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Influence of culture parameters on biological hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC 27021

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Summary

Various medium components (carbon and nitrogen sources, iron, inoculum size) and environmental factors (initial pH and the agitation speed) were evaluated for their effects on the rate and the yield of hydrogen production by *Clostridium saccharoperbutylacetonicum*. Among the carbon sources assessed, cells grown on disaccharides (lactose, sucrose and maltose) produced on the average more than twice (2.81 mol-H_2/mol sugar) as much hydrogen as monosaccharides (1.29 mol-H_2/mol sugar), but there was no correlation between the carbon source and the production rate. The highest yield (2.83 mol/mol) was obtained in lactose and sucrose but the highest production rate (1.75 mmol/h) in sucrose. Using glucose as carbon source, yeast extract was the best nitrogen source. A parallel increase between the production rate and the yield was obtained by increasing glucose concentration up to 40 g/l (1.76 mol-H_2/mol, 3.39 mmol/h), total nitrogen as yeast extract up to 0.1% (1.41 mol/mol, 1.91 mmol/h) and agitation up to 100 rev/min (1.66 mol-H_2/mol, 1.86 mmol/h). On the other hand, higher production rates were favoured in preference to the yield at a neutral initial pH 7 (2.27 mmol/h), 1000 mg iron/l or more (1.99 mmol/h), and a larger inoculum size, 10%, (2.36 mmol/h) whereas an initial alkaline pH of 8.5 (1.72 mol/mol), a lower iron concentration of 25 mg/l (1.74 mol/mol) and smaller inoculum size, 1%, (1.85 mol/mol) promoted higher yield over production rate.

Introduction

The trend of global environmental goals of lower emissions and carbon dioxide reduction, and depletion of known crude oil reserves have intensified the development of non-polluting or clean energy carriers from renewable resources. Among recognized alternatives to fossil fuels, hydrogen is regarded as a clean energy carrier because water is the only product of its combustion. Moreover, its energy value of 231 BTU/mol (244 kJ/mol) is higher than that of any hydrocarbon. Although it can be generated from both renewable and non-renewable resources, more than 96% of the H_2 produced worldwide depends on fossilized energy in one form or another (Wunschiers & Lindblad 2002). By the year 2002, producers were generating about 45 million tonnes of H_2 from fossil fuel (Gorman 2002). The predominant production process of steam reforming (SR) relies heavily on natural gas, a non-renewable feedstock. SR is also an integral part of other major H_2 generation processes being developed such as coal or biomass gasification, biomass pyrolysis and partial oxidation of hydrocarbons. Although it is an efficient H_2 generation process, SR is also capital and energy-intensive. Moreover, large quantities of CO_2 are produced alongside H_2 and as a result the benefits of H_2 energy are diminished. Electrolysis of water to produce H_2 provides a cleaner alternative but, at present, it is not only more expensive than SR processes but it also relies on fossilized energy for operation.

In contrast, microbial production of H_2, also referred to as biohydrogen production, is a much less capital and energy-intensive process. In particular, H_2 production by anaerobic fermentation is technically much simpler and can generate H_2 from a large a number of carbohydrate materials obtained in refuse and waste products (Nandi & Sengupta 1998). It is superior in terms of the rate of production to photosynthetic H_2 production; and it can also make use of the existing infrastructure for biogas and fermentative acetone-butanol production without elaborate adjustments (Das & Nejat Veziroglu 2001; Van Groenestijn *et al.* 2002).

Recent reports on anaerobic H_2 production have involved the use of mixed anaerobic consortia; some of

these studies merely enriched for H_2-producing spore-formers through heating (Oh *et al.* 2003) or by pH adjustment to kill the hydrogenotrophic bacteria (Chen *et al.* 2002). Subsequent analysis of the microbial species in some of these consortia and their soluble metabolic products has revealed *Clostridium* as the dominant anaerobic genus in these cultures (Chen *et al.* 2002; Lin & Chang 2004a; Dinopoulou *et al.* 1988). Clostridia have the capacity to utilize a wide variety of sugars, thus broadening the spectrum of potential carbon sources for H_2 production. Optimizing fermentation conditions that favour clostridial growth should therefore, essentially enhance H_2 production. However, what controls the mechanisms of clostridial H_2 production has not been determined in detail, since the culture conditions that affect H_2 production are only beginning to be ascertained (Logan *et al.* 2002). To date, studies have shown that iron availability, H_2 partial pressure, substrate concentration, temperature (Zhang *et al.* 2003), and the carbon-to-nitrogen ratio (Lin & Lay 2004b) all in some way affect the H_2-producing potential of a microorganism. Nonetheless, most clostridia produce both organic solvents (alcohols and ketones) and acids. Since alcohol production involves the consumption of H_2 in the form reducing equivalents such as NADH, it is inevitable that fermentation conditions that favour the metabolism of sugar to alcohols reduce H_2 production.

Clostridium saccharoperbutylacetonicum ATCC 27021 is a known alcohol-producing microorganism. A number of studies have been conducted using this microorganism for fermentative acetone, butanol and ethanol (ABE) production from defined media (Sirisansaneeyakul *et al.* 1998; Ishizaki *et al.* 1999; Crabbe *et al.* 2001), biomass (Crabbe *et al.* 1998) and biological waste (Bujang *et al.* 1997; Ishizaki *et al.* 1998). Its hydrogen-producing potential, however, has not been reported. Hence this study was conducted to investigate the influence of culture parameters on H_2 production by the microorganism.

Materials and methods

Microorganisms, maintenance and growth conditions

Clostridium saccharoperbutylacetonicum ATCC 27021 was used in this study. The microorganism was grown and maintained in peptone-yeast extract (PY) medium at −78 °C. The PY medium contained per litre of deionized water: tryptone (FisherBiotech, Fair Lawn, NJ), 5.0 g; Bacto peptone (BD, Sparks, MD), 5.0 g; yeast extract (FisherBiotech), Fair Lawn, NJ), 10.0 g; glucose, 5.0 g; Bacto beef extract (BD, Sparks, MD), 5.0 g; Tween 80, 1.0 ml; K_2HPO_4, 2.0 g; salt solution, 40.0 ml (per litre: $CaCl_2{\cdot}2H_2O$, 0.25 g; $MgSO_4{\cdot}7H_2O$, 0.50 g; K_2HPO_4, 1.0 g; KH_2PO_4, 1.0 g; $NaHCO_3$, 10.0 g; NaCl, 2.0 g); haemin solution, 10.0 ml (per litre: 0.5 g haemin, 10 ml 1 M NaOH); 0.5% vitamin K_1, 200 l (per litre: 5 ml vitamin K_1, 970 ml 95% ethanol); and L-cysteine·HCl.H_2O, 0.5 g. Tryptone-yeast extract (TY) medium served as the basal medium for H_2 fermentation studies. It contained the following components per litre of deionized water: tryptone, 6 g; yeast extract, 2 g; K_2HPO_4, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.3 g; and $FeSO_4{\cdot}7H_2O$, 50 mg. Unless stated, the total amount of carbon source in the medium was 20 g/l. Prior to inoculation the pH of the medium was adjusted to 7.5 with 5 M KOH and sterilized at 121 °C for 15 min. The microorganism was activated for fermentation by transferring 1 ml of the stock culture into 10 ml of fresh PY medium followed by anaerobic incubation with the aid of a H_2 and CO_2 generator in a BBL Gaspak jar (BD, Sparks, MD) at 30 °C. Overnight cultures were subcultured in fresh PY medium for 6–9 h and used as inoculum.

Fermentation experiments

Experiments were conducted to determine the effect of the type of sugar on H_2 production. The sources were glucose and fructose as monosaccharides with lactose, maltose and sucrose serving as disaccharides. Subsequent experiments were conducted using glucose as carbon source to determine the effects of other culture parameters on H_2 production. These were glucose concentration (10–60 g/l), initial pH (5–9), nitrogen source and concentration, inoculum size (1–10%), iron concentration (0–2000 mg/l), and agitation speed (0–400 rev/min).

Experimental set-up and H_2 quantification

Batch fermentation was carried out at a working volume of 200 ml in 250 ml media bottles at 30 °C in the respirometer (Challenge Environmental Systems, AK, USA). Unless stated, each medium was seeded with a 5% inoculum and sparged with nitrogen gas (99.9%). All the bottles were tightly closed with open-top screw caps each fitted with a butyl septum. With the exception of the study on the effect of agitation speed, the cultures were agitated at 50 rev/min. The culture pH was not controlled during fermentation. To quantify H_2, gas produced during fermentation was continuously channelled through a needle inserted through the butyl septum into a 30% KOH solution (100 ml) contained in a 150 ml bottle, inserted inline, which stripped CO_2 from the gas stream. The residual gas, essentially H_2, was then channelled into a bubble counter for the measurement of H_2. To ensure that the 30% KOH solution did indeed remove CO_2 from the fermentation gas stream, the H_2 content of the residual gas was also measured with a H_2 sensor (H_2Scan DCH, CA, USA). Each bubble counter was pre-calibrated and linked through an interface to a computer. Therefore, the volume of H_2 measured at any given time by a bubble counter was computed as real-time volumetric data and continuously logged in the computer.

Reproducibility and statistical analysis

All the results presented in this work are the average of a minimum of duplicate experiments. The significance was reported at $P < 0.05$ using the *t*-test.

Analytical methods

Cell density was measured spectrophotometrically as optical density at 600 nm immediately after gas production ceased. Glucose concentration was estimated by the glucose oxidase-peroxidase enzymatic method using the Sigma Diagnostic kit procedure 510 (Sigma Chemical Co., MO, USA). All other sugars were assayed by the dinitrosalicylic acid (DNS) method described by Miller (1959); for disaccharides, 1.5 ml cell-free samples were first pretreated with 100 μl of concentrated hydrochloric acid and boiled for 5 min. The resulting solution was neutralized with 300 μl of 5 M KOH and used for sugar analysis.

Results and discussion

Effects of carbon source and carbon concentration

Five carbon sources, consisting of two monosaccharides, glucose and fructose, and three disaccharides, lactose, sucrose and maltose, were evaluated for their effect on the yield and rate of hydrogen production. All five sugars were nearly completely (> 99%) consumed in H_2 production (Table 1). The highest yield (2.83 mol/mol) was obtained in lactose and sucrose but the least in fructose (1.24 mol/mol). The highest production rate of 1.75 mmol/h was obtained in sucrose and the least, 10.8 mmol/h, from lactose. However, differences were observed in the yield and the H_2 production rate based on the type of sugar used. Disaccharides, on the average, produced twice as much H_2 (2.81 mol/mol) as monosaccharides (1.29 mol/mol), whereas the average production rate obtained from the monosaccharides (1.35 mmol/h) was slightly higher than that of a disaccharide (1.31 mmol/h). These results are higher than the respective yields of 0.98 mol/mol-glucose (Oh *et al.* 2003) and 1.8 mol/mol-sucrose (Logan *et al.* 2002) obtained for a mixed microbial culture, which indicated that mixed microbial cultures did not produce H_2 with any greater efficiency than pure cultures. Furthermore, the carbon sources used in this study are the types of sugar commonly found in food and dairy waste. These industries generate waste with high chemical oxygen demand making treatment and disposal very difficult. As the results indicate these sugars could serve as carbon sources for H_2 production. This is important because converting food or dairy waste into hydrogen provides an alternative means of alleviating the problem and reducing pollution.

To investigate the effect of substrate concentration, glucose was used as the sole carbon source. As shown in Figure 1, H_2 production increased with increasing glucose concentration. Although the highest H_2 production was obtained in 60 g glucose/l, the H_2 yield increased from 1.28 mol/mol in 10 g glucose/l up to 1.76 mol/mol in 40 g glucose/l then decreased to 1.60 mol/mol in 60 g glucose/l. A similar trend was observed between the substrate concentration and the H_2 production rate. The highest production rate of 3.39 mmol/h was also obtained in 40 g glucose/l. The reductions in the H_2 yield and production rate in 60 g glucose/l were attributed to a shift from the acidogenic to the solventogenic pathways where H_2 was consumed to reduce the acids to alcohols (Van Ginkel *et al.* 2001). Wu & Lin(2004) observed a similar trend between the yield and molasses concentration, in which the highest yield was found at a substrate concentration of 40 g COD/l. This trend, however, differed from that of a steady decrease in the H_2 yield with increasing substrate concentration observed for starch in starch wastewater (Zhang *et al.* (2003) and for cellulose (Lay 2001). It was also noted that investigators have reported H_2 yield as mol H_2 per mol substrate, mol H_2 per gramme substrate

Table 1. Effect of various sugars on hydrogen production from by *Clostridium saccharoperbutylacetonicum* ATCC 27021.

[a]Carbon source	H_2 production				
	Residual (g/l)	Duration (h)	Volume (ml)	Yield[b] (mol/mol)	Rate (mmol/h)
Type					
Glucose	0.60	20.0	657.4	1.37	1.47
Fructose	0.67	20.7	575.6	1.20	1.24
Lactose	0.57	29.6	716.5	2.82	1.08
Sucrose	0.15	18.7	734.8	2.83	1.75
Maltose	0.66	28.4	703.0	2.77	1.10

[a] Initial concentration of each carbon source, 20 g sugar/l.
[b] Yield is expressed as mole H_2 per mole sugar.

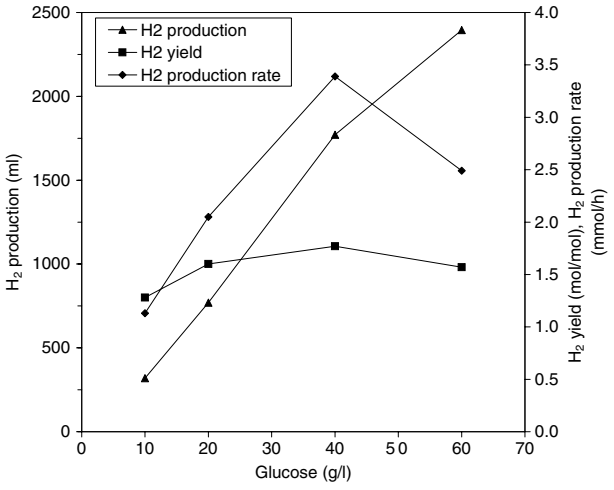

Figure 1. Effect of glucose concentration on the rate and the yield of H_2 production. Results from duplicate run. Symbols: Volume produced, (▲); yield, (■); production rate (◆).

or H_2 produced (ml) per gramme substrate; hence, for ease of comparison with values reported, the H_2 yields were all converted to H_2 produced (ml) per gramme substrate. On that basis, it was found that the maximum yield of 1.76 mol/mol-glucose (219 ml/g-glucose) obtained in this study was higher than the reported values of 132 ml/g glucose (Oh *et al.* 2003), 92 ml/g-starch in starch wastewater (Zhang *et al.* 2003), 193 ml/g-cellulose (Ueno *et al.* 2001), 134 ml/g-molasses (Logan *et al.* 2002), 126 ml/g-sucrose (Lee *et al.* 2002) but less than 314 ml/g-sucrose reported by Lin & Lay (2004b). This indicated that *C. saccharoperbutylacetonicum* is one of the most efficient hydrogen producers.

Table 2. Effect of initial pH on hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC 27021.

PH		H_2 production			
Initial	Final	Duration (h)	Volume (ml)	Yield[a] (mol/mol)	Rate (mmol/h)
5.0	5.03	24.0	0.0	0.00	0.00
6.0	5.95	16.5	367.8	0.74	1.00
6.5	6.10	15.2	650.6	1.31	1.91
7.0	6.05	15.4	784.2	1.58	2.27
7.5	6.04	16.3	708.6	1.43	1.88
8.0	5.95	16.9	699.0	1.40	1.85
8.5	5.71	19.1	859.2	1.72	2.00

[a] Yield is expressed as mole H_2 per mole glucose.
Initial glucose, 20 g/l.

Effect of initial pH

The effect of the initial pH of the medium on H_2 production was investigated by varying the pre-sterilization pH between 5 and 8.5. As shown in Table 2, increasing the pH at intervals of 0.5 units affected H_2 production. Between pH 6 and 8.5, H_2 production was enhanced by every 1 unit increase in pH. While the highest yield, 1.72 mol/mol, was obtained at pH 8.5, the highest production rate of 2.27 mmol/h was observed at pH 7, but there was no clear correlation between initial pH and the H_2 yield or the production rate between pH 7 and 8.5. This suggested that a high production rate did not necessarily translate into a high yield. However, no H_2 production was observed at pH 5 because the organism did not grow whereas production at pH 6 was low, indicating that H_2 production was favoured by a neutral to slightly alkaline initial pH. The optimal pH range of 7–8.5 for H_2 production observed in this study was higher than values reported for similar studies of pH 6.2 using glucose and a mixed culture (Oh *et al.* 2003), pH 5.5–6.0 using sucrose and a heat-treated inoculum (Van Ginkel *et al.* 2001) and pH 6–7 using molasses and a mixed culture (Wu & Lin 2004). They are however in agreement with results which showed that H_2 production by *Clostridia* is completely inhibited below a pH range of 4 to 5 (Bahl *et al.* 1986; Roychowdhury *et al.* 1988; Dabrock *et al.* 1992; Wu & Lin 2004). We concluded that the pH for optimal H_2 production was dependent on the hydrogen-producing microorganism(s).

Effect of nitrogen sources

To study the effect of the nitrogen source on the rate and yield of H_2 production, the fermentation medium was supplemented with various organic and inorganic sources of nitrogen (Table 3). It was observed the nitrogen source also had a marked effect on H_2 production. In general, H_2 production by cultures

Table 3. Effect of nitrogen sources on hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC 27021.

Nitrogen source	H_2 production					
	Duration (h)	Final pH	Residual glucose (g/l)	Volume (ml)	Yield[a] (mol/mol)	Rate (mmol/h)
Organic						
[b]Control	15.3	4.99	0.22	701.0	1.45	2.04
Yeast extract	15.3	4.93	0.16	786.7	1.58	2.30
Corn steep liquor	25.5	5.08	0.10	772.6	1.56	1.35
Casamino acids	20.5	4.78	0.10	700.1	1.44	1.53
Tryptone	16.8	4.88	0.02	678.4	1.43	1.81
Peptone	31.3	4.31	0.19	650.3	1.34	0.93
Cotton seed flour	42.3	4.44	5.78	331.4	0.96	0.35
Beef extract	42.3	4.13	12.20	294.5	1.58	0.31
Soy bean flour	42.0	4.19	9.75	264.5	0.51	0.28
Corn gluten meal	42.0	3.77	11.04	215.2	0.41	0.23
Inorganic						
Ammonium sulphate	22.6	3.89	11.70	205.6	1.08	0.41
Ammonium nitrate	35.8	4.13	15.30	57.6	0.56	0.07
Ammonium chloride	42.6	6.53	19.60	0.3	0.00	0.00
Urea	42.6	38.67	19.10	0.2	0.00	0.00

[a] Yield is expressed as mole H_2 per mole glucose.
[b] Nitrogen source was Tryptone + yeast extract as in the basal medium.
Initial glucose, 20 g/l.

supplemented with organic nitrogen was significantly ($P < 0.05$) higher than those supplemented with inorganic nitrogen sources. Cultures supplemented with yeast extract, corn steep liquor, casamino acids, tryptone and peptone produced higher H_2 yields with near complete sugar consumption (98.9–99.9%). Among these sources, yeast extract was the best source of nitrogen for H_2 production because it facilitated the highest production rate (2.3 mmol/h) and yield (1.58 mol/mol); the H_2 yield was significantly ($P < 0.05$) higher than that of the control which was supplemented with both yeast extract and tryptone (Table 3). The H_2 yields obtained with corn steep liquor (1.56 mol/mol), casamino acids (1.43 mol/mol) and tryptone (1.43 mol/mol) were comparable to that of the control (1.45 mol/mol) indicating the former could be considered as alternative nitrogen sources. However, cultivation with other organic nitrogen sources such as beef extract, cottonseed flour, corn gluten meal or soybean meal was characterized by incomplete sugar consumption (29 and 49%), lower final culture pHs and lower H_2 production than the control (Table 3). Replacing organic with inorganic nitrogen sources resulted in even lower sugar consumption (0–46.5%) and poor H_2 production. Although a number of investigators have used inorganic nitrogen sources such as ammonium hydrogen carbonate (Lay *et al.* 2001; Chen *et al.* 2002; Lee *et al.* 2002; Logan *et al.* 2002; Sung *et al.* 2002; Lin & Chang 2004a) and ammonium chloride (Oh *et al.* 2003; Zhang *et al.* 2003) in H_2 fermentation media, others have shown that when ammonium chloride replaced peptone as a nitrogen source, H_2 yields are halved (Ueno *et al.* 2001). These observations were attributed to the composition of the nitrogen source. Organic nitrogen is a complex nitrogen source composed of a spectrum of peptides and free amino acids. During fermentation these are taken up from the medium by the cell and directly incorporated into proteins or transformed into other cellular nitrogenous constituents (Large 1986). By contrast, the cell spends more energy and time in synthesizing amino acids for protein synthesis from inorganic nitrogen sources (Da Cruz e*t al.* 2002). Among organic nitrogen sources, differences in protein and amino acid composition could have accounted for the differences in the production rates and yields observed in this study. However, the results indicate that organic nitrogen is the preferred nitrogen source.

Following this investigation, the amount of yeast extract was optimized for H_2 production over a range of 0.025 and 0.2% total nitrogen (Figure 2). Increasing the amount of yeast extract enhanced both the rate of H_2 production and the yield. The highest yield (1.41 mol/mol) was obtained with 0.2% yeast extract but this was not significantly different ($P < 0.05$) from 1.39 mol/mol obtained with 0.1%. Therefore, subsequent experiments were conducted with yeast extract (0.1% total nitrogen) as the sole source of nitrogen.

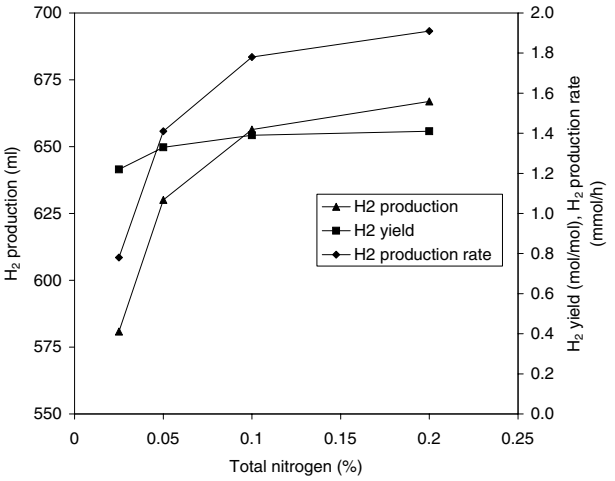

Figure 2. Effect of yeast extract concentration on hydrogen production by *C. saccharoperbutylacetonicum* ATCC 27021 from glucose. Results from duplicate run. Culture volume was 200 ml. Symbols: Volume produced, (▲); yield, (■); production rate (◆).

Effect of inoculum size

H_2 production as affected by the inoculum size (1 to 10%) is presented in Table 4. The H_2 production rate increased from 2 mmol/h at 1% to 2.36 mmol/h with a 10% inoculum. There was no clear correlation between the inoculum size and the H_2 yield although the highest yield of 1.85 was obtained with a 1% inoculum. H_2 evolution by *Clostridium* is primarily a growth-associated process and therefore it is expected that the highest rates of evolution be attained during the growth phase. This implied that for the same concentration of substrate, an initially high substrate to cell ratio would prolong the growth phase and facilitate a longer duration of high rates of H_2 production by the growing cells and that would result in high H_2 production. This however disagrees with the findings of Lay (2001) which showed that a low substrate (cellulose) to cell density facilitated high H_2 generation by a mixed hydrogen-producing culture.

Effect of iron

To determine the effect of iron on H_2 production, ferrous sulphate heptahydrate (0–2000 mg/l) was added to the fermentation medium before sterilization. This

Table 4. Effect of inoculum size on hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC 27021.

Parameter	Inoculum size (%)		
	1.0	5.0	10.0
Duration (h)	20.5	14.8	16.0
H_2 Volume (ml)	920.6	725.6	848.0
Yield[a] (mol/mol)	1.85	1.46	1.70
Rate (mmol/h)	2.00	2.19	2.36

[a] Yield is expressed as mole H_2 per mole glucose.
Initial glucose, 20 g/l.

Table 5. Effect of iron on hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC 27021.

$FeSO_4$ (mg/l)	Residual glucose (g/l)	Biomass (OD at 600 nm)	H_2 production				
			Final pH	Duration (h)	Volume (ml)	Yield[a] (mol/mol)	Rate (mmol/h)
0	9.58	1.83	3.93	24.0	361.7	1.47	0.67
25	0.13	5.19	4.93	26.3	837.8	1.74	1.42
50	0.11	5.68	4.82	19.1	795.8	1.65	1.86
125	0.15	5.86	4.91	19.1	793.5	1.65	1.85
250	0.15	6.53	4.78	19.5	814.1	1.69	1.87
500	0.16	6.56	4.78	19.1	808.1	1.68	1.89
1000	0.15	6.35	4.87	17.9	797.9	1.66	1.99
2000	0.12	6.68	4.98	16.0	808.8	1.68	2.25

[a] Yield is expressed as mole H_2 per mole glucose.
Initial glucose, 20 g/l.

was equivalent to adding between 0 and 401 mg Fe/l to the medium. H_2 production was observed with and without iron supplementation (Table 5). However, iron supplementation significantly ($P < 0.05$) promoted both the H_2 production rate and the yield over that observed in unsupplemented cultures. Increasing the iron concentration also had different effects on the production rate and yield. A sharp increase was observed in the production rate from 0.67 mmol/h (0 mg $FeSO_4{\cdot}7H_2O$/l) to 1.42 mmol/h on supplementing with 25 mg $FeSO_4{\cdot}7H_2O$/l. This was attributed to the almost threefold increase in the cell density as a result of iron supplementation (Table 5). Doubling the iron concentration from 25 to 50 mg $FeSO_4{\cdot}7H_2O$/l resulted in a 0.44 mmol/h (31%) increase in the production rate. This was followed by a 0.03 mmol/h (1.3%) increase in the production rate between 50 and 500 mg $FeSO_4{\cdot}7H_2O$/l. Beyond 500 mg $FeSO_4{\cdot}7H_2O$/l, iron supplementation further increased the production rate. The highest production rate of 2.25 mmol/h was observed at 2000 mg $FeSO_4{\cdot}7H_2O$/l. From this observation, it was apparent that increasing the iron concentration in a culture medium increased the H_2 production rate within the concentration range investigated. Iron supplementation up to 25 mg $FeSO_4{\cdot}7H_2O$/l also significantly enhanced the H_2 yield by 14% over that produced by the unsupplemented culture (Table 5). Between 25 and 50 mg $FeSO_4{\cdot}7H_2O$/l, the H_2 yield decreased slightly from 1.74 to 1.65 mol/mol, which then increased slightly to 1.68 mol/mol at 2000 mg $FeSO_4{\cdot}7H_2O$/l, but the difference was not statistical ($P < 0.05$). The highest yield of 1.74 mol/mol was observed at 25 mg $FeSO_4{\cdot}7H_2O$/l. These results showed that even a small amount of iron greatly affected production and that the major effect of additional iron was to enhance the rate of production. Lee *et al.* (2001) used $FeCl_2$ as the source of iron and reported similar profiles between the yield (or the production rate) and the iron concentration. The maximum H_2 yield reported in that study was at an optimum of 800 mg $FeCl_2$/l (352 mg Fe/l) and that was much higher than 25 mg $FeSO_4{\cdot}7H_2O$/l (5 mg Fe/l) observed in the present study. Both studies, however, underscored the importance of adding iron in greater than limiting levels to maximise H_2 yield from H_2 generating cultures (Hawkes *et al.* 2002). Iron supplementation in hydrogen fermentation media is important because hydrogen-producing Clostridia possess the metalloenzymes, hydrogenase and NADH-ferredoxin reductase, which mediate hydrogen formation. Evidence from hydrogenase purified and characterized from two clostridial species show that these enzymes contain iron clusters and a unique type of Fe–S centre termed the H_2 cluster (Adams 1990; Santangelo *et al.* 1995). The H_2 cluster has been proposed as the site of H_2 oxidation and H_2 evolution (Adams 1987). The iron clusters serve to transfer electrons between the hydrogen cluster and the external electron carrier (Adams 1990). Therefore, it was expected that supplementing with iron would facilitate hydrogenase biosynthesis and hydrogen evolution, whereas iron deprivation could severely affect the enzyme's biosynthesis and function which would then shift the metabolic pathway to alcohol production (Bahl *et al.* 1986; Peguin & Soucaille 1995).

Effect of agitation speed

Table 6 shows the effect of agitation speed (0–400 rev/min) on H_2 production. Agitation was provided by a

Table 6. Effect of agitation speed on hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC 27021.

Agitation speed (rev/min)	Residual glucose (g/l)	H_2 production			
		Duration (h)	Volume (ml)	Yield[a] (mol/mol)	Rate (mmol/h)
0	0.14	20.5	729.9	1.51	1.59
50	0.19	19.1	795.8	1.66	1.86
100	0.15	19.2	795.8	1.65	1.85
150	0.13	20.2	739.0	1.53	1.63
200	19.5	0.0	0.0	0.00	0.00
400	19.5	0.0	0.0	0.00	0.00

[a] Yield is expressed as mole H_2 per mole glucose.
Initial glucose, 20 g/l.

magnetic stirring bar (5 cm × 0.8 cm) placed at the bottom of the flask. The agitation speed affected both the production rate and the yield. Agitation at 50 rev/min resulted in a 10% increase in the yield from 1.51 mol/mol in the unstirred culture to 1.66 mol/mol. The yield remained unchanged at 100 rev/min and dropped thereafter. The highest yield, 1.66 mol/mol, was therefore obtained at 50 and 100 rev/min. There was a wide deviation in the H_2 yield at 150 rev/min (data not shown) indicating that at this speed agitation did not have a uniform effect on the cultures, but that did not affect sugar consumption which was as high (99.4%) as in cultures agitated at lower speeds. At 200 and 400 rev/min, the cultures neither grew nor produced H_2 by the end of the experiment. A similar profile was observed between the production rate and the agitation speed. The maximum production rate of 1.86 mmol/h was also observed at 50 and 100 rev/min. It has been reported that a high dissolved H_2 concentration in the culture medium inhibits H_2 production and favours alcohol production by *Clostridium* (Gapes *et al.* 1982); hence immediate removal of H_2 from the culture medium is recommended to facilitate maximum H_2 yields. In this study, agitation served to remove dissolved gases such as CO_2 and H_2 from the fermentation medium, thereby lowering their concentrations, which enhanced their rates of production. Nonetheless, agitating cultures at high speed could also cause fragmentation and shear damage to the microbial cells (Yerushalmi & Volesky 1985) and therefore there is the need to achieve a balance that ensures maximum productivity without injury to the cells. Based on the results of this experiment, an agitation speed of 100 rev/min was considered as the maximum necessary to attain the highest H_2 production rate and yield by this microorganism. Other investigators, Doremus *et al.* (1985), Yerushalmi & Volesky (1985), Lamed *et al.* (1988) and Lay (2001) have also shown that higher agitation favours H_2 production by clostridial species or a mixed culture containing Clostridia, although the maximum agitation speed reported varied from microorganism to microorganism. In a recent study by Lay (2001), the H_2 yield produced by a mixed hydrogen-producing culture doubled by increasing the agitation speed from 100 to 700 rev/min.

Conclusion

As the world moves toward a H_2 economy, fermentative H_2 production is bound to contribute to our future energy supply. Needless to say, the competitiveness of such fermentative processes would largely depend on the efficiency of hydrogen-producing strains and the ready availability of cheap and locally abundant source of substrate. As a number of preceding studies have demonstrated, identification of clostridial species, in pure cultures or in mixed consortia, holds a key to the success of these H_2 fermentative processes.

In this study, a number of factors were examined for their influence on the rate and yield of H_2 production by *C. saccharoperbutylacetonicum*. The results were useful in predicting the types of sugar and the concentration that would maximize H_2 yields; they showed that impure sugar feedstocks such as food and dairy processing waste could potentially serve as substrates for H_2 fermentation. It also revealed that the microorganism produces H_2 more efficiently from glucose than most of the microorganisms or mixed cultures already reported. Among the medium components, the source of nitrogen was a very important factor, given that organic nitrogen sources were preferred to an inorganic one. The study also pointed out the advantages of employing a small inoculum size and iron concentration to maximize H_2 production and the importance of an initially neutral to slightly alkaline pH coupled with adequate agitation to the success of the fermentation.

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