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Hydrogen production by *Clostridium thermolacticum* during continuous fermentation of lactose

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Abstract

In the production of acetate by *Clostridium thermolacticum* growing on lactose, considerable amounts of hydrogen were generated. Lactose available in large amounts from milk permeate, a wastestream of the dairy industry, appears to be a valuable substrate for cheap production of biohydrogen.

In this study, continuous cultivation of *C. thermolacticum* was carried out in a bioreactor, under anaerobic thermophilic conditions, on minimal medium containing 10 g l^{-1} lactose. Different dilution rates and pH were tested.

C. thermolacticum growing on lactose produced acetate, ethanol and lactate in the liquid phase. For all conditions tested, hydrogen was the main product in the gas phase. Hydrogen specific production higher than 5 mmol H_2 (g cell)$^{-1}$ h^{-1} was obtained. By operating this fermentation at high-dilution rate and alkaline pH, the hydrogen content in the gas phase was maximized.

Keywords: *Clostridium*; Thermophilic; Lactose; Hydrogen; Continuous culture

1. Introduction

Hydrogen gas has high-energy content and thus would have great possibilities as a fuel if the production cost would be low enough. It is a clean fuel producing only water as combustion by-product. As fossil fuel processing and water electrolysis are rather expensive, biological production of hydrogen is potentially more attractive, especially when organic waste as a raw material can be used [1]. Biological hydrogen can be generated by several ways. Hallenbeck and Benemann described the fundamentals of biological hydrogen production: light-driven processes and dark fermentations [2]. Nandi and Sengupta reviewed the fermentative and photosynthetic biological routes of hydrogen production [3]. Wang et al. [4] reported and reviewed production of hydrogen from various wastestreams.

Cheese industry releases large amounts of milk permeate as a by-product (e.g. 150 000 tons are produced per year in Switzerland). This protein free permeate contains about 6% lactose. It is a subject of environmental concern due to its high biological oxygen demand for biodegradation of 50 g l^{-1} attributable to the lactose content [5]. Therefore, this effluent has to be treated before being released into the environment.

Clostridium thermolacticum has been shown to be an appropriate microorganism for efficient lactose fermentation and acetate production in batch culture [6]. We observed and quantified production of hydrogen as a by-product in batch [7] and continuous culture. The objective of the present study was to assess the amount of hydrogen formed during lactose fermentation in continuous culture. In this work hydrogen productivity was monitored at different dilution rates and pH values. The conditions for maximal hydrogen productivity and high hydrogen proportion in the gas phase were defined.

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2. Material and methods

2.1. Microbial species

The heterofermentative anaerobic bacterium *Clostridium stercorarium* subsp. *thermolacticum* DSM 2910 [8], formerly *C. thermolacticum* [9], was used in this study. Stock cultures were maintained in minimal medium containing lactose [10]. The cultures were stored at 4°C and sub-cultured once every month. The purity of the culture was routinely checked by microscopy.

2.2. Culture conditions and analytical techniques

The medium was prepared as described previously [10]. It contained 29 mmol l^{-1} lactose (Merck, Darmstadt, Germany) and was sterilized by filtration through a cartridge Opticap 4″, 0.22 μm pore size (Millipore, Billerica, Massachusetts) into a sterile 50 l tank (Blefa, Kreuztal, Germany). The feed tank was maintained under nitrogen pressure (121 kPa) to avoid any oxygen.

A 2 l bioreactor (Biolafitte, Princeton, New Jersey, USA) was used for these studies. The bioreactor was stirred at 100 rpm, and the temperature was maintained at 58°C. The culture volume was kept constant at 1.0 l by automatic regulation of the culture level. The reactor was autoclaved for 100 min at 121°C, prior filling with 1 l fresh medium. To create anaerobic conditions, the liquid phase of the bioreactor was purged with nitrogen. Twenty milliliters of cell suspension in the exponential phase of growth were added as inoculum. The bacteria were grown in batch for 24 h before the medium flow was started. For each dilution rate, the chemostat was allowed to stabilize until biomass, liquid and gaseous metabolites concentration were constant.

Redox potential (Orbisint CPS12 probe; Endress-Hauser, Reinach, Switzerland) and pH (Mettler-Toledo, Greifensee, Switzerland) were monitored online. The pH of the culture was maintained by automatic addition of 2 M NaOH. Liquid samples were taken out of the reactor, from the liquid exhaust tube, for biomass density determination and HPLC analysis.

Cell density in liquid medium samples was monitored by measuring turbidity at 650 nm using a U-2001 spectrophotometer (Hitachi, Tokyo, Japan). One unit of OD_{650} was found to be equivalent to 0.73 g l^{-1} cell dry weight (CDW) for *C. thermolacticum*. Lactose, lactate, acetate and ethanol were identified and quantified by HPLC on ORH-801 column (Interaction, San Jose, California, USA) at 60°C and a differential refractometer (ERC7517A, ERMA, Sopares, Gentilly, France) at 45°C. Elution was done by 5 mmol l^{-1} sulfuric acid at a flow rate of 0.6 ml min^{-1}.

The gas productivity in the fermentor was quantified using a gas flowmeter (Wohlgroth, Zurich, Switzerland) connected to the gas exhaust tube. Gaseous compounds, including H_2, N_2 and CO_2 were identified and quantified by microGC CP-4900 (Varian Inc., Palo Alto, California, USA). The column used was CP-Cox 1 m (Varian Inc., Palo Alto, California), heated at 80°C. Argon (pressure 145 kPa) was used as a carrier gas. The gas sample was pumped directly from the gas phase of the bioreactor. The injection line was loaded for 15 s with the gas to be analyzed prior to injection. Detection by a thermal conductivity detector heated at 80°C allowed detection of hydrogen concentrations as low as 10 μl l^{-1}.

2.3. Equations

The volumetric productivity, specific productivity and concentration of gas, as well as dissolved carbon dioxide and bicarbonate concentrations in the liquid phase were determined by the following equations:

productivity of gas A [11]:

$$r_A = \frac{F_{\text{gas}}\, p_{\text{A}}}{RT}, \tag{1}$$

concentration of gas A:

$$C_{\text{A}} = \frac{r_{\text{A}}}{D}, \tag{2}$$

determination of dissolved CO_2 [12]:

$$\text{CO}_2(\text{aq}) = K_{\text{H}}\, p_{\text{CO}_2}, \tag{3}$$

determination of HCO_3 concentration [12]:

$$\text{HCO}_3^-(\text{aq}) = \frac{K_1 \cdot \text{CO}_2(\text{aq})}{\text{H}^+}, \tag{4}$$

specific productivity of gas A:

$$q_{\text{A}} = \frac{r_{\text{A}}}{X}, \tag{5}$$

where C_{A} is the concentration of gas A (mol l^{-1}), D the dilution rate (h^{-1}), F_{gas} the gas volumetric flow rate (1 l^{-1} h^{-1}), K_1 the first acidity constant; at 50°C, $K_1(CO_2) = 10^{-6.28}$(mol l^{-1}), K_{H} the Henry's law constant; at 50°C, K_{H} $(CO_2) = 10^{-1.72}$(M atm^{-1}), p_{A} the partial pressure of gas A (Pa), q_{A} the specific productivity of gas A (mol (g CDW)$^{-1}$ h^{-1}), R the gas constant; 8.21×10^{-2} (l atm K^{-1} mol^{-1}), r_{A} the volumetric productivity of gas A (mol l^{-1} h^{-1}), T the temperature; 333 (K) and X the biomass cell dry weight concentration ((g CDW) l^{-1}).

3. Results

During growth of *C. thermolacticum* in chemostat culture on 29 mmol l^{-1} lactose (∼ 10 g l^{-1}), acetate was produced in the liquid phase. Besides, in the gas phase, significant hydrogen production was observed. The productivity of hydrogen was determined as a function of the dilution rate and the pH of the medium.

3.1. Continuous fermentation of lactose at different dilution rates

Dilution rates ranging from 0.012 to 0.19 h^{-1} were tested at neutral pH. Results from continuous fermentation of

Table 1
Biomass, remaining lactose and metabolites concentration for continuous cultures at steady state of *C. thermolacticum* on 29 mmol l^{-1} lactose concentration in the feed at pH 7.0 and different dilution rates. Results from four independent experiments (standard deviation < 17%)

Parameters		Dilution rate (h^{-1})								
		0.013	0.028	0.040	0.058	0.082	0.105	0.130	0.150	0.190
Cell dry weight	(g l^{-1})	0.60	0.70	0.67	0.58	0.48	0.47	0.41	0.39	0.27
Lactose rem.	(mmol l^{-1})	1	3	7	12	16	19	21	23	26
Acetate	(mmol l^{-1})	28	26	25	21	16	13	10	8	4
Ethanol	(mmol l^{-1})	41	36	33	26	20	17	13	10	5
Lactate	(mmol l^{-1})	22	17	8	1	1	1	0	0	0
Hydrogen	(mmol l^{-1})	65	78	61	44	30	22	17	14	8
Total CO_2	(mmol l^{-1})	94	93	82	65	48	43	31	27	18
Carbon balance	(%)	99	98	97	97	97	100	100	101	101
Yield H_2 on lactose	(mol mol^{-1})	2.3	3.0	2.8	2.7	2.4	2.1	2.1	2.3	2.5

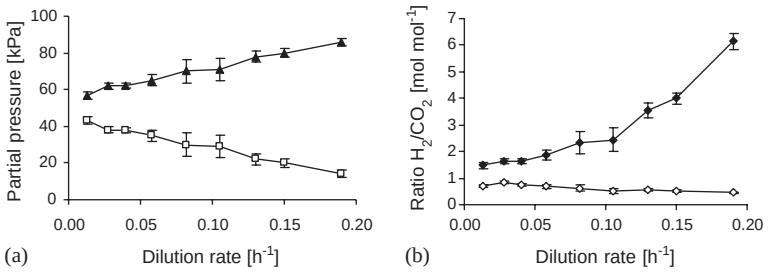

Fig. 1. Gas composition in continuous cultures of *C. thermolacticum* on 29 mmol l^{-1} lactose concentration in the feed, pH 7.0 at different dilution rates. Results from four independent experiments (standard deviation presented as error bars). (a) Hydrogen (black triangles) and carbon dioxide (open squares) partial pressure in gas phase. (b) Ratio H_2(gas)/CO_2(gas) based on H_2 productivity and CO_2 productivity measured in gas phase (black diamonds) and ratio H_2(gas)/CO_2(total) based on total CO_2 productivity, including bicarbonate in liquid phase (open diamonds).

lactose are shown in Table 1. At low-dilution rates, lactose was almost completely degraded. Biomass, acetate, ethanol and lactate were the main products in the liquid phase. The gas phase was composed of hydrogen and carbon dioxide only. Total carbon dioxide (CO_2) represents the sum of gaseous and dissolved CO_2 plus bicarbonate. It was calculated from Eqs. (1)–(4).

The composition of the gas phase and the gas productivity were determined for the different experiments. The percentage of hydrogen and carbon dioxide in the gas phase were measured by GC. The hydrogen and carbon dioxide partial pressures were deduced from these measurements done in the gas phase of the bioreactor, which was at atmospheric pressure. Fig. 1a presents the hydrogen and carbon dioxide partial pressures. The gas flowing out of the reactor had always higher content in hydrogen than in carbon dioxide. The lowest hydrogen proportion (55% v/v) was measured for the lowest dilution rates studied. The hydrogen partial pressure increased linearly with the increase of dilution rate. A maximum of 86% (v/v) hydrogen in the gas phase was achieved for $D = 0.19\ h^{-1}$.

The molar ratio H_2(gas)/CO_2(gas) was calculated based on measurement in the gas phase: it increased from 1.22 for $D = 0.013\ h^{-1}$ to 6.14 for $D = 0.19\ h^{-1}$ (Fig. 1b). It was compared with the ratio H_2(gas)/CO_2(total). It followed a different tendency compared to ratio H_2(gas)/CO_2(gas) over the range of dilution rates studied: H_2(gas)/CO_2(total) was below 1, suggesting that the total production of H_2 and CO_2 remained roughly constant, whereas H_2(gas)/CO_2(gas) increased with the dilution rate. The hydrogen volumetric productivity and hydrogen specific productivity for the different dilution rates tested are shown in Fig. 2a and b, respectively. The calculations were based on results in Fig. 1a, on the gas volumetric flow rate (ml $l^{-1}\ h^{-1}$) and on Eqs. (1) and (5). Maximal hydrogen volumetric productivity was 2.58 mmol $l^{-1}\ h^{-1}$(63 ml $l^{-1}h^{-1}$) for $D = 0.058\ h^{-1}$. The highest hydrogen productivities were measured for dilution rates ranging from 0.04 to 0.08 h^{-1}. Unlike hydrogen volumetric productivity, specific hydrogen productivity increased with dilution rate until reaching a plateau above $D = 0.08\ h^{-1}$. The highest specific productivity obtained was 5.74 mmol g $CDW^{-1}\ h^{-1}$. From

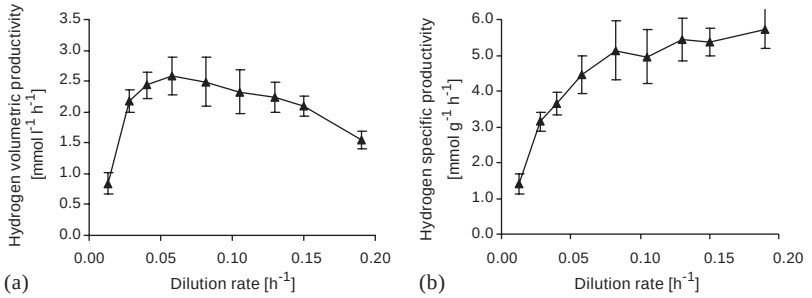

Fig. 2. Hydrogen volumetric productivity (a) and hydrogen specific productivity (b) in continuous cultures of *C. thermolacticum* on 29 mmol l^{-1} lactose concentration in the feed, pH 7.0 at different dilution rates. Results from four independent experiments (standard deviation presented as error bars).

Table 2
Effect of pH on hydrogen and carbon dioxide partial pressure and productivity in a continuous culture of *C. thermolacticum* on 29 mmol l^{-1} lactose concentration in the feed at 58°C and $D = 0.056$ h^{-1}. Results from two independent experiments (standard deviation $\leqslant$ 9%)

Parameters		pH		
		6.4	7.0	7.5
Gas volumetric flow rate	(ml l^{-1} h^{-1})	95	98	94
Cell dry weight	(g l^{-1})	0.57	0.59	0.62
H_2 partial pressure	(Pa)	53×10^3	62×10^3	78×10^3
CO_2 partial pressure	(Pa)	47×10^3	38×10^3	22×10^3
H_2 volumetric productivity	(mmol l^{-1} h^{-1})	2.06	2.48	3.00
Total CO_2 volumetric productivity	(mmol l^{-1} h^{-1})	2.80	3.90	4.89
Ratio H_2 (gas)/CO_2 (gas)	(mol mol^{-1})	1.26	1.82	3.95
Ratio H_2 (gas)/CO_2 (total)	(mol mol^{-1})	0.73	0.64	0.61

results of hydrogen and lactose concentrations, determined for each experiment (Table 1), the yield of hydrogen on lactose was calculated. The yield coefficient of hydrogen on lactose was roughly constant over the range of dilution rates tested. The maximal yield coefficient of hydrogen on lactose obtained was 3.0. Considering that lactose is a disaccharide composed of a glucose and a galactose moiety, this corresponds to a yield coefficient of hydrogen on hexose equivalent of 1.5, which is much lower that theoretical yield.

3.2. Continuous fermentation of lactose at different pH

Results from previous paragraph indicate that dilution rates between 0.04 and 0.08 h^{-1} were optimal for hydrogen productivity at neutral pH. Therefore $D = 0.056$ h^{-1} was chosen to study the effect of pH on hydrogen formation. *C. thermolacticum* has an optimum for growth at pH between 6.8 and 7.4 [9]. Thus three pH values were chosen (pH 6.4, 7.0 and 7.5). pH had a significant effect on the hydrogen partial pressure in the gas phase but also on hydrogen volumetric productivity (Table 2). For the pH range studied, hydrogen proportion was as low as 53% (v/v) at pH 6.4 and increased to a maximum of 78% (v/v) at pH 7.5. In this interval the ratio H_2(gas)/CO_2(gas) increased by three times, whereas the ratio H_2(gas)/CO_2(total) remained roughly constant. Using Eqs. (3) and (4), it could be calculated that the dissolved bicarbonate concentration increased from 12 mmol l^{-1} at pH 6.4 to 70 mmol l^{-1} at pH 7.5. This result shows that large amounts of carbon dioxide were dissolved at pH 7.5. The gas volumetric flow rate remained constant at the different pH tested. Given that the hydrogen partial pressure increased noticeably with the pH (Table 2), it was calculated that the volumetric hydrogen productivity increased with the pH, from 2.06 to 3.00 mmol l^{-1} h^{-1} (73 ml l^{-1} h^{-1}) at pH 7.5, the maximum obtained in this work.

4. Discussion

4.1. Influence of dilution rate

In the production of hydrogen by *C. thermolacticum* grown on lactose, we have shown that hydrogen was the main component of the gas phase (Fig. 1a).

Table 3
Hydrogen productivity and yield by different *Clostridium* species

Microorganism	Substrate	H_2 yield (mol mol^{-1})	H_2 productivity	Reference
Thermophilic species				
C. thermolacticum	Lactose	1.5	2.58 mmol l^{-1} h^{-1} 5.74 mmol(g CDW)$^{-1}$ h^{-1}	This study
C. thermoalcaliphilum	Glucose	1.6		[28]
C. thermobutyricum	Glucose	1.9		[29]
C. thermohydrosulfuricum	Glucose	0.55		[30]
C. thermosaccharolyticum	Glucose	1.72[a]		[31]
		1.22[b]		[31]
C. thermosuccinogenes	Glucose	0.25		[20]
C. thermosulfurigenes	Glucose	0.95		[32]
Mesophilic species				
Clostridium sp. strain X53	Xylan		240 ml l^{-1} h^{-1}	[3]
Clostridium sp. strain No.2	Glucose		20.4 mmol l^{-1} h^{-1}	[23]
	Xylose		21.03 mmol l^{-1} h^{-1}	
C. acetobutylicum	Glucose	1.97	11.3 mmol g^{-1} h^{-1}	[25]
	Glycerol–Glucose	0.42	0.39 mmol g^{-1} h^{-1}	
C. butyricum	Glucose		26 mmol l^{-1} h^{-1}	[26]
	Glucose–Polypeptone	1.4–2.0		[27]
C. paraputrificum M-21	Glucose	1.4		[22]
	Acetylglucosamine	2.4		
C. beijirincki AM21B	Glucose	1.8–2.0		[3]
C. cellobioparum	Glucose	2.73		[18]
C. pasteurianum	Glucose	1.5		[3]
Clostridium sp.	Glucose	0.85	124.8 ml l^{-1} h^{-1}	[1]

[a] $D = 0.1$ h^{-1}, pH=5.4.
[b] $D = 0.03$ h^{-1}, pH = 7.0.

It can be clearly seen, in Fig. 1b, that the ratio $H_2(gas)/CO_2(total)$ remained stable, whereas the ratio $H_2(gas)/CO_2(gas)$ increased with the dilution rate. It appears that all carbon dioxide produced was not entirely released into the gas phase. It can be assumed that a fair amount remained dissolved as carbonic acid and bicarbonate ions in the liquid phase. Indeed, it is known that CO_2 produced by the cells enters the medium in a dissolved form [13].

In our work, as dilution rate increased, medium was renewed faster in the bioreactor, bicarbonate ions were thus continuously washed out, and lower amounts of CO_2 diffused to the gas phase. This could explain the decrease of the CO_2 proportion in the gas phase as dilution rate increased at constant pH. Therefore, the hydrogen proportion in the gas phase increased by working at higher dilution rate.

4.2. Effects of pH

As pH became more alkaline, the proportion of hydrogen in the gas phase increased, as illustrated by the ratio $H_2(gas)/CO_2(gas)$ in Table 2. This difference could be explained by the increase of bicarbonate ions (HCO_3; pK_a = 6.35) [12] formation, as shown in Section 3.2: when pH became more alkaline, the equilibrium was shifted towards the formation of bicarbonate ions. Thus lower amounts of CO_2 diffused to the gas phase. Similar results were obtained by Desvaux et al. with *C. cellulolyticum* [14]. Frick and Junker [15] obtained similar results on pilot scale fermentation and suggested that levels of weak acids (acetate, lactate) and media buffer significantly modify the equilibrium relationship between gaseous CO_2, dissolved CO_2 and bicarbonate.

An increase in hydrogen volumetric productivity was observed when pH was more basic (Table 2). Lee et al. [16] studied hydrogen gas formation from sucrose at different pH. They also reported that hydrogen productivity increases as pH became alkaline.

As a consequence, operating lactose biotransformation at alkaline pH allows both enrichment of the gas phase in hydrogen, from 53 to 78 kPa, and increase of hydrogen volumetric productivity from 2.06 to 3.00 mmol l^{-1} h^{-1}.

4.3. Relationship between ethanol production and hydrogen yield

Degradation of carbohydrate into acetate should yield formation of four hydrogen, if no ethanol is produced [17].

Thus lactose fermentation could be written

$$C_{12}H_{22}O_{11} + 5H_2O \rightarrow 8H_2 + 4CO_2 + 4CH_3COOH. \quad (6)$$

Based on this equation, the theoretical yield of hydrogen on lactose can achieve 8 mol mol^{-1}, corresponding to 4 mol mol^{-1} per hexose. However, the highest yield found in the literature for thermophilic *Clostridium* species was 1.9, for *C. thermobutyricum* growing on glucose (Table 2). In our study, the growth of *C. thermolacticum* on lactose yielded a maximum of 3.0 mol hydrogen per mol lactose (1.5 mol hydrogen per mol hexose). Changes in dilution rate did not affect this yield significantly. Hydrogen partial pressure was 55 kPa and increased with increasing dilution rate (Fig. 1). In other studies, it was described that elevated hydrogen partial pressure prevented the hydrogen production by the bacteria and modified the metabolite pattern towards the production of reduced end-products like ethanol [18,19]. Therefore, it appears that the presence of hydrogen in high concentration could be responsible for reduced hydrogen productivity.

The fact that ethanol was obtained in similar proportion as acetate for all dilution rates tested (Table 1) explains the decrease of hydrogen yield on lactose to less than 4 mol mol^{-1}: NADH used for ethanol production was not available for hydrogen generation. The hydrogen yield could have been 8 mol mol^{-1} theoretically in absence of ethanol formation. Therefore, in case of hydrogen inhibition and associated ethanol production, Eq. (6) could be rewritten

$$C_{12}H_{22}O_{11} + 3H_2O \rightarrow 4H_2 + 4CO_2 + 2CH_3COOH + 2CH_3CH_2OH. \quad (7)$$

4.4. Hydrogen productivity by other organisms on different substrates

Results of hydrogen productivity by other *Clostridium* species are listed in Table 3. Most of the studies were carried out on glucose as a substrate. However it has been reported that many other organic substrates, such as inulin [20], sucrose [16,21], acetylglucosamine and chitin [22], wastestreams containing xylose [23], lignocellulosic waste [24] and even wastewater sludge [4], are potential sources for hydrogen production. According to our knowledge, lactose as the sole carbon source for hydrogen production has never been reported.

5. Conclusion

The elimination of lactose by fermentation using *C. thermolacticum* for acetate production generates biohydrogen as a valuable by-product. At high-dilution rate (0.10–0.20 h^{-1}) and pH above 7.0, the hydrogen content of the gas phase and the specific hydrogen productivity were maximized. Remaining carbon dioxide could be removed using a CO_2-trap. This opens new perspectives for the valorization of the huge amounts of milk permeate, formed by the cheese industry, containing lactose as a free of charge substrate. This wastestream could thus be used for production of both acetate and cheap biohydrogen.

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References

[1] Mizuno O, Dinsdale R, Hawkes FR, Hawkes DL, Noike T. Enhancement of hydrogen production from glucose by nitrogen gas sparging. Biores Technol 2000;73:59–65.
[2] Hallenbeck P, Benemann J. Biological hydrogen production; fundamentals and limiting processes. Int J Hydrogen Energy 2002;27:1185–93.
[3] Nandi R, Sengupta S. Microbial production of hydrogen: an overview. Crit Rev Microbiol 1998;24(1):61–84.
[4] Wang CC, Chang CW, Chu CP, Lee DJ, Chang BV, Liao CS. Producing hydrogen from wastewater sludge by *Clostridium bifermentans*. J Biotechnol 2003;102(1):83–92.
[5] Kisaalita WS, Lo KV. Kinetics of whey-lactose acidogenesis. Biotechnol Bioeng 1989;33:623–30.
[6] Talabardon M, Schwitzguébel JP, Péringer P. Anaerobic thermophilic fermentation for acetic acid production from milk permeate. J Biotechnol 2000;76(1):83–92.
[7] Collet C, Schwitzguébel J-P, Péringer P. Improvement of acetate production from lactose by growing *Clostridium thermolacticum* in mixed batch culture. J Appl Microbiol 2003;95(4):824–31.
[8] Fardeau ML, Ollivier B, Garcia JL, Patel BK. Transfer of *Thermobacteroides leptospartum* and *Clostridium thermolacticum* as *Clostridium stercorarium* subsp. *leptospartum* subsp. nov., comb. nov. and *C. stercorarium* subsp. *thermolacticum* subsp. nov., comb. nov. Int J Syst Evol Microbiol 2001;51:1127–31.
[9] Le Ruyet P, Dubourguier HC, Albagnac G. Characterization of *Clostridium thermolacticum* sp.nov., a hydrolytic thermophilic anaerobe producing high amounts of lactate. Syst Appl Microbiol 1985;6(2):196–202.
[10] Talabardon M, Schwitzguébel JP, Péringer P, Yang ST. Acetic acid production from lactose by an anaerobic thermophilic coculture immobilized in a fibrous-bed bioreactor. Biotechnol Prog 2000;16(6):1008–17.
[11] Atkins PW. Physical chemistry. Oxford: Oxford University Press; 1994.
[12] Stumm W, Morgan JJ. Aquatic chemistry : an introduction emphasizing chemical equilibria in natural waters. New York: Wiley Science; 1981.
[13] Jones RP, Greenfield PF. Effect of carbon dioxide on yeast growth and fermentation. Enzyme Microbiol Technol 1982;4:210–23.

[14] Desvaux M, Guedon E, Petitdemange H. Metabolic flux in cellulose batch and cellulose-fed continuous cultures of *Clostridium cellulolyticum* in response to acidic environment. Microbiology 2001;147:1461–71.
[15] Frick R, Junker B. Indirect methods for characterization of carbon dioxide levels in fermentation broth. J Biosci Bioeng 1999;87(3):344–51.
[16] Lee YJ, Miyahara T, Noike T. Effect of pH on microbial hydrogen fermentation. J Chem Technol Biotechnol 2002;77(6):694–8.
[17] Jungermann K, Thauer RK, Leimenstoll G, Decker K. Function of reduced pyridine nucleotide-ferredoxin oxidoreductases in saccharolytic Clostridia. Biochim Biophys Acta 1973;305(2):268–80.
[18] Chung KT. Inhibitory effects of H_2 on growth of *Clostridium cellobioparum*. Appl Environ Microbiol 1976;31(3):342–8.
[19] de Corte B, Dries D, Verstraete W, Stevens P, Goossens L, de Vos P, de Ley J. The effect of the hydrogen partial pressure on the metabolite pattern of *Lactobacillus casei*, *Escherichia coli* and *Clostridium butyricum*. Biotechnol Lett 1989;11(8): 583–8.
[20] Sridhar J, Eiteman MA, Wiegel JW. Elucidation of enzymes in fermentation pathways used by *Clostridium thermosuccinogenes* growing on inulin. Appl Environ Microbiol 2000;66(1):246–51.
[21] Chen CC, Lin CY, Chang JS. Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. Appl Microbiol Biotechnol 2001;57(1-2):56–64.
[22] Evvyernie D, Morimoto K, Karita S, Kimura T, Sakka K, Ohmiya K. Conversion of chitinous wastes to hydrogen gas by *Clostridium paraputrificum* M-21. J Biosci Bioeng 2001;91(4):339–43.
[23] Taguchi F, Mizukami N, Taki TS, Hasegawa K. Hydrogen production from continuous fermentation of xylose during growth of *Clostridium* sp. strain 2. Can J Microbiol 1995;41:536–40.
[24] Sparling R, Risbey D, Poggi-Varaldo HM. Hydrogen production from inhibited anaerobic composters. Int J Hydrogen Energy 1997;22(6):563–6.
[25] Saint-Amans S, Girbal L, Andrade J, Ahrens K, Soucaille P. Regulation of carbon and electron flow in *Clostridium butyricum* VPI 3266 grown on glucose–glycerol mixtures. J Bacteriol 2001;183(5):1748–54.
[26] van Andel JG, Zoutberg GR, Crabbendam PM, Breure AM. Glucose fermentation by *Clostridium butyricum* grown under a self generated gas atmosphere in chemostat culture. Appl Microbiol Biotechnol 1985;23:21–6.
[27] Kataoka N, Miya A, Kiriyama K. Studies on hydrogen production by continuous culture system of hydrogen-producing anaerobic bacteria. Water Sci Technol 1997;36(6-7):41–7.
[28] Li YH, Engle M, Weiss N, Mandelco L, Wiegel J. *Clostridium thermoalcaliphilum* sp. nov., an anaerobic and thermotolerant facultative alkaliphile. Int J Syst Bacteriol 1994;44(1):111–8.
[29] Wiegel J, Kuk SU, Kohring GW. *Clostridium thermobutyricum* sp. nov., a moderate thermophile isolated from a cellulolytic culture, that produces butyrate as the major product. Int J Syst Bacteriol 1989;39(2):199–204.
[30] Lovitt RW, Shen GJ, Zeikus JG. Ethanol production by thermophilic bacteria: biochemical basis for ethanol and hydrogen tolerance in *Clostridium thermohydrosulfuricum*. J Bacteriol 1988;170(6):2809–15.
[31] Vancanneyt M, de Vos P, Vennens L, de Ley J. Lactate and ethanol dehydrogenase activities in continuous cultures of *Clostridium thermosaccharolyticum* LMG 6564. J Gen Microbiol 1990;136:1945–51.
[32] Schink B, Zeikus JG. *Clostridium thermosulfurogenes* sp. nov., a new thermophile that produces elemental sulphur from thiosulphate. J Gen Microbiol 1983;129:1149–58.