American Journal of Biochemistry and Biotechnology 4 (4): 393-401, 2008
ISSN 1553-3468

Effect of Nitrogen Source and Carbon to Nitrogen Ratio on Hydrogen Production using *C. acetobutylicum*

[1]Mohd Sahaid Kalil, [2]Hisham Salem Alshiyab, and [2]Wan Mohtar Wan Yusoff
[1]Department of Chemical and Process Engineering, Faculty of Engineering,
[2]School of Bioscience and Biotechnology, Faculty of Science and Technology
University Kebangsaan Malaysia 43600 UKM Bangi Selangor, Malaysia

Abstract: Problem statement: One of the main factors influenced the bacterial productivity and total yield of hydrogen is the nitrogen source and its concentration. **Approach:** Using different nitrogen source with different concentration on bacterial productivity of hydrogen showed to affect on both bacterial productivity of hydrogen and biomass concentration. **Results:** Yeast extract as nitrogen source at concentration of 13 g L^{-1} was the best organic nitrogen source and resulted in hydrogen yield $Y_{P/S}$ of 308 mL g^{-1} glucose utilized with biomass concentration of 1.1 g L^{-1}, hydrogen yield per biomass $Y_{P/X}$ of 280 mL g^{-1} L^{-1}, biomass per substrate utilized $Y_{X/S}$ of 0.22 and produced hydrogen in gram per gram of glucose utilized $Y_{H2/S}$ of 0.0275. C/N of 70 enhanced the $Y_{P/S}$ from 308-350 mL g^{-1} glucose utilized with biomass concentration of 1.22 gL^{-1}, $Y_{P/X}$ of 287 mL g^{-1} L^{-1}, $Y_{X/S}$ of 0.244 and $Y_{H2/S}$ of 0.03125. **Conclusion:** Nitrogen source with proper C:N ratio enhanced the hydrogen production.

Key words: Hydrogen production, *clostridium*, anaerobic fermentation, yeast extract

INTRODUCTION

Fossil fuel combustion produces carbon dioxide and other waste gases causing environmental problems. The development of alternative energy resources is desired. Hydrogen is an ideal, clean and sustainable energy resource for the future. Biological hydrogen production processes, including photosynthesis and anaerobic fermentation, are environmentally friendly and less energy intensive compared to chemical processes[1]. Biological hydrogen production is more attractive if organic wastewater or other wastes are used as the raw material. This economical bioenergy-producing process can produce an energy product and simultaneously reduce the pollution strength of these wastes.

The Carbon to Nitrogen (C/N) ratio is important in a biological process. Mixed microfloras from sewage or compost are usually used in biological hydrogen production from organic wastes[2]. Microflora requires a proper nitrogen supplement for metabolism during fermentation. A proper C/N-ratio value for pure culture is necessary to optimize anaerobic hydrogen production from organic substrate.

It is necessary to maintain proper composition of the feedstock for efficient plant operation so that the C:N ratio in feed remains within desired range. It is generally found that during anaerobic digestion microorganisms utilize carbon 25-30 times faster than nitrogen. Thus to meet this requirement, microbes need a 20-30:1 ratio of C to N with the largest percentage of the carbon being readily degradable. Waste material that is low in C can be combined with materials high in N to attain desired C:N ratio of 30:1[3]. Some studies also suggested that C: N ratio varies with temperature.

The Carbon to Nitrogen (C/N) ratio is important in a biological process. Previously, a variety of factors have been found to affect hydrogen production by either mixed or pure cultures. For example, the C/N ratio has been shown to affect fermentative hydrogen by mixed microflora fed with sucrose with an optimal ratio of 47[4]. Similarly, a study where sucrose was varied at a constant ammonium concentration showed that conversion to hydrogen was more efficient at lower substrate loadings[5]. Another study was conducted by Bisaillon *et al*.[6] to investigate some limiting factors in microbial hydrogen fermentation by different strains of *E. coli*. They found that limitation of phosphate or

Corresponding Author: Hisham Salem Alshiyab, School of Bioscience and Biotechnology, Faculty of Science and Technology, University Kebangsaan Malaysia, 43600 UKM Bangi Selangor, Malaysia
Tel: + 60389216419 Ph: +60172210768

sulfate was without great effect. However, strains showed the highest yield of hydrogen per glucose when cultured at limiting concentrations of either ammonia or glucose. They reasoned the enhancement of production to C/N ratio on culture medium.

This study was aimed to improve RCM medium for hydrogen production specifically to investigate the effect of nitrogen source and C/N-ratio of the fermentation medium on hydrogen production using *C. acetobutylicum* NCIMB13357.

MATERIALS AND METHODS

It was shown from the composition of RCM media that RCM have two main kinds of sugar glucose and soluble starch and the hydrogen produced related to both sugars. Due to that variation in the results and we cannot refer the final byproducts to one kind of sugar. For above reason, the first priority was to formulate new medium for hydrogen production using *C. acetobutylicum* NCIMB13357 and it was used RCM medium components as a basic components to formulate new medium for hydrogen production using this bacterium species. It's well known that glucose is the easiest monosaccharide to be used as energy source. New formulated medium have all RCM medium components but with glucose as sole carbon source, with fixed concentration as in RCM medium 5 g L^{-1}. Furthermore in RCM medium, three main organic nitrogen compounds were used as nitrogen source, peptone 10 g L^{-1}, Yeast extract 3 g L^{-1} and meat extract 10 g L^{-1}. For the nitrogen source, different nitrogen source we study to find out the best nitrogen source for hydrogen production by using *C. acetobutylicum* NCIMB 13357.

To study the effect of nitrogen source it was started use this formula have the following composition in g L^{-1}: Glucose (5), one of the following organic nitrogen source (Yeast Extract/ Trypton/ Peptone) (13), Sodium chloride (5), Sodium acetate (3), L-Cystine. HCl 1.0 and bacteriological agar 0.5. In the first step it is used only organic nitrogen source 13 gL^{-1}, then for comparison we used inorganic nitrogen source to find the best source of nitrogen for maximum hydrogen production by *C. acetobutylicum* NCIMB13357. Finally to find out the proper C/N ratio in fermentation medium. C/N ratio determination was determined the nitrogen concentration of proper nitrogen source using Kjeldahl method[9]

Microorganism and culture conditions: *C. acetobutylicum* NCIMB 13357 was purchased from a British culture collection, NCIMB Ltd. Scotland, UK. The bacterium was cultivated in anaerobic condition in Reinforced Clostridial Medium (RCM) for 24 h at 30°C. Liquid medium of RCM was used for inoculum preparation. Measuring an optical density at 600 nm using a spectrophotometer monitored the growth of culture in RCM. Only inoculum with Optical Density (OD) values greater than 0.4-0.6 after 18 h cultivation was used as inoculum. An inoculum of 10% v/v was used throughout this work. Batch fermentation was carried out at a working volume of 100 mL in 500 mL rector bottles at 30ºC, each medium was seeded with a 10% inoculum inside anaerobic cabinet and sparged with nitrogen gas 99.9% then was tightly closed. The culture pH was not controlled during fermentation and the initial pH was fixed to 7.0 before sterilization process. To quantify H_2, gas produced during fermentation was recorded at the end of its production. The evolved gas was collected in a gas collection inverted cylinder, and the volume of evolved gas was measured at room temperature by the water displacement method[7] in a graduated cylinder that had been filled with water of pH 3 or less in order to prevent dissolution of the gas components

Cultivation medium: The medium we start used have the following composition in (g L^{-1}): Glucose (5), one of the following organic nitrogen source (Yeast Extract/ Trypton/ Peptone) (13), Sodium chloride (5), Sodium acetate (3), L-Cystine. HCl (1.0), Agar (0.5).

Analytical methods: The gas composition was determined by gas chromatography (Shimadzu Co., Kyoto, GC-8A) under the following conditions: column: Porapack-Q, carrier gas: Nitrogen, flow rate: 33 m L^{-1} min: Column temperature: 50ºC, injection temperature: 100ºC, detector temperature: 50ºC, detector: Thermal Conductivity Detector (TCD). The cell biomass concentration was estimated as Dry Cell Weight (DCW) by measuring the optical density spectrophotometrically at wavelength of 660nM, and related the optical density to DCW. The reducing-sugar (glucose) content of the medium was also estimated using Miller method[8]. The glucose concentration in the medium was measured using 3, 5 dinitrosalicylic acid (DNS) assay for total reducing sugars. A 1 mL of the sample and 2 mL of the DNS reagent mixture were mixed together in a test tube. The mixtures were placed in a boiling water bath for 5 min and then diluted with 10mL of distilled water. The absorbance at OD 550 nM for all samples was recorded and the glucose concentration was calculated from standard curve.

Individual batch experiments were observed until the hydrogen production from each bottle stopped.

Nitrogen content determination for (C/N) ratio by Kjeldahl method[9]. Final medium pH was measured by pH meter (Mettler Toledo). All of these data were the average (mean) of three trials.

RESULTS

Effect of nitrogen source on hydrogen production: It was noted that investigators have reported H_2 yield as mol H_2 per mol substrate, mol H_2 per gram substrate or H_2 produced mL per gram substrate; hence, for ease of comparison with values reported, the H_2 yields were all converted to H_2 produced mL per gram substrate utilized compared with the H_2 yield produced from glucose utilized.

Gas analysis by the GC-TCD showed that the percentage of hydrogen in produced gas was 64.5%. Following this percentage, the results shown in Table 1 indicated that using RCM medium for hydrogen production by *C.acetobutylicum* NCIMB13357 gave maximum hydrogen of 1400 mLL^{-1} and this value was for two main carbon sources which were 5 gL^{-1} glucose and 1 g L^{-1} soluble starch with maximum H_2 productivity of 63.5 mL L^{-1} h^{-1}. Compared with above results obtained using RCM medium, the new medium as shown in Table 2 and 3 using 5 g L^{-1} of glucose gave maximum of 308 mL g^{-1} glucose utilized with maximum productivity of 55 mL L^{-1} h^{-1}. Hydrogen yield was higher than obtained from RCM medium whereas hydrogen productivity was lower than RCM medium due to carbon source in its composition (glucose and soluble starch) whereas the new medium used have only glucose with same concentration as in RCM. The results shown in Table 2 and 3 indicated that the hydrogen production was affected by the source of organic nitrogen. By using 13 g L^{-1} of organic nitrogen, yeast extract gave the highest H_2 yield with maximum productivity than other organic source. For comparison the results shown in Table 4 and 5 illustrated that the organic nitrogen source enhanced bacterial growth as well as hydrogen production than inorganic source.

The maximum hydrogen yield as shown in Fig. 1a indicated that the highest H_2 yield (308, 258 and 228 mLg^{-1} glucose utilized) was obtained by using 13 gL^{-1} yeast extract, peptone and trypton respectively. Compared with inorganic nitrogen source, the results shown in Fig. 1d and f indicated that organic nitrogen source was better for hydrogen production as well as bacterial growth. The highest hydrogen yield of 308 mL g^{-1} glucose utilized was obtained using 13 g L^{-1} yeast extract with maximum biomass concentration of 1.1 g L^{-1}. It was observed from the above results that nitrogen source had a marked effect on H_2 production.

In general, H_2 production by cultures supplemented with organic nitrogen as shown in Fig. 1e was higher than those supplemented with inorganic nitrogen sources.

Cultures supplemented with yeast extract, peptone and tryptone produced higher H_2 yields. Among these sources, yeast extract was the best source of nitrogen for hydrogen production and these results agreed with the finding of Lay[10] he found that the cultures supplemented with yeast extract, tryptone and peptone produced higher H_2 yields with near complete sugar consumption among these sources, yeast extract was the best source of nitrogen for hydrogen production, they reasoned that yeast extract facilitated the highest production rate.

Table 1: Hydrogen production by *C.acetobutylicum* NCIMB13357 using RCM medium.Y_{H2} (mL L^{-1}), [Biomass] (gL^{-1}). Biomass production L^{-1} culture, $Y_{P/X}$ (mL g^{-1}): (H_2 mL g^{-1} Biomass/). H_2 P (mL L^{-1} h^{-1})

	H_2 P	Y_{H2}	[Biomass]	$Y_{P/X}$
RCM	63.5	1400	1.4	200

Inoculum size 10% (v/v), I pH. 7.0, Temp 30ºC

Table 2: Hydrogen production by *C.acetobutylicum* NCIMB13357 with different organic nitrogen source

Nitrogen source	f pH	Glucose consumed (%)	H_2 P
Peptone	4.53	78	46
Trypton	4.55	80	41
Yeast extract	4.49	78	55

[Glucose] .5 g L^{-1}, Nitrogen source concentration 13 g L^{-1}, inoculum size 10% (v/v), I pH. 7.0, Temp 30ºC

Table 3: Hydrogen production by *C.acetobutylicum* NCIMB13357 with different organic nitrogen source

Nitrogen source	$Y^1_{P/S}$	$Y^2_{P/S}$	[Biomass]	$Y_{P/X}$	$Y_{X/S}$	$Y_{H2/s}$
Peptone	201	258	1.24	208	0.25	0.024
Trypton	182	228	1.11	205	0.22	0.020
Yeast extract	240	308	1.10	280	0.22	0.028

$Y^1_{P/S}$ (H_2 mL g^{-1} glucose supplied) (mL g^{-1})), $Y^2_{P/S}$ (mlg^{-1}) (Utilized): (H_2 mL g^{-1} glucose utilized), [Biomass] (g L^{-1}). Biomass production gL^{-1} culture, $Y_{P/X}$ (mL g^{-1} L^{-1}): (H_2 m L g^{-1} Biomass L^{-1}), $Y_{X/S}$: (Biomass production per g glucose supplied), $Y_{H2/s}$ (conversion of H_2 (mL) to H_2 (g) per g glucose utilized) [Glucose] .5 g L^{-1}, inoculum size 10% (v/v), I pH. 7.0, Temp 30ºC

Table 4: Effect of organic and Inorganic nitrogen source addition on glucose consumption and H_2 P (m L^{-1} h^{-1}) by *C.acetobutylicum* NCIMB13357

Nitrogen source	f pH	Glucose consumed (%)	H_2 P
Organic			
Peptone	4.51	78	46
Trypton	4.53	80	41
Yeast extract	4.49	78	55
Inorganic			
Ammonium sulfate	4.21	70	30
Ammonium nitrate	4.67	65	33
Ammonium chloride	4.58	60	28

[Glucose] .5 g L^{-1}, Nitrogen source concentration 13 g L^{-1} inoculum size 10% (v/v), I pH. 7.0, Temp 30ºC

The results of present study also agreed with Mongi *et al.*[11] they found the yeast extract using 0.1% was the best nitrogen source for hydrogen production and finally with Morimoto *et al.*[7] they reported that by using 0.2% of yeast extract, the hydrogen yield was the best among the nitrogen source they used. Lower final culture pHs for yeast extract as shown in Table 2 and 4 indicated that more acids was produced suggested that the substrate utilization was better for hydrogen production than other nitrogen sources.

Replacing organic with inorganic nitrogen sources resulted in poor H_2 production as well as bacterial growth. The results shown in Fig. 1a and e were fully agreed with a number of investigators that they have used inorganic nitrogen sources such as ammonium hydrogen carbonate[10,12-14] and ammonium chloride[15,16] in H_2 fermentation media, their results indicated that the lower yield they obtained it might be due to the nitrogen source and the microorganism(s) they were used, others have shown that when ammonium chloride replaced peptone as a nitrogen source, H_2 yields are halved[17]. These observations were attributed their lower hydrogen yield to the composition of the nitrogen source in fermentation medium they used.

Table 5: Effect of Organic and Inorganic nitrogen source on H_2 production by *C.acetobutylicum* NCIMB13357

Nitrogen source	$Y^1_{P/S}$	$Y^2_{P/S}$	[Biomass]	$Y_{P/X}$	$Y_{X/S}$	$Y_{H2/s}$
Organic						
Peptone	201	258	1.24	208	0.25	0.024
Trypton	182	228	1.11	205	0.22	0.020
Yeast extract	240	308	1.10	280	0.22	0.028
Inorganic						
Ammonium sulfate	130	186	1.03	181	0.2	0.016
Ammonium nitrate	143	220	1.00	220	0.2	0.020
Ammonium chloride	120	200	1.01	198	0.2	0.0018

$Y^1_{P/S}$ (H_2 mL g^{-1} glucose supplied) (mL g^{-1})), $Y^2_{P/S}$ (mL g^{-1}) (Utilized): (H_2 mL g^{-1} glucose utilized), [Biomass] (g L^{-1}). Biomass production g per L culture, $Y_{P/X}$ (mL g^{-1} L^{-1}): (H_2 ml per g Biomass per L), $Y_{X/S}$: (Biomass production per g glucose supplied), $Y_{H2/s}$: (conversion of H_2 (mL) to H_2 (g) per g glucose utilized) [Glucose] .5 g L^{-1}, Nitrogen source concentration 13 g L^{-1} inoculum size 10% (v/v), I pH. 7.0, Temp 30°C

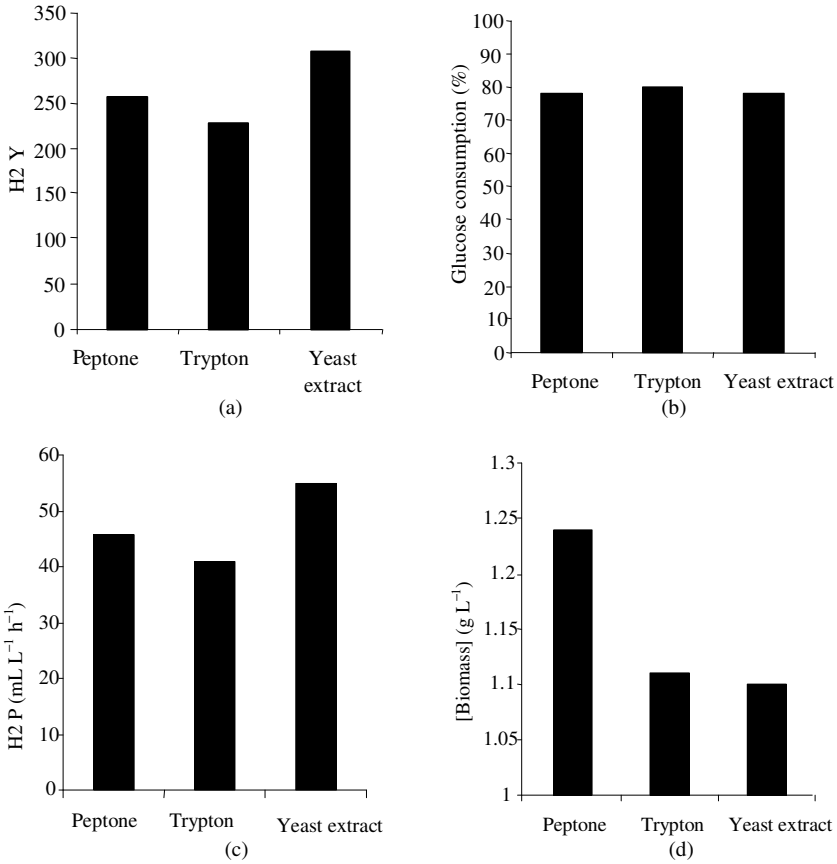

Fig. 1: Effect of Organic Nitrogen Source (13 gL^{-1}) on (a) H_2 yield (mLg^{-1}) (Utilized), (b) glucose consumption (%), (c) H_2P (m L^{-1} h^{-1}) and (d) Biomass concentration (g L^{-1}). [Glucose] .5 g L^{-1}, Nitrogen source concentration 13 g L^{-1}, inoculum size 10% (v/v), I pH. 7.0, Temp 30ºC

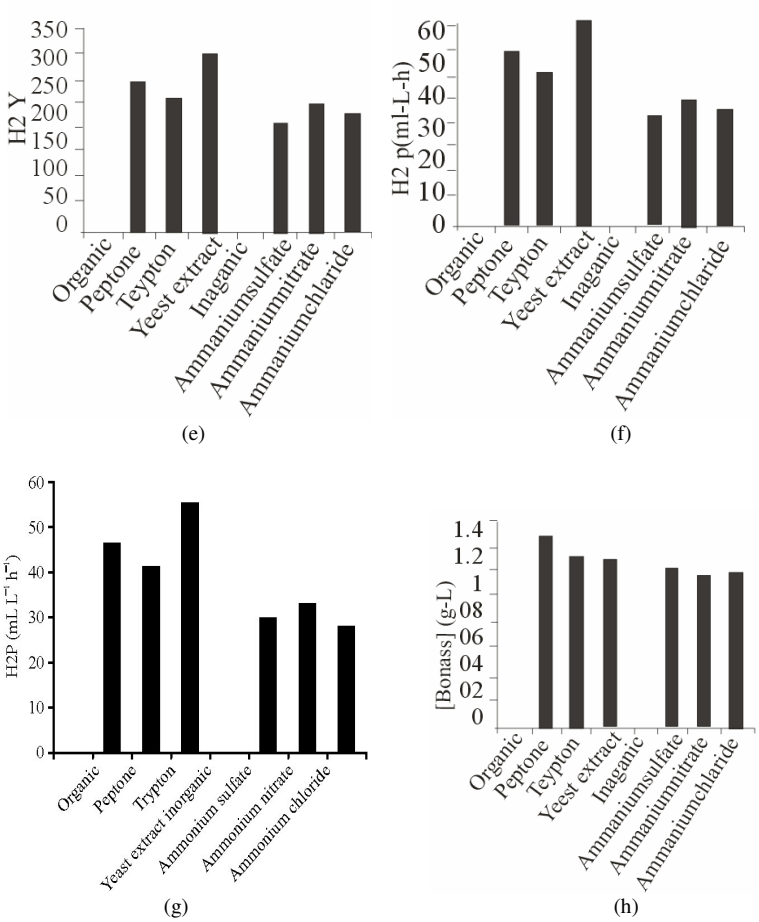

Fig. 1: Effect of Nitrogen Source (13g $^{-}$l each) on (e) H_2 yield (mLg^{-1}) (Utilized), (f) Glucose consumption (%), (g) H_2P ($mlL^{-1}h^{-1}$) and (h) Biomass concentration (gL^{-1}).[Glucose] .5 gL^{-1}, Nitrogen source concentration 13 gL^{-1} inoculum size 10% (v/v), I pH. 7.0, Temp 30ºC

It was reported by Stanbury *et al.*[18] that ammonium salt such as ammonium sulfate will usually produce acid conditions as the ammonium ion is utilized and the free acid will be liberated, whereas nitrates will normally cause an alkaline drift as they are metabolized like ammonium nitrate will first cause an acid drift as the ammonium ion is utilized, and nitrate assimilation is repressed. When the ammonium ion has been exhausted, there is an alkaline drift as the nitrate is used as an alternative nitrogen source. These reports showed that inorganic nitrogen source did changes in

Effect of medium C/N ratio on hydrogen production: The results had shown in previous section that organic nitrogen was better for hydrogen production as well as bacterial growth. The results shown in Table 6 and 7 demonstrated that the nitrogen concentration affect in both ways (increasing or decreasing) on hydrogen production as well as bacterial growth.

The results shown in Table 7 indicated that nitrogen source and its concentration (measured according to Kjeldahl method[9] have affected on the quantity of hydrogen production and showed that yeast extract was the best nitrogen source and 5.0 g L^{-1} 70 mg g^{-1} N_2 was the best concentrations for maximum hydrogen production. Following the Kjeldahl method[9], nitrogen concentration in 5g of yeast extract has only 70 mg of nitrogen concentration (1g of YE have only 14 mg N_2. According to this ratio, the optimum C/N ratio of 70 C = 5 g L^{-1} was the best for maximum hydrogen production by *C.acetobutylicum* NCIMB13357.

Microflora requires a proper nitrogen supplement for metabolism during fermentation. However, the results shown in Table 5 indicated that organic nitrogen is the preferred nitrogen source. Whereas the results shown in Fig. 2a suggested that at C/N ratio of 70 the hydrogen yield was enhanced from 308 to 350 mLg^{-1} glucose utilized. This finding fully agreed with the finding of Tanisho *et al*.[19] they reported that *Enterobacter aerogenes* st.E.82005 yielded 0.5 mole hydrogen from 1 mole glucose under glucose-peptone culture but when they change the peptone from 5-10 gL^{-1}, the yield was enhanced to 1.16 mole H_2/mole of glucose, they reasoned that for the substrate they used (Molasses) it might not contain sufficient nitrogen source for bacterial growth. Suggested that proper C/N ratio enhance the bacteria for more growth and substrate utilization, also with Aiyer[20] he was studying the effect of C/N ratio on *Bacillus licheniformis* SPT 27 to produce alpha amylase.

Table 6: Effect of different C/N (Glucose/ Yeast Extract) ratio and its effect on glucose consumption and H_2 P ($mlL^{-1}h^{-1}$)

(C/N) ratio	f pH	Glucose consumed (%)	H_2 P
36	4.51	77	49
71	4.46	80	70
143	4.46	75	52
238	4.47	77	49

[Glucose] (5 gL^{-1}), inoculum size 10% (v/v), I pH. 7.0, Temp 30ºC

Table 7: Effect of different C/N (glucose/ yeast extract) ratio and its effect on hydrogen production

(C/N) ratio	$Y^1_{P/S}$	$Y^2_{P/S}$	[Biomass]	$Y_{P/X}$	$Y_{X/S}$	$Y_{H2/s}$
36	225	292	1.15	254	0.23	0.028
71	280	350	1.22	287	0.24	0.032
143	227	303	1.03	294	0.20	0.028
238	214	278	0.84	331	0.17	0.024

$Y^1_{P/S}$ (H_2 mL^{-1} g glucose supplied) (mL $g^{-1)}$), $Y^2_{P/S}$ (mLg^{-1}) (Utilized): (H_2 mL^{-1} g glucose utilized), [biomass] (g L^{-1}). Biomass production g per L culture, $Y_{P/X}$ (mL $g^{-1}L^{-1}$): (H_2 mL per g Biomass per L), $Y_{X/S}$: (Biomass production per g glucose supplied), $Y_{H2/s}$ (conversion of H_2 (ml) to H_2 (g) per g glucose utilized) [Glucose] .5 g L^{-1}, Nitrogen source concentration 13 g L^{-1}, inoculum size 10% (v/v), I pH. 7.0, Temp 30ºC

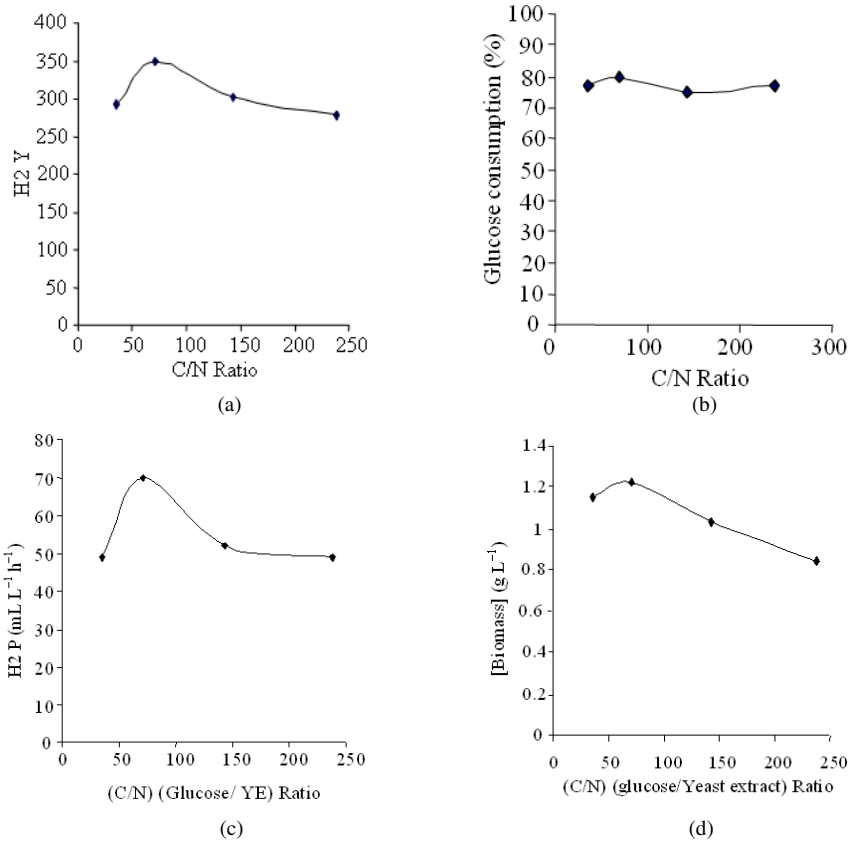

Fig. 2: Effect of (Glucose/ Yeast Extract) Ratio on (a) H_2 yield (mL g^{-1}) (Utilized), (b) Glucose consumption (%),(c) H_2P (ml L^{-1} h^{-1}) and (d) Biomass concentration (g L^{-1}). [Glucose] .5 g L^{-1}, inoculum size 10% (v/v), I pH. 7.0, Temp 30ºC

He found that peptone and ammonium hydrogen phosphate as nitrogen source were the best among all organic and inorganic source they used with optimum C/N ratio of 1:1 was the sufficient to maximize the bacterial productivity of alpha amylase. Whereas, Gottschalk and Morris[21] reported that using ammonium as nitrogen source for solvent production, they stated that they failed to obtain significant levels of solvents, reasoned that to the ammonia/glucose ratio of the fermentation medium. They claimed that C/N ratio of the medium cannot induce the bacterium they used for solvent production. All of these finding suggested that proper C/N ratio should be used to get the maximum production of such product.

The results of present study agreed with Morimoto *et al*.[7] they reported by increasing the yeast extract from 0.2-0.4%, the hydrogen yield of 2.1 mol^{-1} mole of glucose was increased by 30%, but with further increase to 0.8%, the hydrogen yield was decreased by 50%. They reasoned that to the nitrogen concentration in fermentation medium, since they used POME as substrate with mixed culture under thermophilic condition. Whereas Yokoi *et al*.[22] reported that, hydrogen was not produced by *C. butyricum* when they cultivate without nitrogen source, but with organic nitrogen but not from inorganic, above 0.1% of polypeptone, the hydrogen was produced and the amount of hydrogen was maximized to 2.4 mol/mole glucose, but when they reduce the polypeptone to 0.05%, the hydrogen yield decreased markedly suggested that the addition of this concentration 0.1% was necessary for maximum hydrogen production by *C. butyricum*.

Hydrogen productivity was enhanced as shown in Fig. 1c, g and Fig. 2c by using proper nitrogen source with proper C/N ratio from 55 m L^{-1} h^{-1} using 13 g L^{-1} of yeast extract to maximum of 70 m L^{-1} h^{-1} using 5 g L^{-1}.of yeast extract. Enhancement of bacterial productivity of hydrogen attributed to the biomass concentration which also was increased as shown in Fig. 1d and Fig. 2d from 1.1 g L^{-1} using 13 g L^{-1} of yeast extract to max of 1.22 g L^{-1} using 5 g L^{-1} of yeast extract suggested that proper C/N ratio enhanced the bacterial growth.

The results of this study indicated that by using Yeast extract as nitrogen source and with C/N ratio of 70, hydrogen yield by *C. acetobutylicum* NCIMB13357 was the best. Furthermore, increasing or decreasing of this ratio would adversely affect on both hydrogen production and bacterial growth.

DISCUSSION

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[11]. By contrast, the cell spends more energy and time in synthesizing amino acids for protein synthesis from inorganic nitrogen sources[11]. Among organic nitrogen sources, differences in protein and amino acid composition could have accounted for the differences in the production rates and yields observed. Yeast Extract comprises the water soluble components of the yeast cell, the composition of which is primarily amino acids, peptides, carbohydrates and salts. Yeast Extracts are rich in nitrogen, vitamins and other growth stimulating compounds and therefore are used as an ingredient in media for the cultivation of microorganisms

C/N ratio of 70 obtained in this study was higher than 47 obtained by Lin & lay[4] who found at a C/N-ratio of 47, the hydrogen yield reached 600 mL g^{-1} sucrose. They attributed this increased by 500%, compared with the blank to proper C/N-ratio and that lead to enhancement of hydrogen production since they used mixed culture but for the bacterium we used in this study we found that at optimum C/N ratio of 70, hydrogen yield was the maximum and reached to 350 mL g^{-1} glucose utilized 280 mL g^{-1} glucose; 2.24 mol H_2/ mol glucose with maximum increase in the yield of 308 to 350 mLg^{-1} glucose utilized (240 to 280 mL g^{-1} glucose) of 14%. Since sucrose is disaccharide and gave two times than glucose.

Finally the final H_2 yield obtained by using the new medium was 280 mL g^{-1} glucose supplied 2.24 mol H_2 mol^{-1} glucose supplied): (According to Wooshin *et al*.[23]: Each 125 mL of $H_2 \approx$ 1 mole H_2, and that was higher than reported values in the literature for mesophilic species of clostridia as reported by Collet et al [24], and using glucose as substrate.

CONCLUSION

This study demonstrated that nitrogen source and proper C/N-ratio enhances hydrogen production. The hydrogen-producing microorganism activity exhibits a C/N-ratio-dependent characteristic. According to our results, a C/N-ratio of 70 gave the maximum hydrogen yield

ACKNOWLEDGMENT

The author would like the thank Universiti Kebangsaan Malaysia for financial assistance under grant No UKM-OUP-BTK-14/2007.

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