clostridium tyrobutyricum fermentation

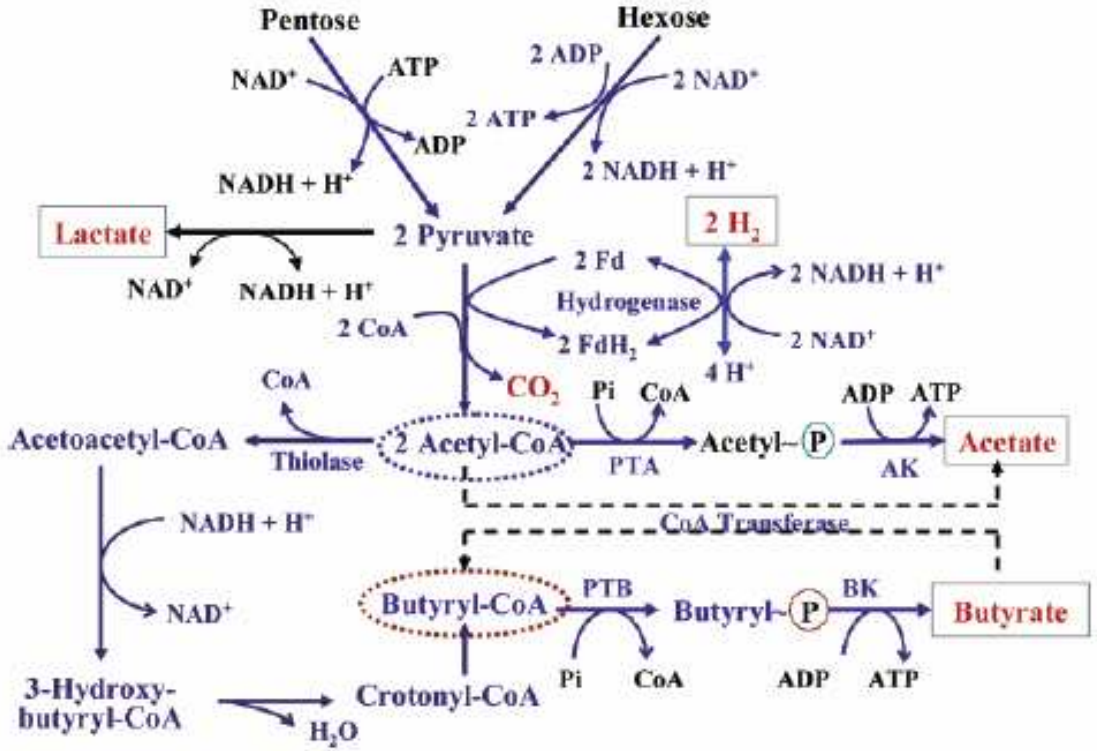

Figure 1. The metabolic pathway in *Clostridium tyrobutyricum*. In general, glucose and xylose are catabolized to pyruvate by the EMP pathway and the HMP pathway, respectively. Pyruvate is oxidized to acetyl-CoA and carbon dioxide with concomitant production of hydrogen. Acetyl-CoA is either oxidized to acetate through the PTA-AK pathway or butyrate through the PTB-BK pathway. Abbreviations: PTA, phosphotransacetylase; AK, acetate kinase; PTB, phosphotransbutyrylase; BK, butyrate kinase.

Liu et al.2006

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Dark fermentation

Faculative anaerobic bacteria

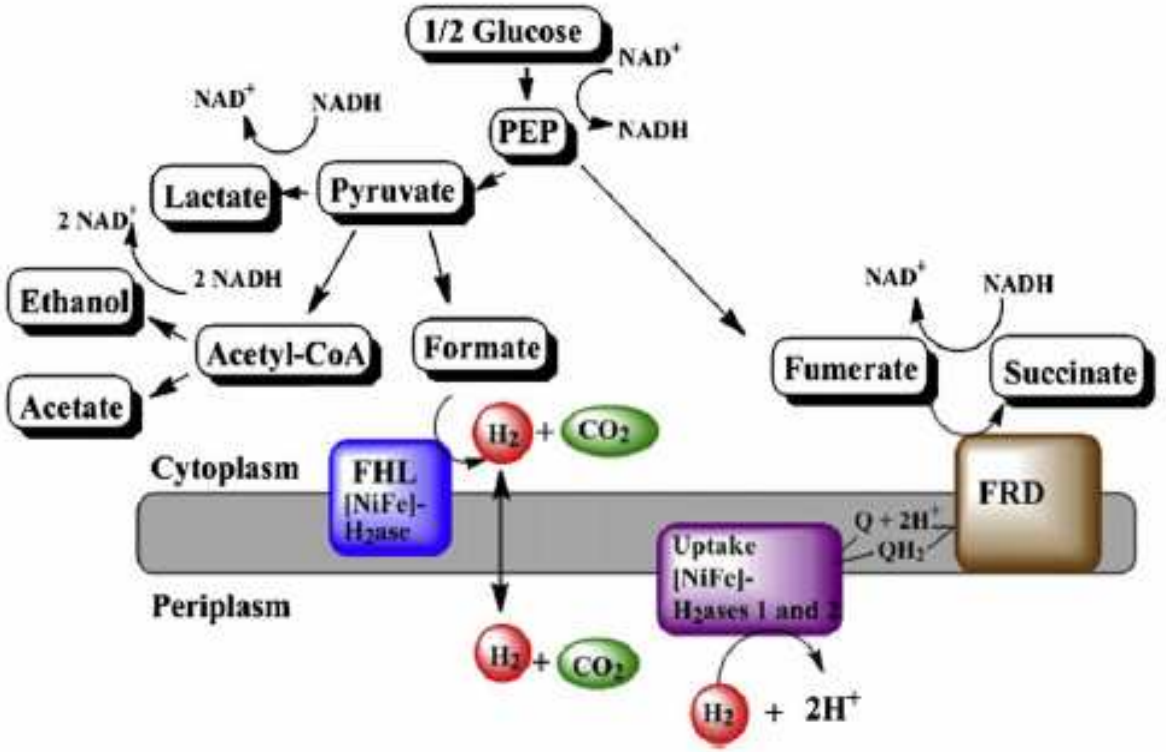


Fig. 4 – Mixed acid fermentation for biohydrogen production in E. coli. The metabolic pathways for the break down of glucose are shown with arrows indicting one or more reactions in series. The orientation of the hydrogen-evolving formate–hydrogen lyase (FHL) complex in the membrane is shown along with the uptake hydrogenases and fumarate reductase (FRD). Abbreviations: PEP, phosphoenol-pyruvate; Q, quinine pool; QH_2, quinol. Adapted from Murarka 2008 [119].

Mathews and Wang 2009

Dark fermentation

Fermentation have ben optimised by evolution to produce cell biomass and not hydrogen.

P.C. Hallenbeck, J.R. Benemann (2002)

As a result, the oxidation–reduction state has to be balanced through the NADH consumption to form some reduced compounds, i.e., lactate, ethanol and butanol, resulting in a lowered hydrogen yield.

Fermentation parameters	max
temperature	37-55°C
pH	4.0-7.0 optimal 5.5
Butyric acid	19 -30 mM (maximal stress)
Fe^{2+}	20-1800 mg/L
Nitrogen	0.5-10 g N/L
Cu > Ni–Zn > Cr > Cd > Pb	Toxic order

Wang et Wan 2008

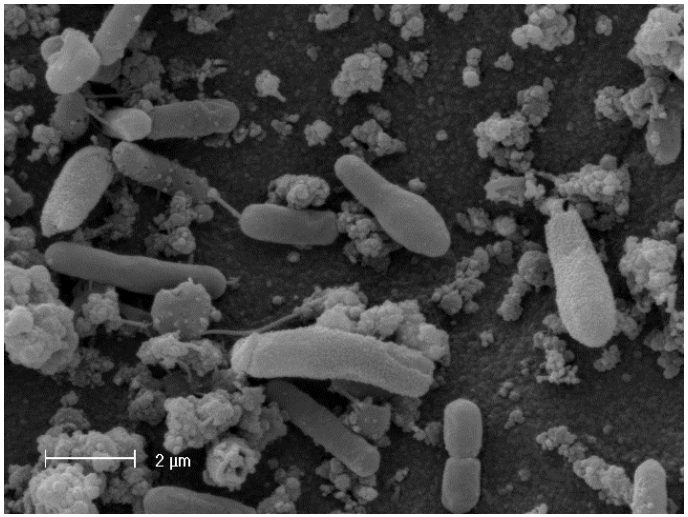
Dark fermentation

$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 + 2CO_2 + 4H_2$

glucose acetic acid

$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2CO_2 + 2H_2$

glucose butyric acid



Hydrogen clostridial producers
Clostridium acetobutylicum
Clostridium beijerinckii
Clostridium butyricum
Clostridium tyrobutyricum
Clostridium saccharoperbutylacetonicum
Clostridium thermolacticum
Clostridium cellulolyticum

Hydrogen production

Thermodinamics of hydrogenase reaction

Maximal theoretical yield hydrogen production:

- 4 moles of H_2/mole of hexose (i.e. glucose)
- H_2 Partial pressure **< 60 Pa**
- Production of: **NADH**, reduced ferredoxin

NADH ferredoxin reductase

- 2 moles of H_2/mole of hexose (i.e. glucose)
- H2 Partial pressure **> 60 Pa**
- ~~Production of: **NADH**, reduced ferredoxin~~

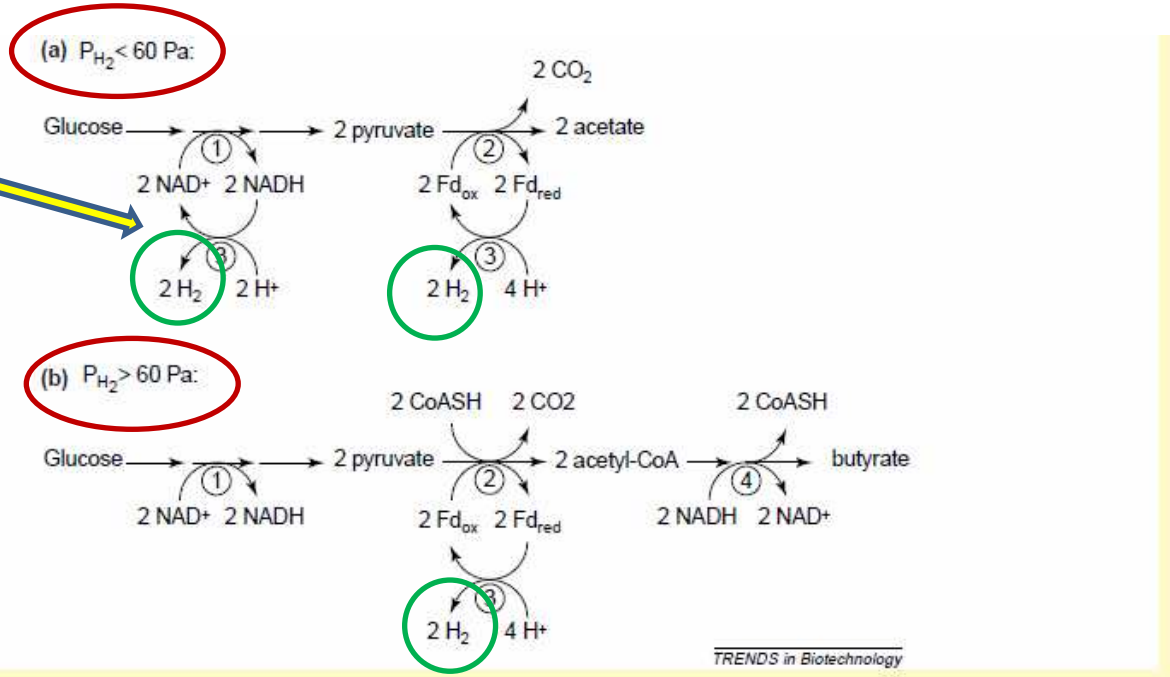


Figure II. Effect of hydrogen partial pressure on biological production of hydrogen. **(a)** Oxidation of NADH by production of hydrogen is thermodynamically favorable only when the hydrogen partial pressure is less than 60 Pa, otherwise; **(b)** other fermentation products must be formed. Reactions [(a) and (b)]: 1, glucose metabolism through glycolysis or the Entner-Doudoroff pathway; 2, oxidative decarboxylation of pyruvate by pyruvate:ferredoxin oxidoreductase; 3, formation of hydrogen by hydrogenase; 4, butyrate fermentation.

Angenent et al 2004

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Dark fermentation

➢ Pure culture

Table 1 – The pure bacterial cultures for fermentative hydrogen production.

Inoculum	Substrate	Reactor type	Maximum hydrogen yield	References
Clostridium acetobutylicum	Glucose	Batch	2.0 mol/mol glucose	[9]
Clostridium acetobutylicum ATCC 824	Glucose	Continuous	1.08 mol/mol glucose	[10]
Clostridium butyricum CGS5	Xylose	Batch	0.73 mol/mol xylose	[11]
Clostridium butyricum CGS2	Starch	Batch	9.95 mmol/g COD	[12]
Clostridium pasteurianum CH_4	Sucrose	Batch	2.07 mol/mol hexose	[11]
Clostridium paraputrificum M-21	Chitinous wastes	Batch	2.2 mol/mol substrate	[13]
Clostridium thermocellum 27405	Cellulosic biomass	Batch	2.3 mol/mol glucose	[14]
Clostridium thermolacticum	Lactose	Continuous	3.0 mol/mol lactose	[15]
Clostridium sp. strain no. 2	Cellulose	Continuous	0.3 mol/mol glucose	[16]
Clostridium sp. Fanp2	Glucose	Batch	0.2 mol/L medium	[17]
Enterobacter aerogenes HO-39	Glucose	Batch	1.0 mol/mol glucose	[18]
Enterobacter aerogenes NBRC 13534	Glucose	Batch	0.05 mol/L medium	[19]
Enterobacter aerogenes	Glucose	Batch	–	[20]
Enterobacter aerogenes HU-101	Glycerol	Batch	0.6 mol/mol glycerol	[21]
Enterobacter aerogenes	Starch	Batch	1.09 mol/mol starch	[22]
Enterobacter aerogenes E 82005	Molasses	Continuous	3.5 mol/mol sugar	[23]
Enterobacter cloacae IIT-BT 08	Glucose	Continuous	–	[24]
Enterobacter cloacae IIT-BT 08	Sucrose	Batch	6 mol/mol sucrose	[25]
Enterobacter cloacae IIT-BT 08	Cellobiose	Batch	5.4 mol/mol cellobiose	[25]
Escherichia coli MC13-4	Glucose	Batch	1.2 mol/mol glucose	[26]
Escherichia coli	Glucose	Batch	2.0 mol/mol glucose	[27]
Escherchia coli	Glucose	Continuous	2.0 mol/mol glucose	[28]
Pseudomonas sp. GZ1	Waste sludge	Batch	0.007 mol/g TCOD	[29]
Thermoanaerobacterium thermosaccharolyticum KU001	Glucose	Batch	2.4 mol/mol glucose	[30]
Thermococcus kodakaraensis KOD1	Starch	Continuous	–	[31]
Thermotoga elfii	Glucose	Batch	84.9 mmol/L medium	[32]
Hydrogen-producing bacterial B49	Glucose	Batch	0.1 ml/L culture	[33]
Ruminococcus albus	Glucose	Batch	2.52 mol/mol glucose	[34]
Hafnia alvei	Glucose	Batch	–	[35]
Citrobacter amalonaticus Y19	Glucose	Batch	8.7 mol/mol glucose	[36]
Ethanoligenens harbinense YUAN-3	Glucose	Continuous	1.93 mol/mol glucose	[37]

Wang et Wan 2009

Dark fermentation

- **Mixed culture:**

Advantages

- Compost, anaerobic sludge, municipal sewage sludge
- Thermal treatment to select clostridial community
- Wide range of substrate utilisation

Disadvantages

- The hydrogen produced by the bacteria can be consumed by other bacteria (hydrogenotrophic methanogenesis, $4H_2 + CO_2 \equiv CH_4 + 2H_2O$)

Pretreatment methods to enrich the community of H_2 producing bacteria			
Heat-shock			
Acid	Base	Heat-shock	
Acid	Base	Heat-shock	Aeration
Acid	Base	Heat-shock	chloroform
Acid	Base	Heat-shock	2 bromoethanesulfonate

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Dark Fermentation

Operating conditions

- pH
- biohydrogen partial pressure
- Temperature
- inhibitors

temperature,
pH and H2 partial pressure regulate metabolic pathways of hydrogen producing microorganisms

In addition,
substrate types and their pre-treatment methods, bioreactor configurations, inoculum sources and enrichments also influence the biohydrogen production.

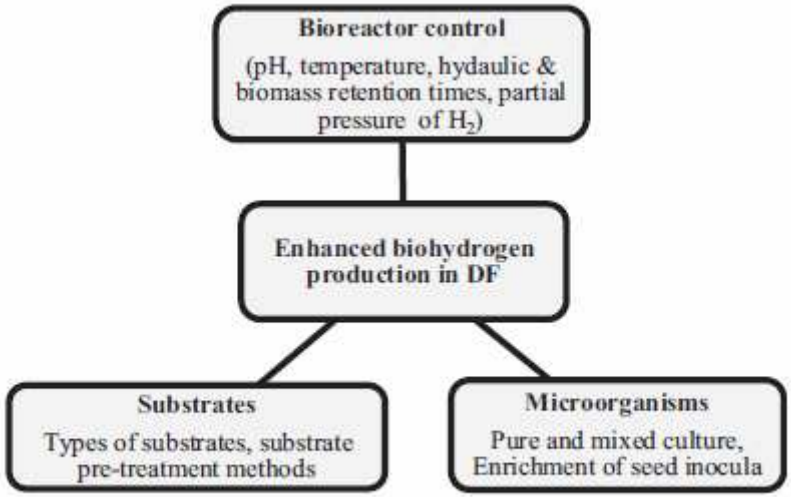

Fig. 4. Strategies to enhance the biohydrogen yield in DF of organic biomass.

A. Ghimire et al./Applied Energy 144 (2015) 73–95

Bio-hydrogen and Food waste – by products

- the waste generated from direct agricultural
- production, i.e. crop residues
- livestock waste, i.e. animal manure
- food waste.

- Deproteinised whey

Parameter	Value
Fat	0.59 g L^{-1}
Protein	3.44 g L^{-1}
Lactose	113 g L^{-1}
COD	157 g L^{-1}
Phosphate	820 mg P L^{-1}
Total phosphorous	1040 mg P L^{-1}
Ammonia	27.0 mg N L^{-1}
Total nitrogen	759 mg N L^{-1}

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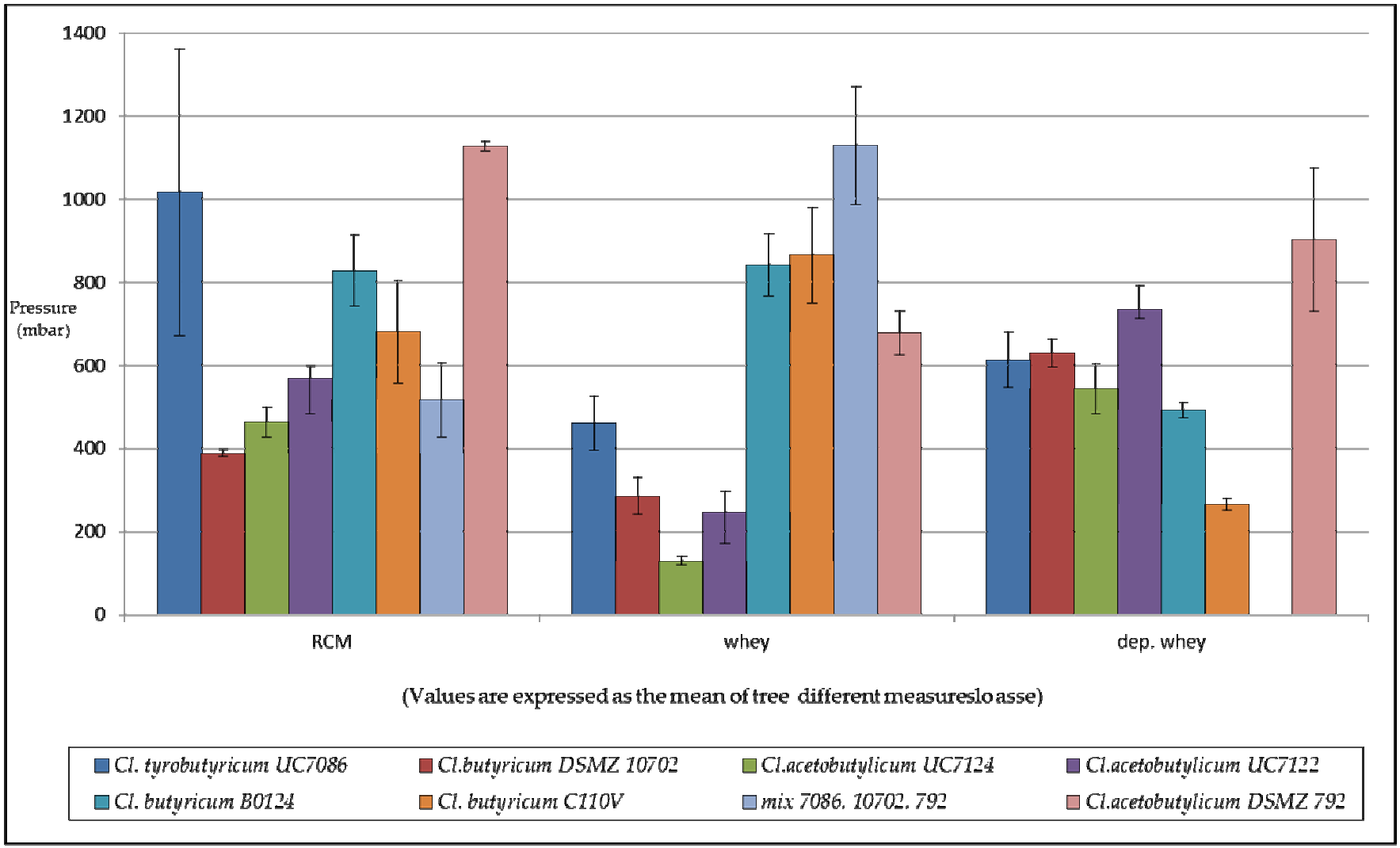
Selection of clostridial strains based on gas production. Experiments were performed by culturing the bacteria in shyntetic medium, whey, deproteinised whey



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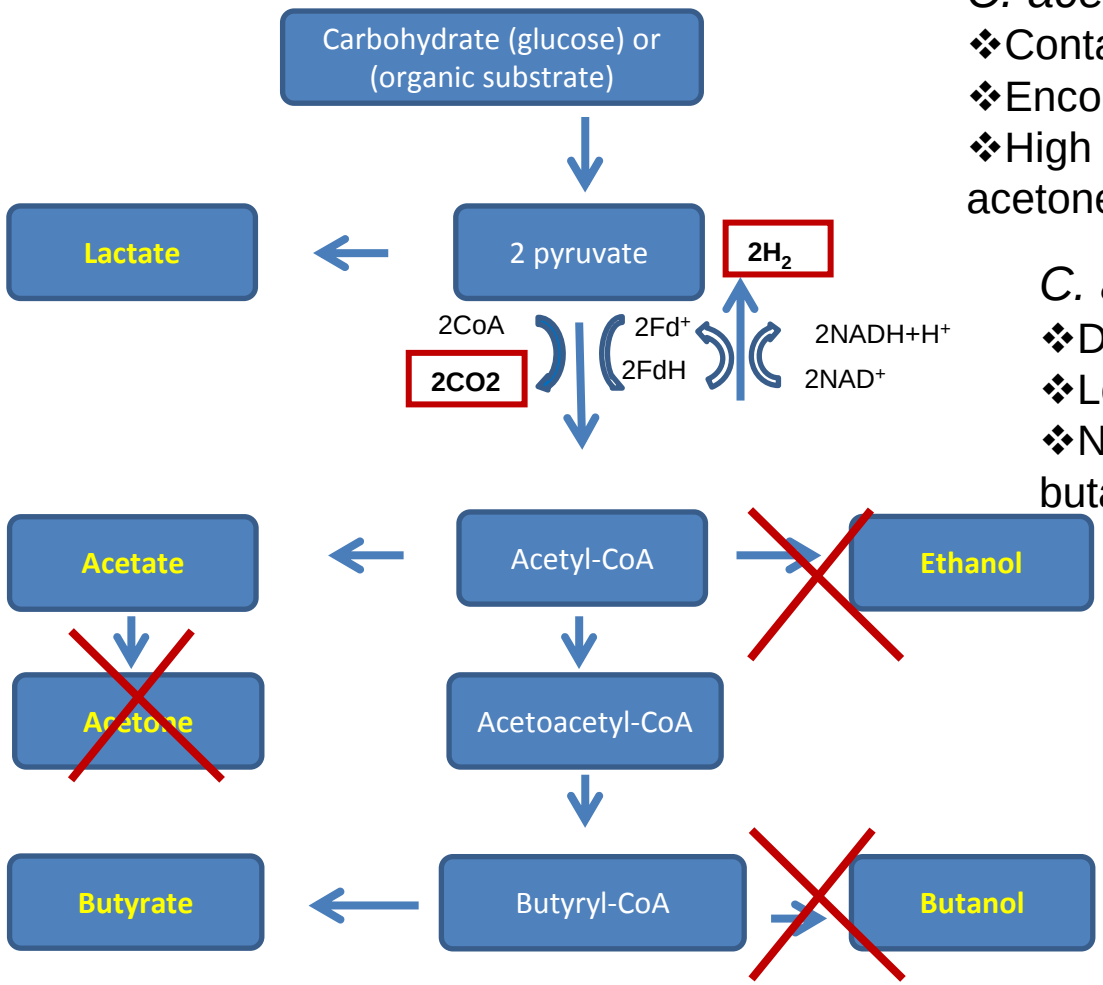
Clostridium acetobutylicum Mu56 mutant strain



C. acetobutylicum DSM792 type strain:

- ❖Contains pSOl1 megaplasmid
- ❖Encodes genes for solventogenesis
- ❖High energetic metabolic products: acetone, butanol, ethanol

C. acetobutylicum Mu56:

- ❖Degenerate strain
- ❖Loss pSol1 megaplasmid
- ❖No production of acetone and butanol

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Clostridium acetobutylicum Mu56 mutant strain

***C. acetobutylicum* Mu56**:

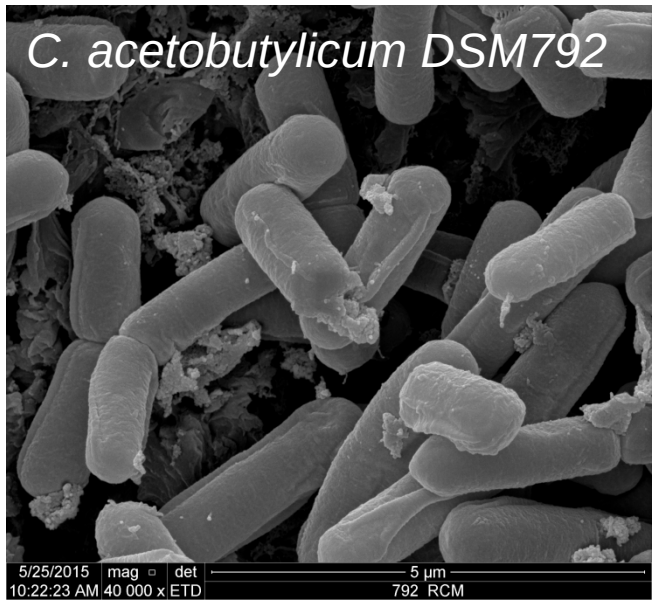
❖Loss of pSol1 was checked by aldehyde/alcohol dehydrogenase gene detection using PCR technique

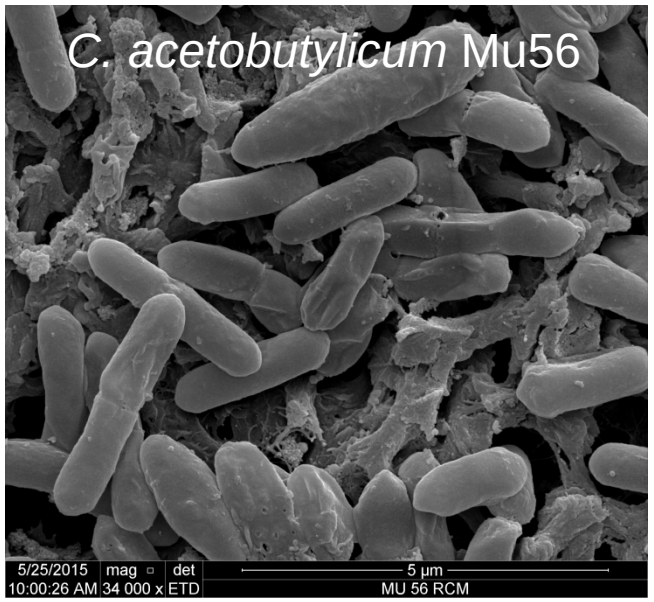
❖Southern blot was used to confirm the loss of pSOL1 megaplasmid

❖Complete genome analysis was carried out of Mu56 strain

❖production of acetone and butanol was detected

❖Hydrogen production and VFA were evaluated in *C. acetobutylicum* type strain 792 and mutant Mu56.





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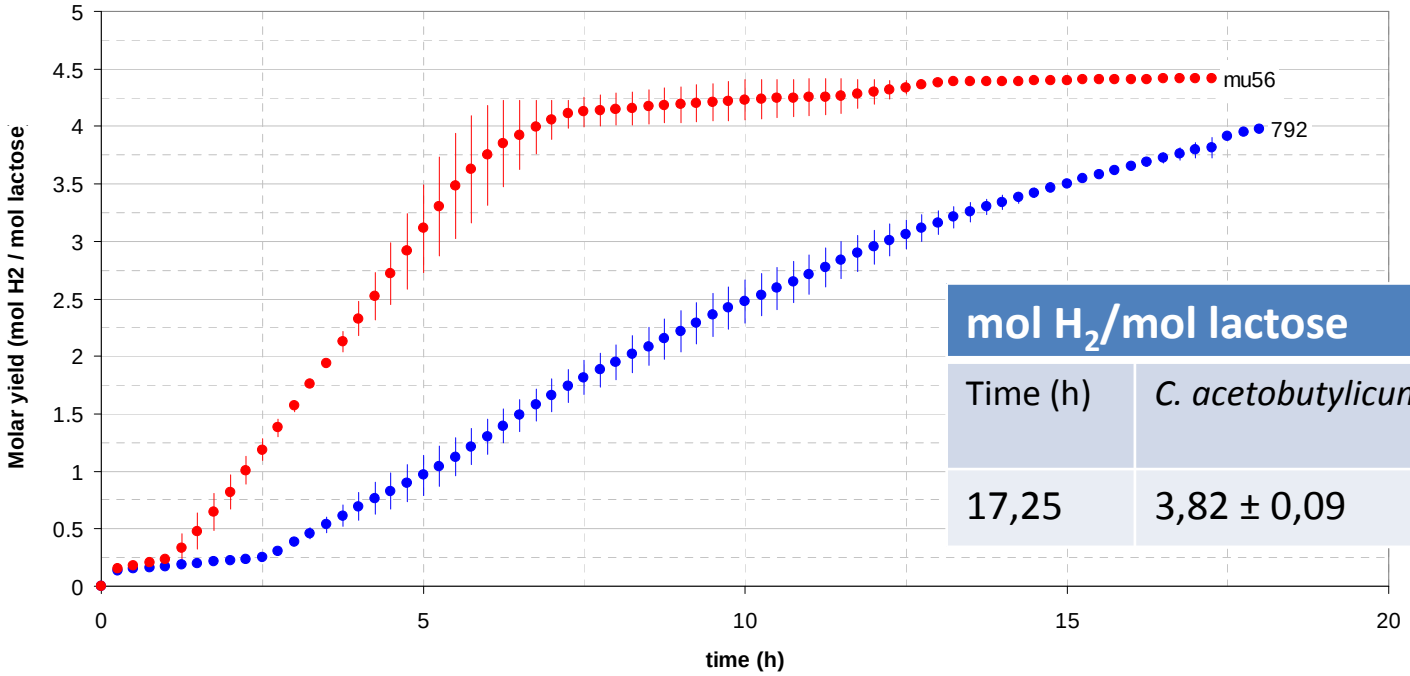
Hydrogen production in deproteinised whey
BHP analysis



mol H_2/mol lactose

Time (h)	*C. acetobutylicum* 792	*C. acetobutylicum* Mu56
17,25	3,82 ± 0,09	4,42 ± 0,01

Concentration (mmol/L)of VFA and alcohols after 48 h incubation

	Acetic	Propionic	Isobutyric	Butyric	Isovalerianic	Valerianic	Ethanol	Caproic	Butanol	Total
Mu56	73,4	0,00	0,00	39,04	0,00	0,00	0,43	0,00	0,00	112,87
DSM 792	69,38	0,00	0,00	34,58	0,00	0,00	0,80	0,00	2,18	106,93

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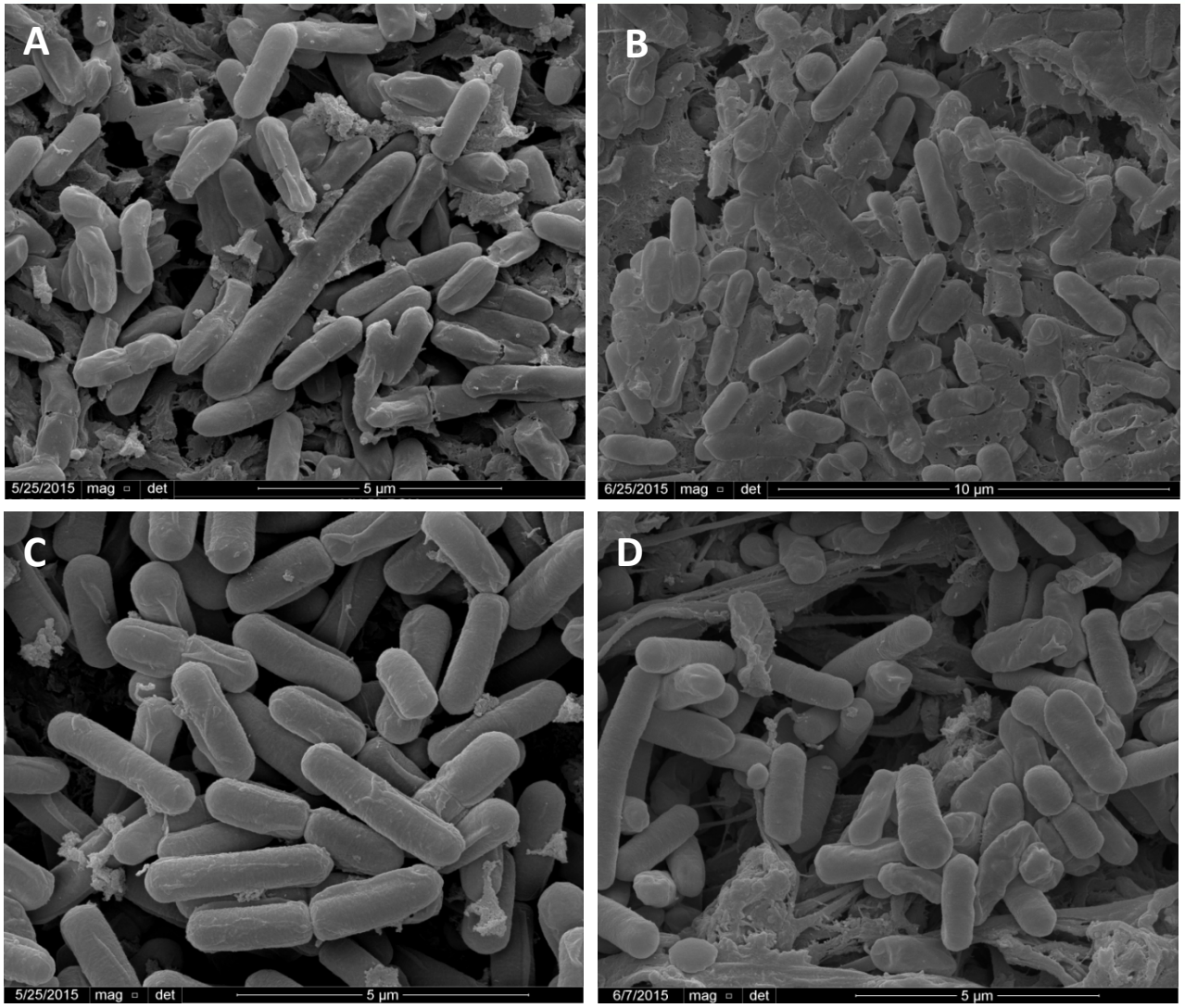
Scanning Electronic Analysis of *C. acetobutylicum* DSM792 and Mu56

A: Mu56 growth in RCM
B: Mu56 growth in whey

C: DSM792 growth in RCM
D: DSM792 growth in whey



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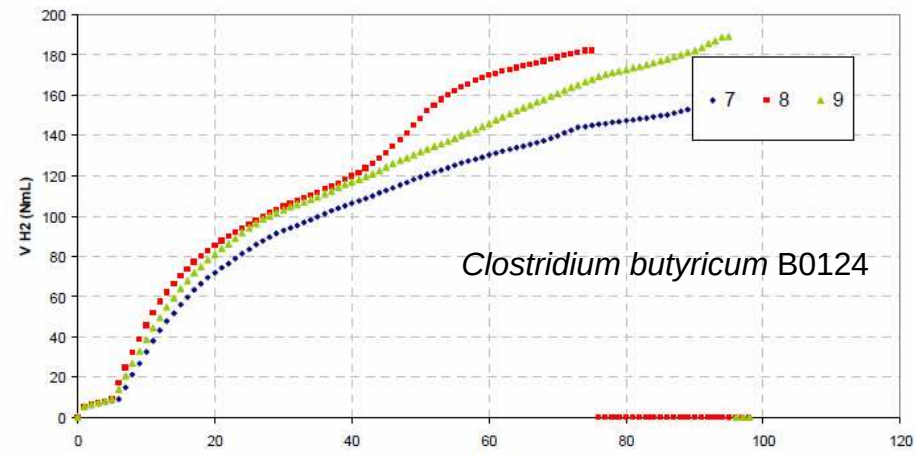
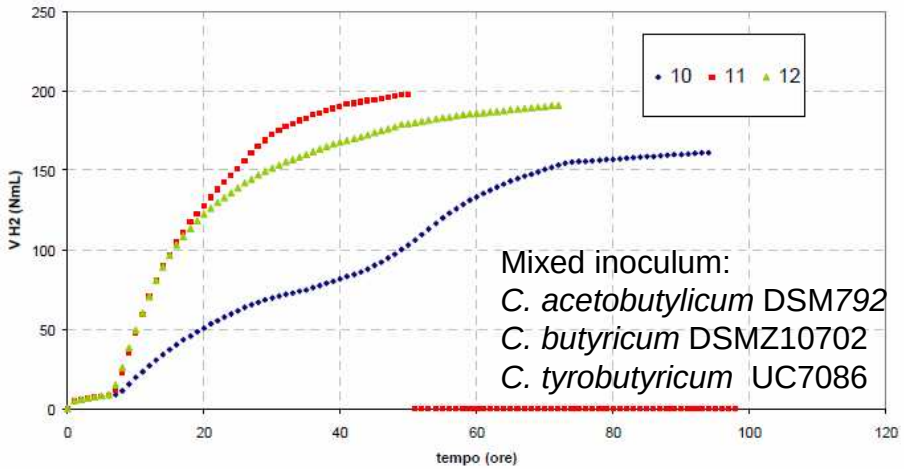
Hydrogen production in deproteinised whey
BHP analysis

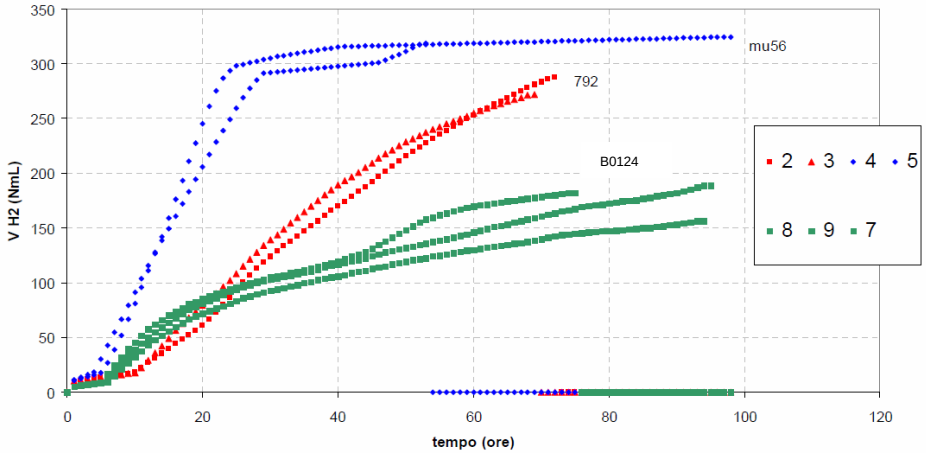


Clostridium butyricum B0124



Mixed inoculum:
C. acetobutylicum DSM*792*
C. butyricum DSMZ10702
C. tyrobutyricum UC7086

C. acetobutylicum DSM*792* (red)
C. acetobutylicum Mu56 (blue)
C. butyricum DSMZ10702(green)



Conclusions

- ✓ The Dark Fermentation is more than a promising technique for the production of bio-hydrogen that may already have the application outcomes
- ✓ The dark fermentation is a relevant process to valorize by products food or food waste
- ✓ The Clostridia are efficient producers of bio-hydrogen but research must be continued
- ✓ The Mixed culture of bacteria is a promising technique to increase the final hydrogen yield
- ✓ The natural culture for dark fermentation are a world to explore

Thanks for the attention!

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