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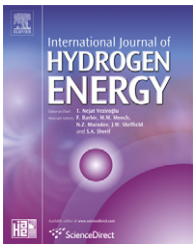

Impact of carbon and nitrogen sources on hydrogen production by a newly isolated *Clostridium butyricum* W5

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ABSTRACT

Whilst biological process has been recognised as a promising approach for hydrogen production, the high production cost is still a key issue for moving this technology on an industrial step. Carbon and nitrogen feedstock represents 30–40% total costs of fermentative hydrogen production. This work was to investigate the impacts of carbon, and nitrogen sources and their concentrations on hydrogen fermentation by a newly isolated *Clostridium butyricum* W5. Biochemical performance of a batch fermentation process was evaluated by hydrogen production and yield, bacterial biomass and volatile fatty acids. Six raw or waste carbon sources and six organic or inorganic nitrogen sources were employed. Experimental data revealed that molasses and NH_4NO_3 were technically and economically suitable carbon and nitrogen sources for hydrogen production. The highest hydrogen yield of 1.63 mol H_2/mol hexose was obtained using 100 g/L molasses and NH_4NO_3 1.2 g/L.

1. Introduction

The depletion of global fossil fuels and associated environmental problems are driving the need for the alternative forms of energy [1]. Combustion of hydrogen produces a high energy yield which is 2.75 times greater than that of hydrocarbon fuels [2], and only water as by-product, making it a highly efficient and non-pollutant energy alternative. Hydrogen production techniques include biological, photoelectrochemical and thermochemical processes. Electrochemical or thermochemical processes consume fossil fuel and electricity. They are energy intensive and not environmental friendly [3]. Biological hydrogen production is favoured on the basis of both economic and environmental concerns due to its low costs for process capital and operation [4].

The production of hydrogen by dark fermentation has attracted great attention recently because of its advantages for industrialization. It has very high evolution rate compared to photosynthetic hydrogen production, and is not limited by light and can produce hydrogen constantly [4]. Valuable by-products and biomass also make this application more favoured by commercialization [5,6]. Many renewable waste materials can be used as carbon sources for hydrogen production, including starch and cellulose-containing agricultural or food industry by-products and wastes, carbohydrate-rich industrial wastewaters and waste sludge from wastewater treatment plants [7,8]. Those applications not

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only produce hydrogen but also can be an environmental friendly waste treatment processes [9–11]. Many previous investigations focused on special waste materials in continuous processes with activated sludge, such as starch wastewater [12] and waste biosolids [13]. Recently, more researches have been afforded to isolation or gene modification of hydrogen producers [5,14,15]. However, a little information on how carbon and nitrogen sources impact on production of hydrogen and associated by-products in hydrogen fermentation is available in the literature. Considering that 30–40% of total production costs are associated with the substrates [16], detailed investigations on searching suitable carbon and nitrogen sources, and evaluating carbon and nitrogen concentrations in fermentation broth for certain bacterial hydrogen fermentation are clearly necessary.

The relatively low yield of biological hydrogen production drove researchers focused on seeking highly yielded hydrogen-producing microorganisms, genetically modifying existing microorganisms, or optimizing fermentation process optimization [17–19]. Clostridia have been established as the main hydrogen-producing bacteria in many hydrogen production processes [20,21]. An earlier study from this laboratory has reported the isolation of a *Clostridium butyricum* W5, an efficient hydrogen producer, from hydrogen-producing activated sludge [15]. Objectives of this study were to select suitable carbon and nitrogen sources for hydrogen fermentation, including waste potato starch (WPS), molasses, glucose, NH_4NO_3 and yeast extract. A laboratory-scale batch fermentation process was carried out to find out how the carbon and nitrogen sources and their concentrations affect fermentation performances in terms of hydrogen production and yield, bacterial growth and production of by-products.

2. Methodology

2.1. Microorganism and maintenance

C. butyricum W5 (GenBank accession number: DQ831124) was isolated from hydrogen-producing sludge and identified by 16S rDNA sequence and the RapID™ ANA II System (Remel, Inc., Lenexa, KS) in our previous research [15]. The strain was routinely maintained anaerobically on blood agar medium (PP2001, Columbia, HBA, Oxoid) at 35 °C for 48 h, and was monthly transferred in a MK3 Anaerobic Work Station (Don Whitley Scientific Limited, West Yorkshire, England) with a 10% hydrogen and 90% nitrogen gas atmosphere. It was then stored at 4 °C within an anaerobic jar (AG0025, Columbia, HBA, Oxoid). The anaerobic gas atmosphere is created by ANAEROGEN (AN2005, Columbia, HBA, Oxoid) in the anaerobic jar.

2.2. Culture media and cultivation methods

Tryptone Soya Broth (CM0129, Columbia, HBA, Oxoid) was used as the seed culture medium. It was autoclaved at 121 °C for 20 min. A 100 mL bottle containing 100 mL of the broth was inoculated with a single colony picked up from stored blood agar and then cultivated in an anaerobic jar at 35 °C for 12 h before inoculation into the bioreactor.

2.3. Preliminary screening of carbon and nitrogen sources

Six carbon sources (glucose, fructose, lactose, sucrose, WPS and molasses) and six nitrogen sources (yeast extract (LP0021, Columbia, HBA, Oxoid), neutralised soya peptone (NSP) (LP0044, Columbia, HBA, Oxoid), urea [$CO(NH)_2$], ammonium sulphate [$(NH_4)_2SO_4$], potassium nitrate (KNO_3) and ammonium nitrate (NH_4NO_3)) were employed in the screening investigations for hydrogen production. WPS was provided by Smiths Chips Ltd (Australia). The WPS contained 90% (w/w) starch and 0.04055 % total Kjeldahl nitrogen (TKN), as determined by previous research [22]. Molasses was obtained from Magill Grain Store Pty Ltd (Australia). The hexose concentration in the molasses was 44.4% (w/w) and the TKN was 0.7086%. Yeast extract was used as nitrogen source for the carbon source screening experiments. Glucose was used as carbon source for the trials of nitrogen source screening. The fermentation media contained carbon sources equal to 10 g/L hexose and nitrogen sources equal to 3 g/L yeast extract. Preliminary screening experiments were carried out at 35 °C with initial pH 7.0 in refitted 1 L bottles, which have two tubes fitted into the rubble stopper to allow inert gas purging and biogas emission. The working volume was 800 mL and 10% (v/v) seed culture was used as inoculum. Biogas was collected by the water release method.

2.4. Comparison of different carbon and nitrogen sources in bioreactor

Batch fermentation was performed in a laboratory-scale batch bioreactor BioFlo 110 (New Brunswick Scientific, USA), at 35 °C, pH 6.5, with a working volume of 1.5-L, to compare the effects of carbon (glucose, molasses and WPS) and nitrogen sources (yeast extract and NH_4NO_3). The agitation was 300 rpm. The anaerobic environment was maintained by sealing all the connections with silicone gel. Temperature, pH and agitation rate were controlled during fermentations according to experimental design. The inoculum was transferred with a sterile hypodermic disposable syringe. Nitrogen purging for keeping the anaerobic environment of the medium was carried out after the inoculation. Biogas produced was collected by the water release method.

Carbon and nitrogen source concentrations in fermentation media were determined in the same way as bottle test. Fermentation performed at 35 °C, pH 6.5, 300 rpm with an inoculum size of 100 mL seed culture, and fermentation duration is 20 h.

2.5. Effects of molasses and NH_4NO_3 concentrations

The effects of molasses and NH_4NO_3 concentrations were evaluated in the bioreactor. Bioreactor operation conditions were the same as described in Section 2.4. Fermentation was performed with 100 mL seed culture at 35 °C, pH 6.5, 300 rpm. A series of molasses concentrations from 20 to 120 g/L were tested to evaluate the effect of carbon source concentration. The optimal concentration was then used afterwards to evaluate NH_4NO_3 concentration (0–1.5 g/L).

2.6. Sample preparation and analysis

For the bottle test, total biogas volume and hydrogen concentration were measured and 20 mL of fermentation broth in the bottle were collected after 16 h for analysis of hydrogen production and biomass. For batch fermentation, biogas volume and hydrogen concentration were measured and 20 mL of fermentation broth in the bioreactor were collected at 4 h intervals for investigation of the kinetics of hydrogen accumulation, cell growth and formation of volatile fatty acids (VFAs).

Fermentation broth samples were centrifuged at 4 °C, 3000 rpm for 20 min. Pellets were washed twice with Mill-Q water and weighed after drying at 60 °C for 4 h for biomass measurement. Supernatant (100 μL) was diluted 10 times and further filtered through a 0.22 μm membrane for analysis of VFAs by HPLC. The HPLC analysis used an ROA Organic Acid Column (Phenomenex, 300 × 7.8) and a refractive index detector (Varian, Model 350). The mobile phase was 4 mM H_2SO_4 at a flow rate of 0.5 mL min^{-1} and the column temperature was 50 °C. The biogas was sampled using a glass syringe and analyzed on a CP-3800 gas chromatograph (Varian Inc. CA, USA) equipped with a thermal conductivity detector as described previously [15]. The working temperatures of injector, detector, and column were maintained at 50, 140 and 40 °C, respectively. All results were obtained from the means of triplicate determinations.

3. Results and discussion

3.1. Screening of carbon and nitrogen sources

Glucose was the most commonly used carbon source for determining hydrogen production ability in laboratories [14,23]. WPS and molasses were also considered by previous researchers as a potential fermentation raw material because of their wide availability, low cost, high carbohydrate concentration and high biodegradability [24,25]. In consideration of lowering the production costs for further industrial purposes, several inorganic nitrogen sources were also selected. Among six organic or inorganic nitrogen sources, urea has the highest proportion of nitrogen content (46.7%), followed by NH_4NO_3 (35%), $(NH_4)_2SO_4$ (21%), KNO_3 (13.9%), yeast extract (9.8%) and NSP (8.7%). To compare the requirement of nitrogen sources, nitrogen content in this study was set up as 0.30 g/L, leading to different concentrations of nitrogen sources as shown in Table 1. Experimental data of screening carbon and nitrogen sources for hydrogen production are given in Table 1.

The results from carbon source screening experiments showed that the highest concentration of hydrogen (950 mL/L) and biomass (1.45 g/L) was measured in the fermentation system using molasses, flowed by WPS which produced 648 ml/L hydrogen and 1.35 g/L biomass. The high biomass and hydrogen production compared with monosaccharide and disaccharide are possibly due to the bio-accessible nitrogen sources existing in WPS and molasses [22,26,27]. A similar hydrogen production performance was given in the fermentation system using glucose, fructose and sucrose. These showed that the newly isolated *C. butyricum* W5 has a capability to use those monosaccharides and disaccharides, which also are the main sugar content of hydrolysed WPS and molasses [14]. Lactose fermentation resulted in a poor performance in terms of hydrogen production and bacterial growth, indicating that lactose-containing substrate may not be a suitable carbon source for the hydrogen production by *C. butyricum* W5. The results of nitrogen source screening revealed that the highest hydrogen production could be achieved by using yeast extract (950 mL/L). Inorganic nitrogen sources $(NH_4)_2SO_4$ and NH_4NO_3 also produced a relatively high yield (460 and 483 mL/L, respectively), which showed great industrial potential because of their low cost. Urea and KNO_3 appeared not to be a favoured nitrogen source for *C. butyricum* W5.

3.2. Effects of glucose, WPS and molasses

According to the results obtained from carbon source screening, two promising carbon sources, WPS and molasses, were further investigated by batch bioreactor process. As a comparison trial, a batch fermentation using glucose as carbon source was carried out under the same operation

Table 1 – Production of hydrogen and biomass using different carbon and nitrogen sources

Carbon source	Carbon source concentration (g/L)	Nitrogen source	Nitrogen source concentration (g/L)	Biomass (g/L)	Cumulative hydrogen production[a] (mL/L)
Glucose	10.0	Yeast extract	3.00	1.28	550
Fructose	10.0	Yeast extract	3.00	1.22	518
Lactose	9.5	Yeast extract	3.00	0.45	138
Sucrose	9.5	Yeast extract	3.00	1.30	558
WPS	10.0	Yeast extract	3.00	1.35	648
Molasses	22.5	Yeast extract	3.00	1.45	950
Glucose	10.0	NSP	3.38	1.02	482
Glucose	10.0	Urea	0.63	0.56	256
Glucose	10.0	$(NH_4)_2SO_4$	1.40	0.97	460
Glucose	10.0	KNO_3	2.12	0.80	380
Glucose	10.0	NH_4NO_3	0.84	1.09	483

a Cumulative hydrogen production calculated as mL hydrogen per litre fermentation broth.

condition. Analytical data revealed that lactic acid, acetic acid and butyric acid were end by-products, and other VFAs and alcohols either were measured at a very low level or were undetectable. Fig. 1 presents kinetic profiles of biochemical parameters during 20 h fermentation, showing the effect of the three carbon sources on production of hydrogen (A), bacterial biomass (B), lactic acid (C), acetic acid (D) and butyric acid (E). A shorter lag phase and higher hydrogen production and faster biomass growth were given in the fermentation using molasses as carbon source (Fig. 1A and B). The use of WPS corresponded to a relatively longer lag phase but higher hydrogen production and biomass growth than glucose. The shortest lag phase (6 h) was obtained by using molasses, followed by glucose (8 h) and WPS (12 h).

VFAs produced along with hydrogen are valuable by-products. In theory, 4 or 2 mol of hydrogen per mole hexose can be produced if acetic acid or butyric acid is the sole end-product, respectively [28]. It is worthwhile to note that the hydrogen fermentation using pure culture of *C. butyricum* W5, as hydrogen producer, produced mainly lactic acid, acetic acid and butyric acid as by-products, and lactic acid was found to be the dominate VFA in the fermentation using molasses and glucose. Compared with glucose, molasses fermentation resulted in a high production of hydrogen and biomass, while accumulating more acetic acid and butyric acid (Fig. 1D and E). Angenent and the co-workers reported that the formation of other reduced organic compounds like lactic acid, propionic acid and ethanol will lead to a much low hydrogen yield [28]. They stated that these metabolic pathways bypass the major hydrogen-producing reaction in carbohydrate fermentations [28]. A small amount of propionic acid (less than 0.1 g/L) was observed at 8 h when fermented molasses consumed by bacteria (data not shown). No ethanol production was observed during the fermentations using *C. butyricum* W5. Fig. 1C shows that a higher level of lactic acid was produced by glucose compared to molasses. The WPS fermentation demonstrated an unfavoured performance in terms of hydrogen yield, production of VFAs (Table 2).

3.3. *Effects of NH_4NO_3 and yeast extract*

Using cheap inorganic nitrogen source instead of expensive organic nitrogen sources is favoured in an industrial process. NH_4NO_3, a widely used inorganic nitrogen source, was tested in comparison with yeast extract. The results from experiments of nitrogen source impact are shown in Fig. 2. Similar lag phases (8 h) were obtained from the fermentation using both nitrogen sources. It is reasonably to note that NH_4NO_3 fermentation led to a slow bacterial growth. A slightly higher butyric acid concentration was obtained in the fermentation using NH_4NO_3 than the fermentation using yeast extract. Comparing with the experimental data in the first 12 h fermentation, the use of NH_4NO_3 demonstrated a weaker performance in terms of production of hydrogen (Fig. 2A), biomass (Fig. 2B), and lactic acid (Fig. 2C) and acetic acid (Fig. 2D) than those fermentations using yeast extract.

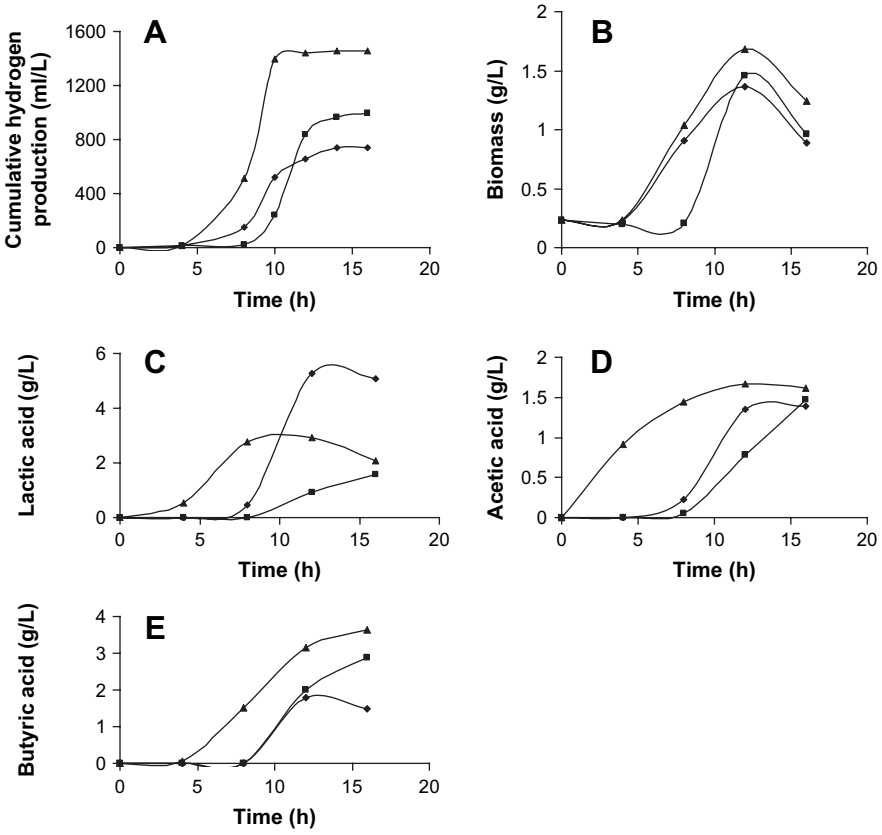

Fig. 1 – Kinetic profiles of hydrogen (A), biomass (B) and lactic acid (C), acetic acid (D) and butyric acid (E) produced using glucose (◆), WPS (■) and molasses (▲) as carbon sources.

Table 2 – Effect of carbon and nitrogen source on production of VFA and hydrogen yield

Experiment Number	Carbon source	Nitrogen source	Lactic acid (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Sugar usage (%)	Hydrogen yield (mol/mol hexose)
E1	Glucose	Yeast extract	5.30	1.35	1.79	97	0.61
E2	WPS	Yeast extract	1.60	1.47	2.87	75	1.11
E3	Molasses	Yeast extract	2.92	1.62	3.65	96	1.22
E4	Glucose	NH_4NO_3	4.09	0.81	1.72	97	0.51

Obviously, yeast extract was the favoured nitrogen source for the growth of *C. butyricum* W5, leading to a high production of the hydrogen and by-products. Data in experiment E2 and E3 in Table 2 show that the extra nitrogen content in WPS and molasses may be responsible for the high hydrogen yield and VFA production.

3.4. *Effects of molasses concentration*

The carbon source concentration is crucial to a fermentation process. It affects both production rate and yield, and the cost of whole process [16]. High carbon source concentration can promote the production rate and improve the efficiency. However, high hexose and oligosaccharides concentration will modify metabolic pathways and lead to production of unwanted by-products [14]. Fig. 3. shows the hydrogen production and yield, and biomass growth at different molasses concentrations. The hydrogen production increased with the increase in molasses concentration, while the yield reached 1.63 mol H_2/mol hexose at 100 g/L molasses and kept a similar level (1.62 mol H_2/mol hexose) at the molasses concentration of 120 g/L. Moreover, the lag phase was prolonged from 8 to 18 h when molasses concentration increased from 100 to 120 g/L (data not shown), which was supposed to be introduced by high hexose initial concentration. Therefore, 100 g/L could be regarded as the highest tolerant molasses concentration of *C. butyricum* W5. Molasses has been used in many previous investigations of hydrogen production and a similar long accumulation time of more than 20 h was observed by previous researchers when a molasses concentration was higher than 60 g COD/L [29]. It can be seen that the molasses concentration significantly affected the hydrogen production and bacterial growth. Statistical data analysis showed that the hydrogen production and biomass growth have a lineal relationship with the carbon source concentration. Lineal equations were $y = 1780.6x - 887.42$ with $R^2 = 0.9948$ and $y = 0.9739x - 0.2443$ with $R^2 = 0.9872$ (Fig. 3).

As shown in Fig. 4, a noticeable variation in VFA portion can be observed using molasses and NH_4NO_3 as substrates compared with glucose and yeast extract (Figs. 1 and 2). When molasses concentration was 20 g/L, butyric acid was the main product, while lactic acid was not detectable. As increasing

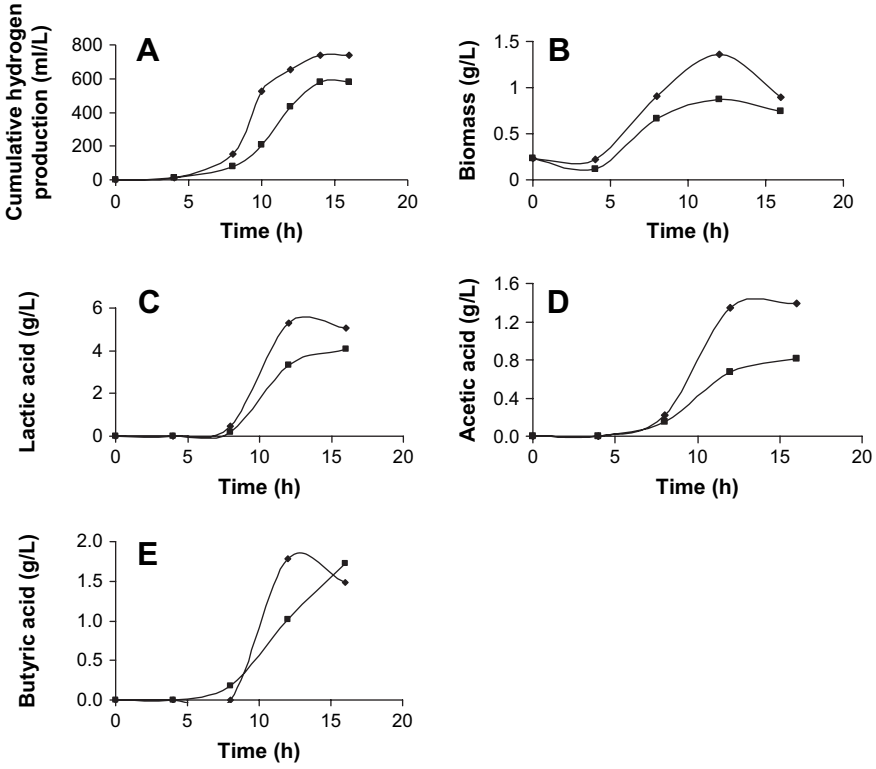

Fig. 2 – Kinetic profiles of hydrogen (A), biomass (B) and lactic acid (C), acetic acid (D) and butyric acid (E) produced using yeast extract (◆) and NH_4NO_3 (■) as nitrogen sources.

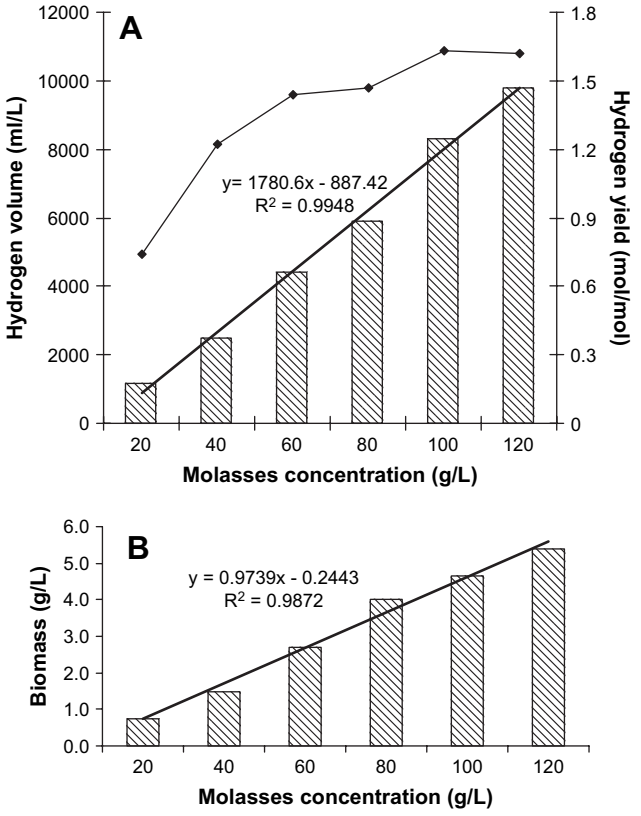

Fig. 3 – Hydrogen production (▨) and yields (◆) (A), and biomass (B) at different molasses concentrations.

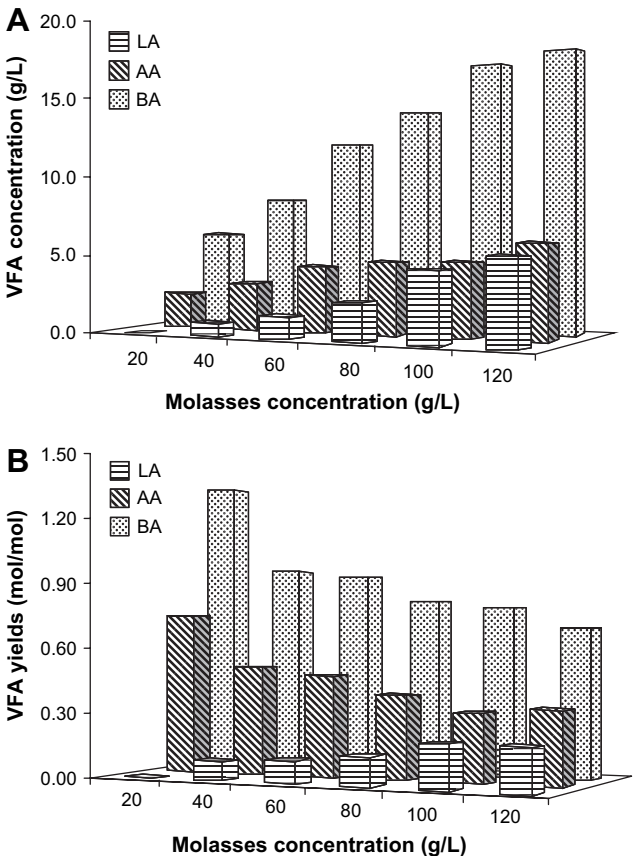

Fig. 4 – Lactic acid (LA), acetic acid (AA) and butyric acid (BA) concentrations (A) and yields (B) at different molasses concentrations.

molasses concentration, the acetic acid and butyric acid increased, but the corresponding hydrogen yield decreased. Comparing the results shown in Figs. 1 and 2 with those showed in Fig. 3, a high lactic acid production was found in the fermentations which used either yeast extract as carbon source or a high concentration of molasses. No propionic acid was detected during the fermentation.

3.5. *Effects of NH_4NO_3 concentration*

NH_4NO_3 is a cheap and high nitrogen content (35%) nitrogen source and has been widely used for many fermentation processes. As the molasses employed in this research contains 0.7086% nitrogen which is mainly organic nitrogen [30]. NH_4NO_3 was used as a supplementary nitrogen source. Impact of nitrogen source concentration of hydrogen production/yield, biomass growth and VFA yield is shown in Figs. 5 and 6. Production of both hydrogen and biomass increased with the increase in NH_4NO_3 from 0 to 1.2 g/L. No further production improvement was found as NH_4NO_3 concentration increased up to 1.5 g/L. The lag phase decreased from 16 to 12 h as NH_4NO_3 concentration increased which indicated that a fast cell growth rate needs a high nitrogen source concentration (data not shown). No statistical relationships between NH_4NO_3 concentration and other biochemical parameters were found.

It was noted that NH_4NO_3 concentration had a little impact on lactic acid production. This confirmed our hypothesis in Section 3.4. Our results indicate that lactic acid formation may be affected by organic nitrogen source, which might be metabolic products of amino acids or other organic compounds [31]. In this case, lactic acids produced in hydrogen fermentation appeared to have a little impact on hydrogen yield from hexose. Acetic acid and butyric acid increased with the increase in NH_4NO_3 concentrations, while the ratio of acetic acid/butyric acid did not change greatly. Our results suggested that VFA productions were significantly affected by substrates. Further investigation could be done to profile the metabolic pathways of this three VFA and to manipulate the metabolic pathway towards hydrogen production.

4. Conclusion

The experimental data revealed that the newly isolated *C. butyricum* W5 is a promising hydrogen producer which can use a wide range of carbon and nitrogen sources in either raw or waste basis. Molasses and waste potato starch can be used as carbon sources for a large scale hydrogen production. While yeast extract is the most favoured nitrogen source for bacterial growth and hydrogen fermentation, the NH_4NO_3 was proven to be a suitable nitrogen source for fermentative

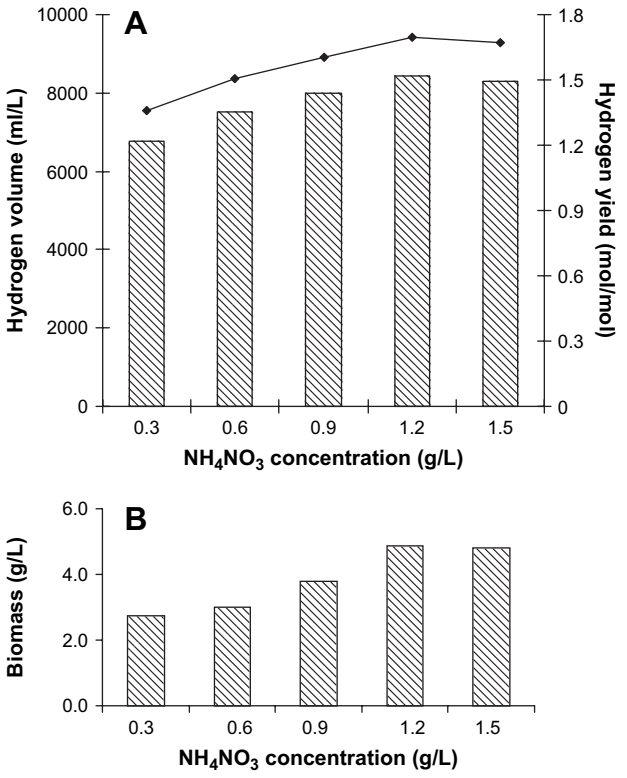

Fig. 5 – Hydrogen production (▨) and yields (◆) (A), and biomass (B) at different NH_4NO_3 concentrations using molasses as carbon source.

hydrogen production. The most suitable molasses and NH_4NO_3 concentrations for this application were 100 and 1.2 g/L respectively. The use of the cheaper carbon and nitrogen sources of molasses, waste starch and NH_4NO_3 shows great potential for a commercial hydrogen production process.

In association with hydrogen production, the fermentation using *C. butyricum* W5 produced lactic acid, acetic acid and butyric acid as by-products. Differently, lactic acid was found to be the main VFA produced when using glucose as carbon source, while butyric acid was the main VFA produced using molasses and WPS. A high level of acetic acid and butyric acid was associated with a high level of hydrogen and biomass, but the yields of these two VFAs decreased as the hydrogen production yield increased. Preliminary investigation of a laboratory-scale fermentation system using molasses and NH_4NO_3 as substrates resulted in a comparably high hydrogen yield of 1.63 mol H_2/mol hexose, while a maximum yield of 0.61 mol H_2/mol hexose was given in glucose fermentation.

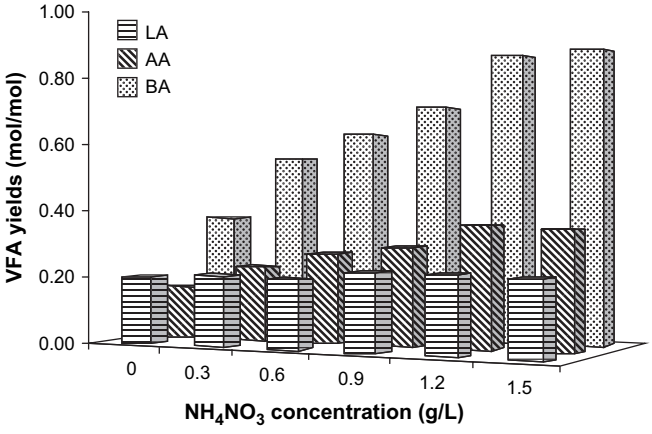

Fig. 6 – Lactic acid (LA), acetic acid (AA) and butyric acid (BA) yields at different NH_4NO_3 concentrations using molasses as carbon source.

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