World Academy of Science, Engineering and Technology
International Journal of Biotechnology and Bioengineering
Vol:7, No:9, 2013

A Study of Fatty Acid Production in the Batch Reactor via the Carbohydrate Fermentation by *C. butyricum*

H. Azan T., R. W. Lovitt, Nur K. T., N. Azwa. M. B.

***Abstract*—**Carbohydrate can be used as a substrate that can be consumed by *C. butyricum* and converted to useful chemicals such as acetic and butyric acid. Influence of concentration and types of carbohydrate to cell growth, carbohydrate consumed, productivity and carbon balance have been explored. Batch reactor was selected in this study to avoid contamination due to simpler operation system. Glucose was preferred as first types of carbohydrate to be tested. Six concentrations were studied from 0 to 28g/L. Eventually, 15g/L has shown the best concentration for glucose in term of growth rate ($2.63h^{-1}$) and carbon balance (99.76% recovery). Comparison for types of carbohydrate was also conducted. 15g/L of xylose (monosaccharide) and starch (complex carbohydrate) was tested. In term of growth rate and productivity, glucose showed the best carbohydrates. Results for this study showed that glucose and xylose produced more than 80% of acetic acid and less than 20% of butyric acid. Meanwhile, 63.1% of acetic acid and 36.9% of butyric acid were produced from starch.

Keywords*—C. butyricum*, glucose, starch, xylose, carbohydrate.

I. Introduction

BATCH reactor of which the reactor is filled with organic matter and microorganisms and the process of decomposition is allowed to proceed for a predetermined time or until gas production decreases to a predetermined (low) rate. Normally, in this type of operation, 10 to 20% of material is left as seed when the reactor is reloaded and the operation repeats [1]. Many researchers have reported the application of this operation to culture *C. butyricum* [2]-[4]. In batch cultures, the growth of microorganism will pass through a number of phases and typically starts with the lag phase in which the cells adapt to their new environment. The specific growth rate (μ) then slowly accelerates until it reaches the log or exponential phase. In this phase, the growth is only limited by the capacity of biomass to grow (the maximum specific growth rate (μ^{max}) and the cell concentration (x)). When the microorganisms have been growing in the exponential phase for some time, nutrient depletion and possible end product inhibition will occur causing the growth rate to slow down and finally stop as the culture enters the stationary phase. Here, the growth rate (μ) for the microorganism is equal to the death rate (kd) of the microorganism represents by dx/dt=0. Sometimes after the stationary phase, the microorganism enters the death phase where there is a net loss of active biomass. Fig. 1 shows the graph for the concentration of cell versus time in this system. The shaded area is the cell productivity (g/Lh^{-1}) and the slope of graph gives $\mu(h^{-1})$.

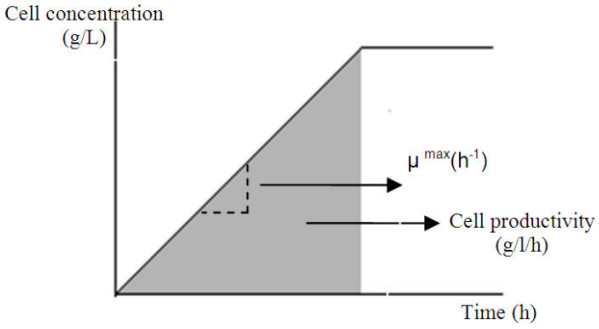

Fig. 1 Cell concentration versus time in the batch system Cell productivity will be dependent on the period in which the culture is harvested

Meanwhile, *C. butyricum* is capable of metabolizing a wide variety of carbohydrates most notably hexose sugars and polymers (i.e. glucose, fructose, sucrose, lactose and starch), pentose sugars such as xylose and ribose. Therefore, *C. butyricum* possesses some capability to produce extracellular enzymes such as amylase. However, this organism is not cellulolytic, lipolytic and proteolytic and cannot ferment sugar alcohols like glycerol and mannitol. These properties indicate that they are saccharolytic organisms that can ferment a wide variety of wastes including waste sugar streams such as molasses into acetate, butyrate, carbon dioxide and hydrogen. Many researchers have been studied the capability of *C. butyricum* in sugar substrates in the limited concentration [3], [5], [6]. However this study, was investigate in variety of glucose, started from the lower to the higher concentrations and it will compared with xylose and starch concentration. The biochemistry of fermentation can be viewed as a process by which sugar is reconfigured within the cell to give a mixture of reduced and oxidized end products and by doing so, it generates energy for the growth. A key aspect of this is the maintenance of the redox balance of electron flow between electron that generating the reactions and electron consuming reactions as the cell must recycle the electron carriers to

H. Azan T. is with the School of Industrial Technology, Universiti Sains Malaysia (phone: +6046534302; fax: +6046573678; e-mail: azan@ usm.my).
R.W. Lovitt is with the Department of Chemical and Biological Engineering, Swansea University (e-mail: r.w.lovitt@swansea.ac.uk).
Nur K. T. is with the Faculty of Agriculture, Universiti Teknologi Mara (e-mail: nisya1087@yahoo.com).
N. Azwa M. B. is with the Faculty of Civil Engineering, Universiti Teknologi Mara (e-mail: nurazwa.bashar@ppinang.uitm.edu.my).

maintain rapid metabolism. If there is a redox imbalance then the carbon flow (and hence energy generation) through metabolism slows, reducing growth rates and cell yields. Fig. 2 shows the decomposition of carbohydrate and its transformation to acetic and butyric acid, carbon dioxide and hydrogen. As shown, the organism can ferment a wide variety of carbohydrates and these are taken up by the cell and metabolized to 2 mole of pyruvate plus 2 moles of reducing equivalent (NADH) and 2 moles of ATP via the Emden-Myerhof-Parnas pathway. This is the most common carbohydrate pathway in anaerobic bacteria. From pyruvate, the carbon metabolism is more specialized and restricted to strict anaerobes because of the involvement of electron transfer processes involving ferredoxin (Fd) [7]. A series of enzymes allow the electrons to flow from NADPH and NADH and thus allows electron transfers between the key parts of metabolism within these organisms. The end result is the generation of Hydrogen derived from electrons being transferred to hydrogenise to form molecular hydrogen and reduced end products such as butyrate. Both ferredoxin and hydrogenise are easily poisoned by oxygen and cause serious metabolic problems within the anaerobes where electron balances are easily disturbed. The carbon flow from the pyruvate is via a further decarboxylation to release carbon dioxide and form Acetyl CoA. This can then be either metabolized to acetate with the generation of ATP or it is condensed and is further metabolized to 4 carbon compounds that are further reduced to form butyrate plus ATP (Fig. 2). It should be noted that the amount of ATP formed by substrate phosphorylation is regulated by the relative proportions of acetate and butyrate. It is to the advantage of the growth of the organism to produce as much acetate as possible from glucose. In theory, if more hydrogen is released then more acetate (and hence more ATP) can be generated per mole of glucose metabolized. For this to occur, the electron flow has to be very efficient, however, this is dependent on the type and characteristics of the enzymes associated with the ferredoxin reduction either directly or indirectly from NADPH and NADH. The extreme situation of acetate, CO_2 and hydrogen production is not observed in reality as the thermodynamics of electron transfer to hydrogen are not favorable. However, external electron acceptors (oxygen and some organic materials) can shift the balance towards acetate away from butyrate.

II. Methodology

A. Preparation Inoculum

C. butyricum NCMB 7432 used in this research was supplied by NCIMB LTD (23 St. Machar Drive, Aberdeen, U.K). Recitation media for culture *C.butyricum;* Yeast Extract 10g/L, glucose 1g/L, rezurin 0.05% (w/w) and $(NH)_4SO_4$ 0.5g/L. The inoculum was started with 30mL serum vial cultures containing 25mL of growth media. Then, 4mL was taken and transferred to 50mL serum vials containing 40mL of growth media. This was incubated for 24 hours.

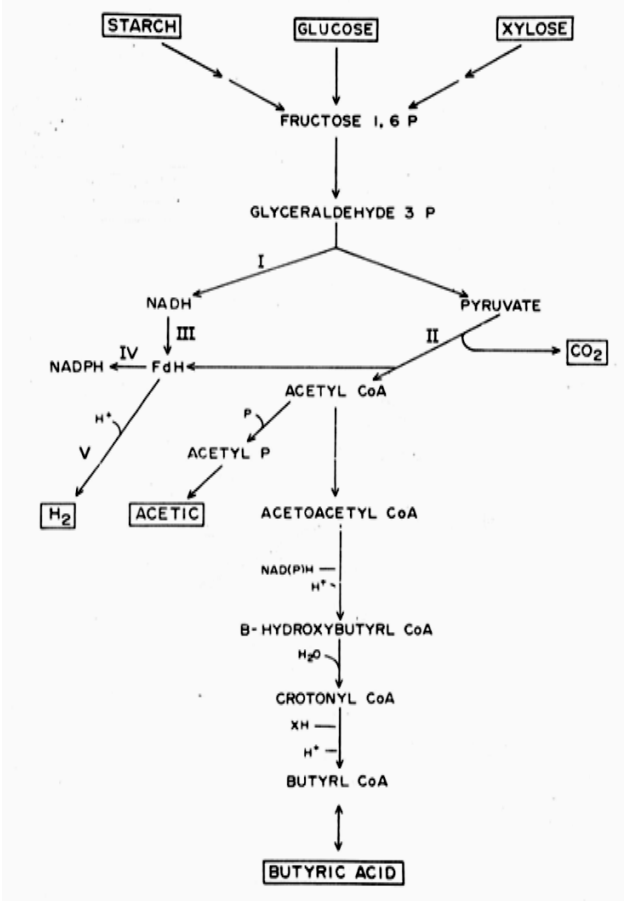

Fig. 2 The proposed carbon flow pathways for *C.butyricum* in carbohydrate fermentation [8]

B. Preparation of Batch Culture

A 2L jar fermenter with a diameter of 12.5cm and a height of 21cm was prepared. The top of the fermenter consisted of a ground glass flange on to which a silicon rubber sheet of 15 cm in diameter was clamped. A series of 12 x 1.2 cm holes were cut to place ports or glass tubing to allow for addition of material and probes to measure variables within the reactor. Fig. 3 shows the batch fermenter complete with arrangements. The fermenter was connected to other equipment via the ports, for example the alkali addition system including pump and pH meter; gas in and out, and addition ports for inoculations and sample together with temperature control by thermostatic water tank and heat exchanger.

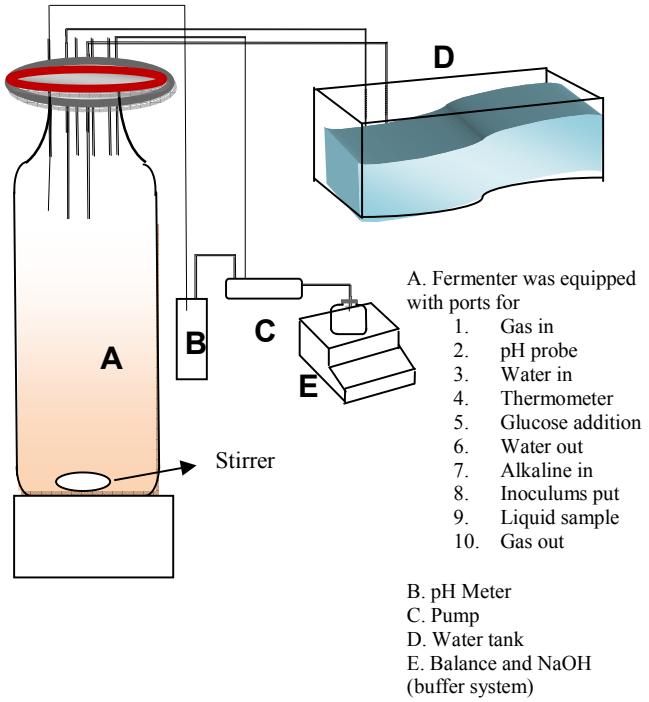

Fig. 3 The batch process system

C. Analysis

1. Acetic and Butyric Concentrations

A headspace method was used to analyze the concentration of acetic and butyric acid using GC. A 10mL sample was taken and was added to 5g NaCl in the 30mL serum bottle. The bottle was sealed and placed in the water bath at a temperature of 50°C for 10 minutes. Once equilibrated, 0.5 mL of gas was taken with a glass gas-tight syringe from the headspace of bottle and injected into the GC for analysis. The responses of the GC were recorded and the concentrations of the fatty acids were calculated from a standard calibration. A VARAIN ProStar GC-3800 (USA) fitted with flame ionization detector (FID) was used. Hydrogen gas for the FID was produced in-situ by a Hydrogen generator: UHP-20H NITROX (Swan Hunter, UK). While the air and the carrier gas, helium, were supplied as compressed gases from cylinders (BOC, UK). A 15m x 0.25mm x 0.25µm fused silica capillary column coated high-quality polyimide was fitted (Sigma, UK).

2. Cells

Absorbance using UV spectrophotometer was used to directly measure the cell concentration expressed as dry weight. Optical density (O.D) can be used to measure biomass or dry weight (g/L) [9]-[11]. A calibration of absorbance for correction factor at 660nm, 1cm light path versus cell concentration was carried out. To obtain the dry weight of the culture, 50ml of fermentation broth was centrifuged at 4°C and 10000rpm (MSE, SANYO) for 20 minutes to produce a cell pellet. The cells were then washed three times using distilled water and weighed after drying at 105°C for 24 hours. The relationship between dry weight and absorbance at 660 nm for *C. butyricum* was $y = 0.0959x - 0.0006$.

3. Glucose, Xylose, and Starch Analysis

Glucose and starch were analyzed by assay kit. The assays were supplied as a kit from Sigma (GACO-20 and STA 20). Meanwhile, xylose was determined using 3-5 dinitrosalicylic acid (DNS) reagent.

III. Results

A. Effect of Glucose Concentration to *C.butyricum*

In the first step, batch experiment with a range of glucose concentrations were investigated from 0 to 28g/L. Fig. 4 shows the growth rate of *C. butyricum* versus concentration of glucose (g/L). The growth rate was the lowest at 0g/L and increased up to 15g/L but further increased in glucose will reduce the growth rate. The graph in Fig. 4 shows the peak growth rate to be 0.263µ at 15g/L and the lowest growth rate at 0g/L was 0.094µ.

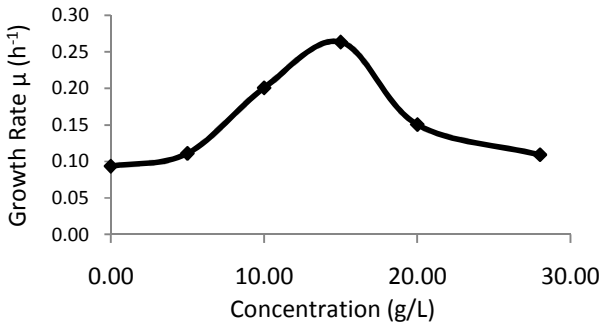

Fig. 4 The effect of glucose concentration to growth rate of *C. butyricum*

Fig. 5 shows the graph of concentration for acetic and butyric acid formed during the fermentation. For the acetic acid, the concentrations increased from 0g/L to 15g/L of initial glucose. At 0g/L of initial glucose, the production of acetic acid was 0.002 mol/L and it further increased to 0.20 mol/L at 15g/L. From 15g/L to 28g/L, the production of acetic acid decreased probably due to spectrum changes of fermentation products such as solvents or other non-volatile products. At 28g/L of initial glucose, the amount of acetic production was 0.104mol/L. Fig. 5 also shows the pattern of butyric acid concentration. Butyric acid concentrations increased from 0g/L to 28g/L of initial glucose concentration. However, the increase in butyric acid concentration was stimulated between 5g/L to 15g/L glucose concentration. However, from 15g/L to 28g/L the increase slowed. Presumably, high glucose concentrations alter the end products spectrum or the products themselves shift the fermentation away from the acetate and butyrate. Fig. 5 also shows the ratio for acetic and butyric acid levels for each concentration of glucose. The ratios have been calculated by dividing the amount of acetic acid (mol/L) by the amount of butyric acid (mol/L). 0g/L of glucose concentration produced 14 mol of acetic and 1 mol of butyric

acid. Meanwhile, 5g/L of glucose produced 21 mol of acetic acid and 1 mol of butyric acid. Then, 10g/L of glucose produced 6 mol of acetic acid and 1mol of butyric acid. Next, 15g/L produced 3 mol of acetic acid and 1 mol of butyric acid. Lastly for 20g/L and 28g/L of glucose have produced 2.4 mol and 2.2 mol of acetic to 1 mol of butyric acid. The explanation for the shift in the ratio could be due to the presence of an excess of electron acceptors at low glucose concentration (from the yeast extract for example) while it shifts to low ratios at high glucose concentration where the relative electron acceptor concentrations are lower. An alternative explanation may be due to the high glucose concentrations that force the production of butyrate with a shift away from hydrogen formation. Unfortunately, hydrogen production has not been measured in this research.

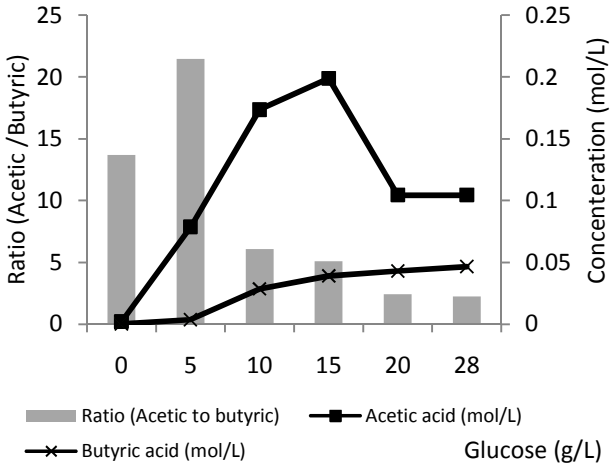

Fig. 5 The effect of glucose concentration on acetic and butyric acid production and the acetate butyrate ratio in 2L batch culture at pH 6.5 and 37°C

Table I shows the fermentation balance for variety of glucose concentrations. Fermentation balances on the glucose fermentations of *C butyricum* were carried out to account for the carbon that can be found in the end products of the fermentation. The assumptions made for these calculations were that all the carbon in yeast extract should be considered, that for every mole of acetate, 1 mole of carbon dioxide was formed. For every mole of butyrate two moles of CO_2 were formed. Biomass was assumed to be 50% C.

Table I shows that good results for carbon balances were identified at 10 and 15g/L while at 5, 20 and 28g/L between 40 to 60% due to other fermentation products were produced or yeast extract was not utilized effectively in these conditions. The most probable additional products are solvents; however, in this study no analysis of solvents was considered. The most probable products are diols, however this has to be confirmed. Due to 15g/L of glucose showed almost the best concentration in a few aspects; therefore 15g/L of concentration was chosen for comparison of variety carbohydrate.

TABLE I
CARBON FERMENTATION BALANCES FOR A RANGE OF GLUCOSE CONCENTRATIONS

Initial Glucose mM	ΔS in C mM	Yeast in C mM	Total C input	Cell in C	A + CO_2 (mM) in C	B + 2CO_2 (mM) in C	Total C output	% C Rec.
0.00 (0 g/L)	0.00	410.0	410.0	5.8	5.47	0.6	11.9	2.9
27.80 (5g/L)	122.4	410.0	532.4	7.8	214.9	20.1	242.8	45.6
55.50 (10g/L)	255.6	410.0	665.7	10.1	487.5	129.3	626.9	94.2
83.30 (15g/L)	381.3	410.0	791.3	12.1	542.7	234.6	789.4	99.8
111.00 (20g/L)	461.0	410.0	871.0	15.3	285.0	258.7	559.0	64.2
155.40 (28g/L)	690.1	410.0	1100.1	15.6	284.6	280.3	580.4	52.8

A= Acetic acid; B=Butyric acid; C=carbon; Rec.=Recovery
*Note
ΔS= Initial glucose-final glucose in the reactor
1. Cell mass was 50% C by weight. This is a typical value for bacterial cells [12].
2. Yeast extract was also 50% C by weight [13].
3. For each mole of acetate produced 1 mole of CO_2 (see biochemical pathways.This assumption can be illustrated in Fig. 1 which shows the metabolic pathway for the fermentation of glucose in this organism).
4. For each mole of butyrate produced 2 moles of CO_2 (see also Fig. 1).

A. Comparison between Variety of Carbohydrate

Table II shows the comparisons of growth rate (μ), doubling time (t_d) and biomass (max) from the data in Fig. 6. The specific growth rate for glucose ($0.263h^{-1}$) was faster than the starch ($0.192h^{-1}$) and xylose ($0.123h^{-1}$). Presumably, starch hydrolysis reduced the specific growth rate while xylose was most probably low because of xylose uptake and the metabolism was fundamentally slower than that of hexose sugar, glucose. Biomass (max) showed the same pattern as the growth rate. Higher biomass (max) produced by glucose at

0.291g/L, followed by starch at 0.178g/L and xylose at 0.151g/L.

TABLE II
THE GROWTH RATE (μ), T_D (H) AND OD MAX FOR STARCH, XYLOSE, AND GLUCOSE

Carbohydrate	Starch(15g/L)	Xylose(15g/L)	Glucose(15g/L)
Growth rate μ (h^{-1})	0.192	0.123	0.263
t_d(h)	3.59	5.61	2.62
Biomass (max) (g/L)	0.178	0.151	0.291

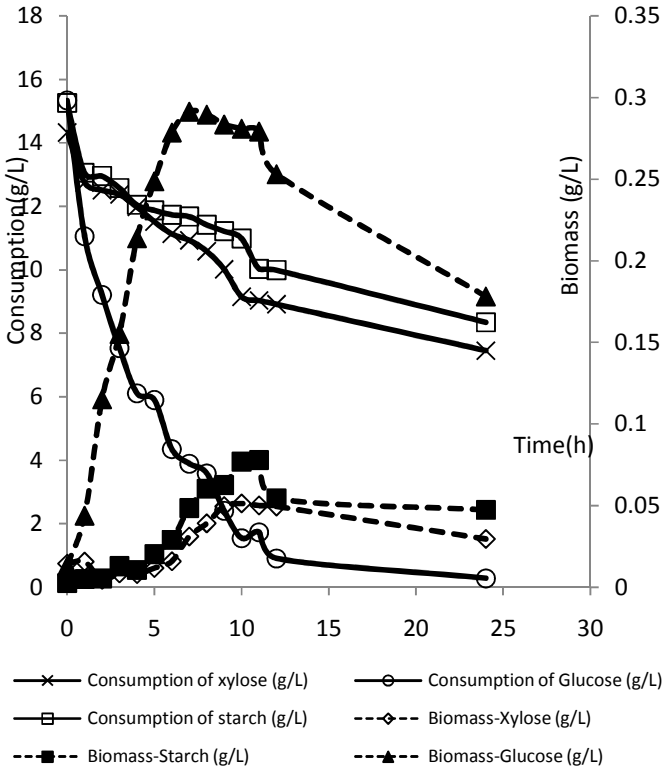

Fig. 6 A comparison of three cultures growth of glucose, starch and xylose in 2L batch cultures at pH 6.5 and 37°C. The substrate consumption and biomass formation were also shown

Fig. 7 shows the pattern of acetic and butyric acids that were produced by glucose, starch and xylose. For all fermentations, acetate acid was the major end product with some production of butyrate. Final acetate levels were 0.2 mol/L for glucose, 0.038 mol/L for starch and 0.075 mol/L for xylose. While final butyrate concentrations were 0.040 mol/L for glucose, 0.02 mol/L for starch and 0.015 mol/L for xylose. From comparison between Figs. 6 and 7, all of the substrates were recorded for their maximum production of acetic and butyric acids at the same time as biomass concentrations were in the maximum stage.

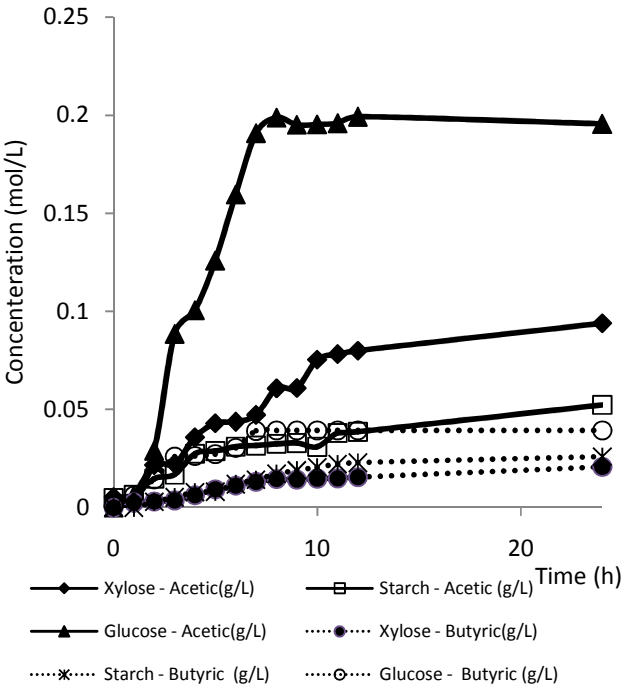

Fig. 7 Production of acetic and butyric acids in the variety of carbohydrates

Table III shows the comparison of glucose, starch and xylose in term of production and carbohydrate consumed. The most notable in this comparison was the difference between starch and the monosaccharide glucose and xylose, in which the butyrate production was enhanced on starch and that the acetate:butyrate ratio was much lower than that observed with xylose or glucose

TABLE III
A COMPARISON OF FATTY ACID ON GLUCOSE, XYLOSE AND STARCH

Type of Car.	Car. Cons.	A mol/L	B mol/L	Total acid mol/L	% total acid production A	% total acid production B	Ratio (A/B)	Ratio (total acid /Car.)
Glucose 15g/L	0.06	0.20	0.04	0.24	82.2	17.8	4.94	3.75
Xylose 15g/L	0.03	0.075	0.015	0.09	83.6	16.4	5.10	2.65
Starch 15g/L	0.03	0.038	0.022	0.06	63.1	36.9	1.71	1.88

A= Acetic acid; B=Butyric acid; Car.=Carbohydrate; Cons. = Consumed

IV. CONCLUSION

Glucose can be considered as an easier carbohydrate to be consumed compared to xylose and starch. This is because glucose is a simple sugar. 15g/L of glucose concentration was identified as the highest carbon recovery. Acetic acid was produced by glucose and xylose more than 80% and less than 20% for butyric acid. However for starch, the percentage of butyric acid was more than 35% and acetic acid less than 65%. This situation occurred as starch is a complex carbohydrate; therefore it has a potential to produce high percentage of butyric acid compared to xylose and glucose. Finally,

C.butyricum has a potential to consume carbohydrates and produce useful chemicals. Probably, this technology can be applied in the leachate, sludge or waste water for treatment and produce useful chemicals due to all of those materials have very high concentration of carbohydrate waste or organic matter.

UNITS

L
g/L
mol/L
mol/mol
h^{-1}

HELPFUL HINTS

A=Acetic acid
B=Butyric acid
Car.=Carbohydrate
Cons.=Consumed
Rec.= Recovery
μ=Growth rate
Td= Doubling time
CO_2=Carbon dioxide
h=hour

ACKNOWLEDGMENT

This studied was funded by Universiti Sains Malaysia and was operated in Swansea University, Wales, UK.

REFERENCES

[1] Chongrak, P. (1996). Organic Waste Recycling, 2nd edition. John Willey and Son Inc.
[2] Abbad A., S., Amine, J., Gerard P & Petitdemange, H. (1998). "Effect of glucose on glycerol metabolism by *Clostridium butyricum* DSM 5431.", Journal Application Microbiology. 84(4):515-22.
[3] Wang, X. & Jin, B. (2009). "Process optimization of biological hydrogen production from molasses by a newly isolated Clostridium butyricum." Journal of Bioscience and Bioengineering, 107(2):138-144..
[4] Vandak, D., Zigova, J., Sturdik, E. & Schlosser, S. (1997). "Evaluation of solvent and pH for extractive fermentation of butyric acid.", Proc. Biochemical, 32:245–251.
[5] Miyake, J., Mao, X.Y. & Kawamura, S. (1984). "Hydrogen photoproduction from glucose by co-culture of photosynthetic bacteria and Clostridium Butyricum.", J Ferment Technol, 62:531-5.
[6] Yoki, H., Mori, S., Hirose, J., Hayashi, S. & Takasoki, Y. (1998). "H_2 production from starch by a mixed culture of Clostridium Butyricum and Rhodobacter sp. M-19.", Biotechnol Lett., 20:895-9.
[7] Gerhard, G. (1979). Bacterial Metabolism. 1st ed., Springer Verlag, New York Inc.: pp 167-221.
[8] Petitdemange, H., Cherrier, C., Bengone, J.M. & Bray, R. (1977). "Etude des activites NADH et NADPH- ferredoxine oxydoreductasiques chez Clostridium acetobutylicum.", Canadian J. Microbiol., 23:152-160.
[9] Willis P.A. (1977), Anaerobic Bacteriology, Clinical and Laboratory Practise. 3rd ed., Butterworth and CO: pp 34-110.
[10] Demain L. A. & Davies, J. E. (1999) Manual of Industrial Microbiology and Biotechnology. 2nd ed., ASM Press Washington D.C: p 29-45, 49-60, 61-75, 80-93,183-147, 151-163,165-179.
[11] Avonts L., Van Uyten, E. & De Vuyst, L. (2004). "Cell growth and bacteriocin production of probiotic Lactobacillus strain in different media.", International Dairy Journal, 14:947-955.
[12] Atkinson, B., & Mavituna, F. (1991). Biochemical Engineering and Biotechnology Handbook. Macmilan, Stockton Press, 2nd Edition.
[13] Bridson E.Y. (1998). The Oxoid Manual. 8th Edition. Oxoid Limited, Wade Road, Bassingstoke.