Study the Suitability of Cheese Whey for Bio-Butanol Production by Clostridia

Mervat I. Foda[1*] Hong Joun2 and Yin Li[2]

[1*]Dairy Science Dept., National Research Center, Cairo, Egypt, [2]Institute of Microbiology, Chinese Academy of Sciences, Beijing, China
*mervat1m@yahoo.com

Abstract: The aim of the present paper is to investigate the feasibility of bio-butanol production by batch fermentation of cheese whey a dairy industry waste characterized by lactose concentration ranging from 4.5% to 5.0 % (w/w). The microorganisms used to carry out the fermentation processes was *Clostridium acetobutylicum* DSM 792 and *Clostridium acetobutylicum* AS 1.224. Preliminary experiments, performed in aerobic conditions on lactose medium lab experiments, have shown that *Clostridium acetobutylicum* DSM 792 was the best in the solvents production compared with AS 1.224. The bio reactor batch experiments were carried out on lactose and cheese whey media. The experimental data have demonstrated the process feasibility that cheese whey is an excellent substrate for fermentation and exhibits better performance with respect to lactose medium. [Journal of American Science 2010;6(8):39-46]. (ISSN: 1545-1003).

Key words: cheese whey, lactose, bio-butanol, bio-ethanol, *Clostridium acetobutylicum*

1. Introduction

When oil price suddenly increased in 1973, intensive research interests returned on the conversion of agricultural products into fuels and chemicals.

Biofuels used in transport are typically bioethanol which is used as a petrol substitute and biodiesel which is used as a diesel substitute. Also, butanol has sufficiently similar characteristics to gasoline to be used directly in any gasoline engine without modification and/or substitution. Butanol is superior to ethanol as a fuel additive in many regards: higher energy content, lower volatility, less hydroscopic (thus does not pick up water), and less corrosive (Dürre, 2007). Branched chain 4-carbon alcohols including isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol have higher octane numbers compared with n-butanol (Atsumi *et al*., 2008) and thus are good candidates as fuel additives (Lee *et al*., 2008).

During the early 1980s, many problems that have prevented butanol fermentation from being commercially viable were presented. These obstacles can be enumerated as: (1) use of dilute sugar solutions due to butanol inhibition; (2) low product concentration in fermentation broth which is attributed to butanol inhibition and results in cost intensive recovery; (3) low product yield, usually of the order of 0.3 due to conversion of approximately 53% of substrate into CO_2 and H_2; and (4) low reactor productivity. In spite of these problems, it has been stressed that butanol can be economically produced if cheaper substrates are used. Substrates that require minimum processing and fall in this category are by-products or waste products of agricultural industries such as molasses, and whey permeate (Dürre, 2007).

On the other hand, an estimated 90.5 billion pounds of whey was generated as a byproduct of cheese production in 2006, comprising about 85.8 billion pounds of sweet whey and 4.7 billion pounds of acid whey. Over the last 5 years from 2001 to 2006, the volume of whey increased by 15 % commensurate of whey increases in the production of cheeses. Besides the liquid carrier, the composition of whey is approximately 0.3 percent butterfat, 0.8 percent whey proteins, 4.9 percent lactose, and 0.5 percent minerals. Cumulatively, there were 4.4 billion pounds of lactose contained in the whey produced that year (Charles, 2008).

In Egypt, more than 70% of milk produced is used in cheese making to produce about 336939 tons fresh cheese annually (CAPMS, 2004), in addition to, all cheese varieties. In China, cheese production is increased rapidly from 10.000 tons (2006) to 15.000 tons (2008), which increased the amount of whey from 90.000 to 135.000 tons respectively. These are no plans for the utilization of whey in Egypt and China as in the most countries in the world. So, increasing cheese production will create pollution problems in addition to its fuel interest.

The present study is intended to investigate the possibility of using cheese whey as a source for bio-butanol production, evidencing the differences existing between lactose medium and cheese whey, which – in principle – could be used as raw materials to achieve fermentation processes aimed at bio-butanol production.

2. Material and Methods

Microorganisms

Clostridium acetobutylicum DSM 792, which corresponds to ATCC strain 824 was donated by Institute of Microbiology, Beijing, China. While, *Clostridium acetobutylicum* AS 1.224 was isolated from soil by Laboratory of Molecular Physiology and Systems Biotechnology, Department of Industrial Microbiology and Biotechnology, Institute of Microbiology Chinese Academy of Sciences, Beijing, China.

Preparation of lactose medium

The medium contained per L of deionized water: KH2PO4, 0.75 g, K2HPO4, 0.75 g, Mg SO4 – H2O, 0.4 g, MnS04, - H2O, 0.01g, Fe SO4 –7 H2O = 0.01 g, Na CI, 1 g , Yeast extract, 5 g, Asparagine, 2 g, (NH4) 2 SO4 , 2 g, Cysteine-HCL , 1g, Rezurium , 50 g lactose and l ml. CaCO3, 2 g (only for pre cultures). The pH value was adjusted to 7 using Na OH (2 N). After sterilization, the medium was sparged with nitrogen to remove the oxygen, and maintained under nitrogen pressure until its inoculation.

Preparation of cheese whey as fermentation medium

Cheese whey (containing 45 – 50 g/l lactose) was obtained from Inner Mongolia Yili Industrial Group Co., Ltd., Mongolia, China. Whey was boiled for 30 min to denature and precipitate most of the whey proteins (Chen and Zall, 1982). The precipitated whey protein was separated and the supernatant was filtered through cheese cloth to remove the balance of any minute amounts of protein.

Lab batch experiment:

The 3 ml of inoculum were grown in 100-ml screw-cap bottles with rubber septa under nitrogen using 50 ml of degassed lactose medium and inoculated at 37□C for 75 hrs with the agitation rate was 150 rpm; samples were taken after 50 and 75 hrs for analysis.

Bio-reactor batch experiments:

Best strain for butanol production was used to run bio-reactor batch experiments. Pre cultures were grown in 100-ml screw-cap bottles with rubber septa under nitrogen using 50 ml of degassed lactose medium and inoculated at□37 At least two transfers were prepared before fermentor inoculated. A fermentor 7.5-liter (New Brunswick Scientific Co., Edison, NJ) with pH and temperature control was used in batch fermentation. Two different batch experimentswere done using *C. acetobutylicum* DSM 792 with lactose medium and deproteinized whey (without adding yeast extract). Lactose medium (containing 50 g of lactose /liter) reached volume of 3.0 liter was inoculated with 300 ml of the pre culture. The pH for the fermentation was regulated at 5.0 by automatic addition of ammonia solution (NH3-H2O), the temperature was maintained at 37°C with150 rpm agitation rate. Samples were taken every 12 hrs for 5 days to determine different biofuels production.

HPLC analysis

The concentration of substrate and fermentation products was measured by a High-Pressure Liquid Chromatography (HPLC, Agilent 1200) with refractive index detector. The separation was obtained with an Aminex HPX-87H (Bio-Rad) column (300 by 7.8 mm). Elution was done at 15°C with 0.05 mM H2SO4 at a flow rate of 0.5 ml/min.

3. Results and Results

Bio-butanol formation in Lab batch experiment

Figure-1 and -2 show comparative formations of biofuels in lab batch experiment (lactose medium) using *Clostridium acetobutylicum* DSM 792, and *Clostridium acetobutylicum* AS 1.224 after 50 hrs of fermentation process.

It could be noticed that *Clostridium acetobutylicum* DSM 792 showed obvious differences from *Clostridium acetobutylicum* AS 1.224 by lactose utilization which has reflected to fermentation products. The intermediate accumulation of acetic and butyric acid was very low in *Clostridium acetobutylicum* AS 1.224 compared to *Clostridium acetobutylicum* DSM 792. These results are in agreement with those obtained by Bieb, (1999), found that butanol and acetone-producing strain DSM 2152, invalidly described as '*Clostridium saccharoperbutyl- acetonicum* ' was compared with the type strain *C. acetobutylicum*, *DSM 792*, with respect to solvent and acid formation at varying pH values and growth rates. Under all conditions strain DSM 2152 produced much lower amounts of butyric and acetic acids than the type strain. The pH optimum for solvent formation was higher, ie 5.5 instead of 4.5. Solvent formation occurred at higher dilution rates, but below 0.1 h^{-1} a lower solvent concentration was obtained, indicating that acid production was too low to provide a sufficient amount for acetone formation. The strain (*Clostridium acetobutylicum* AS 1.224) is a candidate for genetic improvement supplementary to the type

strain of *C. acetobutylicum* which is the subject of a rapid development in DNA recombination.

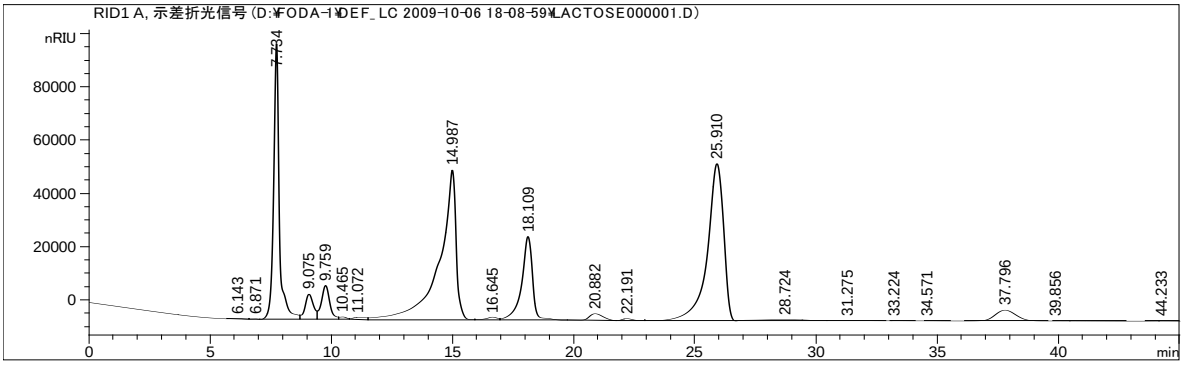

Fig. (1): HPLC-chromatogram of biofuels formation by *Clostridium acetobutylicum* DSM 792 using lactose medium (37^0 C, shaking velocity 150 rpm, pH 7.0) after incubation time for 5 hrs.

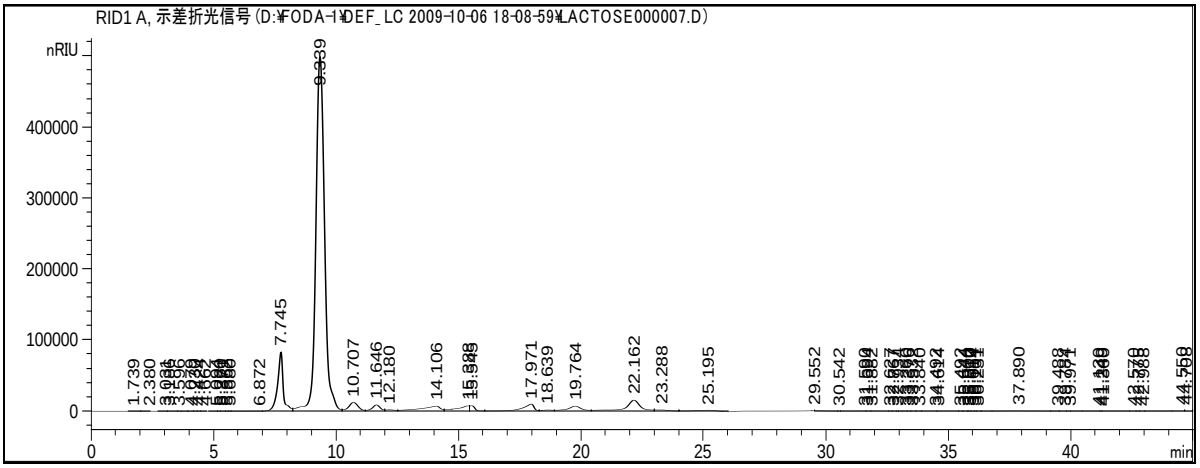

Fig. (2): HPLC-chromatogram of biofuels formation by *Clostridium acetobutylicum* AS 1.224 using lactose medium 37^0 C, shaking velocity 150 rpm, pH 7.0) after incubation time for 5 hrs.

Francisco and Russell (1996) reported that *Clostridium acetobutylicum* had phosphotransferase systems for glucose and lactose, and the lactose system was inducible. When *C. acetobutylicum* was provided with glucose and lactose, the cultures grew in a diauxic fashion, and glucose was used preferentially. Cells grown on lactose took up thiomethyl galactoside, and retained this non-metabolizable lactose analog for long periods of time. Because glucose inhibited thiomethyl galactoside uptake and caused the efflux of thiomethyl galactoside that had already been taken up, it appeared that *C. acetobutylicum* had inducer exclusion and inducer expulsion mechanisms similar to those found in lactic acid bacteria.

Table (1) shows lactose consumption and related biofuels production by the two Clostridium strains in lactose medium with initial pH 7 after 75 hrs. It can be seen that production of acetic and butyric acid by *Clostridium acetobutylicum* DSM 792 was higher amounts and slightly increased by prolonging the incubation time from 50 to 75 hrs. Ethanol and acetone production were decreased, while butanol amount was not changed. By *Clostridium acetobutylicum* AS 1.224, acetic acid and ethanol production was increased after 50 hrs, while other compounds did not detect. These results are in agreement with those reported by Lee *et al.*, (2008) who mentioned that the clostridia solvent production is biphasic fermentation, the first phase is the acidogenic phase, during which the acids forming pathways are activated, and acetate, butyrate, hydrogen, and carbon dioxide are produced as major products.

Table (1): Production of biofuels in lactose medium (37^0 C, shaking velocity 150 rpm, pH 7.0) by *Clostridium acetobutylicum DSM* 792 and AS 1.224 after 75 hrs.

Components (g/l)	*Clostridium acetobutylicum* DSM 792		*Clostridium acetobutylicum* AS 1.224	
	Incubation Time (Hours)			
	50	75	50	75
Lactose	5.28 ± 0.85	2.57 ± 1.84	38.28 ± 1.69	35.02 ± 1.80
Acetic acid	13.10 ± 1.00	13.41 ± 0.35	0.66 ± 0.35	2.56 ± 2.10
Ethanol	6.42 ± 0.18	4.94 ± 2.24	2.13 ± 1.52	4.38 ± 2.57
Butyric acid	10.47± 0.15	10.87 ± 0.53	0.59 ± 0.62	0.00
Acetone	0.26 ± 0.12	0.00	0.52 ± 0.40	0.00
Butanol	0.71 ± 0.06	0.71 ± 0.13	0.01± 0.01	0.00

During the first phase, the cells grow rapidly and form carboxylic acids, mostly acetate and butyrate; the excretion of these acids lowers the external pH. These acids are suggested to act as inducers for the biosynthesis of the solventogenic enzymes during a second fermentative phase (Ballongue *et al.,* 1985). The acids formed earlier re-enter the cells and act as co-substrates for the production of neutral solvents (Fond *et al.*, 1985). At this point, the production of the acids ceases as well as cell growth, and the medium pH increases slightly due to the acid uptake (Terracciano and Kashket, 1986). It has been suggested that the switch to solvent production is an adaptive response of the cells to the low medium pH resulting from acid production (Bahl *et al.*, 1982). The major end product of the fermentation is butanol, with acetone and ethanol being minor products. The bacterium, *C. acetobutylicum* is able to metabolize a great variety of carbon resources. Depending on the nature of the carbohydrate and the culture conditions, the extent of solvent conversions can vary (Compere and Griffith, 1979).

The transition from acidogenic to solventogenic phase is the result of a dramatic change in gene expression pattern (Dürre *et al.*, 1987). Bryant and Blaschek, (1988) found that the pH of the medium is very important to the biphasic acetone–butanol fermentation. In acidogenesis, rapid formation of acetic and butyric acids causes a decrease in pH. Solventogenesis starts when pH reaches a critical point, beyond which acids are reassimilated acetone and butanol, are produced. Therefore, low pH is a prerequisite for solvent production (Kim *et al.*, 1984). However, if the pH decreases below 4.5 before enough acids are formed, solventogenesis will be brief and unproductive. Increasing the buffering capacity of the medium is a simple way to increase growth and carbohydrate utilization as well as butanol production (Lee *et al.*, 2008).

Desai *et al.,* (1999) constructed a stoichiometric model for the flux analysis of acid and solvent formation pathways. As expected, higher acetate and butyrate forming fluxes were observed in acidogenesis compared with those in solventogenesis. The direction of the butyrate forming pathway was reversed in solventogenesis. During solventogenesis, the fluxes toward acetone, butanol and ethanol formation were greatly elevated. In addition, fluxes linked to the acetone forming pathway (e.g., uptake fluxes of acetate to acetyl-CoA and butyrate to butyryl-CoA) increased in solventogenesis. Transcriptome profiling strongly supported that this flux pattern was driven by phase-dependent gene expression (Alsaker *et al*., 2004).

2- Bio-butanol formation in bio-reactor batch experiments

Figure-3 shows the lactose consumption by *Clostridium acetobutylicum* DSM 792 in lactose medium and deproteinized cheese whey after 5 days of fermentation process. Lactose consumption in lactose medium goes to completion after 3 days only, while with cheese whey almost (20%) of lactose was remained. These results are similar to that obtained by Sansonetti, *et al.*, (2009) and Salman &Mohammad (2005) who found that lactose consumption goes to completion within 13 h only, i.e. much earlier than it was reported for raw cheese whey fermentation, which might also affect the process performance.

The highest fermentation product on lactose medium (50g/l) was acetic acid as shows in Fig. (4) reached the maximum formation after 3 days then decreased. The production of butyric acid and ethanol was almost constant after 2 days.

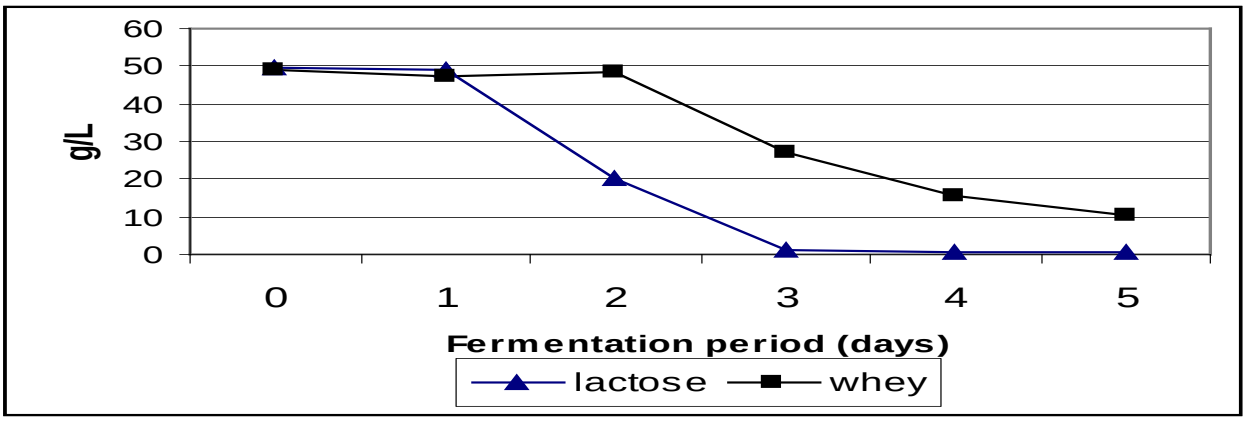

Fig. (3): Effect of fermentation media on lactose consumption by *Clostridium acetobutylicum DSM* 792 after fermentation process (37°C, shaking velocity 150 rpm, pH 5.0) for 5 days.

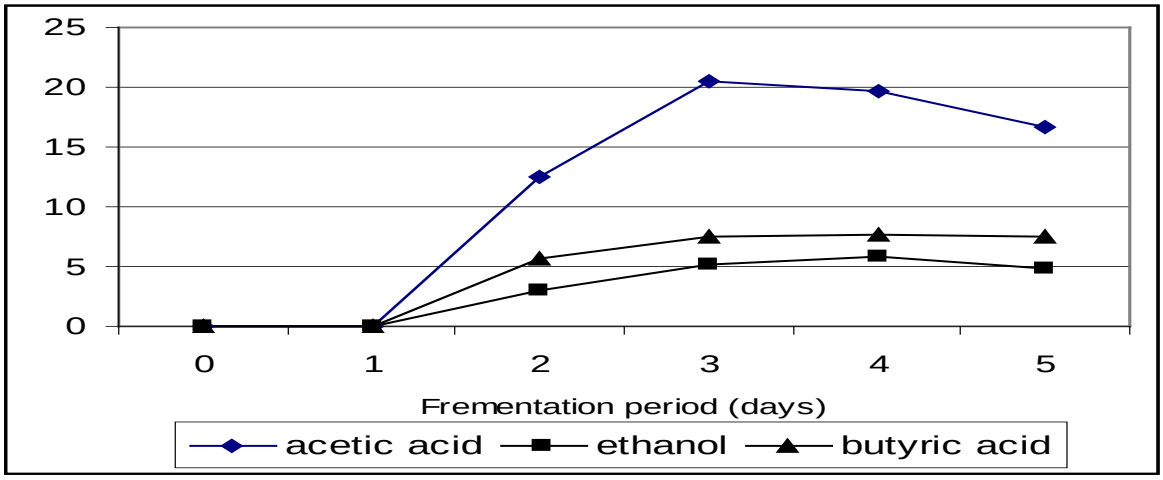

Fig. (4): Fermentation products on lactose media (37°C, shaking velocity 150 rpm, pH 5.0) by *Clostridium acetobutylicum DSM* 792 after fermentation process for 5 days.

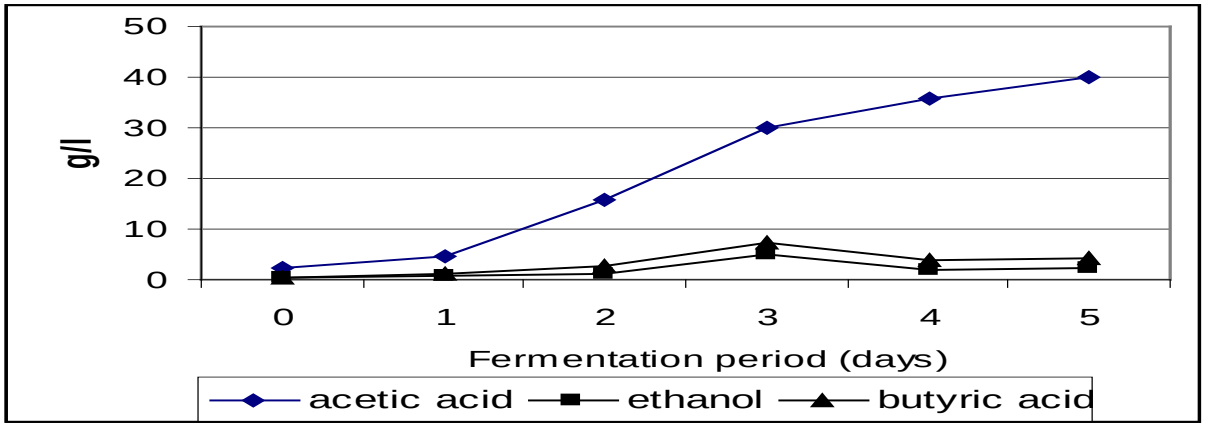

Fig. (5): Fermentation products from cheese whey medium (37°C, shaking velocity 150 rpm, pH 5.0) by *Clostridium acetobutylicum DSM* 792 after fermentation process for 5 days.

Figure (5) shows the production of acetic acid in cheese whey, which increased to reached the double amount compared to lactose medium. Ethanol and butyric acid were reached the maximum after 3 days then decreased to constant amount almost similar to lactose medium. These results are in agreement with those obtained by Ezeji *et al.*, (2004) who found that it takes 2–6 days to complete batch fermentation depending on the condition and the type of substrate employed. The final total concentration of solvents produced ranges from 12 to 20 g L^{-1} in batch fermentation, which can be separated from the fermentation broth by distillation. Classical fed-batch and continuous cultivation do not seem to be economically feasible, because of solvent toxicity and the biphasic nature of acetone–butanol fermentation, respectively. To overcome this problem, fed-batch culture has been coupled with an in situ recovery process.

Sansonetti *et al.*, (2009) studied the feasibility of ricotta cheese whey (scotta) fermentation process to produce bio-ethanol by *K. marxianus*. Furthermore, it was showed that scotta represents an excellent substrate since it allows attaining an ethanol yield of 97%, very close to the theoretical one. Complete lactose consumption was observed after 13 h for scotta as compared to 18 h for raw cheese whey. As far as the fermentation process is concerned, scotta is to be considered as a substrate completely different from traditional raw cheese whey and, also, from deproteinized whey, thus representing a valid alternative source to produce bio-ethanol.

Qureshi and Maddox (2005) produced acetone-butanol- ethanol (ABE) from whey permeate medium, supplemented with lactose, in a batch reactor using *Clostridium acetobutylicum* P262, coupled with ABE removal by perstraction. ABE were produced from lactose at a yield of 0.44. The ratio of acids to solvents was significantly lower in the perstraction experiment compared to the control batch process suggesting that acids were converted to solvents. Qureshi and Maddox **(**1987) immobilized cells of *Clostridium acetobutylicum* by adsorption onto bonechar and used in a packed bed reactor for the continuous production of solvents from whey permeate. A maximum solvent productivity of 4.1 g l^{-1} h^{-1}, representing a yield of 0.23 g solvent/g lactose utilized, was observed at a dilution rate of 1.0 h^{-1}. The reactor was operated under stable conditions for 61 days. High concentrations of lactose in the whey permeate favored solventogenesis, while low concentrations favored acidogenesis.

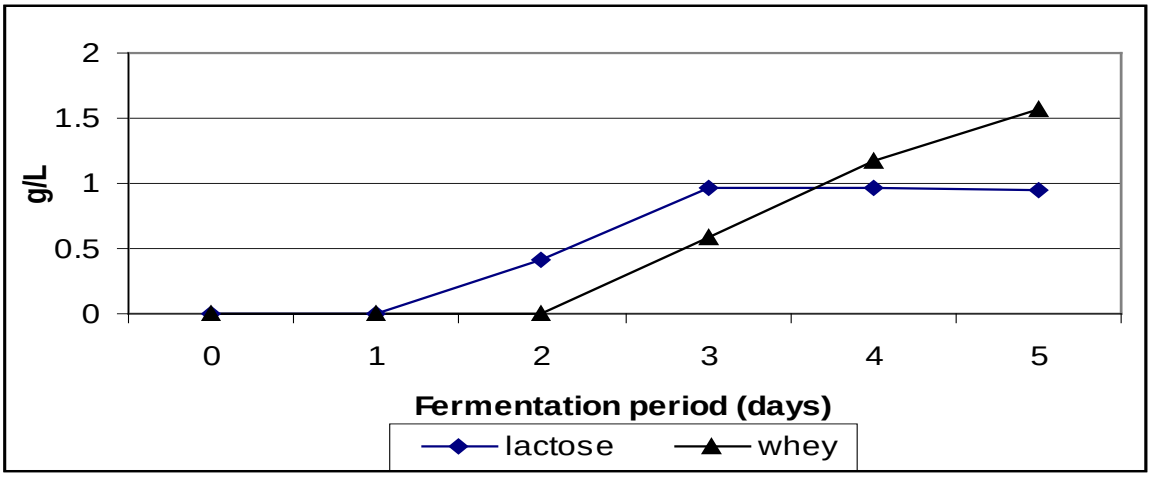

Fig (6): Butanol production from lactose and cheese whey media (37°C, shaking velocity 150 rpm, pH 5.0) by *Clostridium acetobutylicum DSM* 792 after fermentation process for 5 days.

Butanol was observed after 1 day with cheese whey medium and after 2 days when lactose medium was used as shown in Fig (6). Butanol production was increased dramatically with cheese whey during the fermentation period till the end. While, with lactose medium, the production was constant after 3 days till the end of fermentation period. This could be due to highest lactose consumption in the lactose medium compared to cheese whey (Fig-3).

These results are in contrarily with those obtained by (Qureshi *et al.*, 1988) who reported that unused lactose was toxicity due to ABE, in particular butanol. Using *C. acetobutylicum* in a batch reactor a total concentration of ABE of 20 g L^{-1} is rarely reached. At various stages of fermentation lactose

utilization was fluctuated. A maximum lactose utilization rate of 0.47 gL^{-1} was recorded between 56 and 75 h of fermentation. A yield o f solvent of 0.33 was calculated from this run. During this fermentation 0.70 gL^{-1} acetic acid and 0.25 g L^{-1} butyric acid was also produced. It was reported by Zhao *et al., (*2005) that the initiation of butanol formation corresponded to the time when butyryl-P concentration reached its peak. It was suggested that the concentration of butyryl-P should be higher than 60– 70 pmol g DCW1 for butanol production. The higher butyryl-P peak concentration corresponds to higher butanol formation fluxes. Supporting this finding, the butyryl-P peak concentration never exceeded 50 pmol gDCW1 in the non-butanol producing strain lacking the megaplasmid pSOL1.

One of the most critical problems in ABE fermentation is solvent toxicity. Clostridial cellular metabolism ceases in the presence of 20 g/L or more solvents (Woods, 1995). This limits the concentration of carbon substrate that can be used for fermentation resulting in low final solvent concentration and productivity. The lipophilic solvent butanol is more toxic than others as it disrupts the phospholipid components of the cell membrane causing an increase in membrane fluidity (Bowles and Ellefson, 1985).

Moreira *et al.*, (1981) had attempted to elucidate the mechanism of butanol toxicity in *C. acetobutylicum*. They found that 0.1–0.15 M butanol caused 50% inhibition of both cell growth and sugar uptake rate by negatively affecting the ATPase activity. Increased membrane fluidity causes destabilization of the membrane and disruption of membrane-associated functions and membrane-bound ATPase activity (Bowles and Ellefson, 1985).

In batch bio-reactor experiments it could be noticed the absent of acetone production in either lactose medium or cheese whey. This result could be due to the acetone-forming enzymes as mentioned by Mermelstein *et al.*, (1993). Who reported that the acetone producing pathway is coupled with that leading to the formation of the precursor of butanol, butyryl-CoA, from butyrate. In the fermentation of recombinant *C. acetobutylicum* with amplified *adc* (encoding acetoacetate decarboxylase) and ctfAB (encoding CoA transferase) genes, the acetone-forming enzymes became active earlier, which led to earlier induction of acetone formation.

4. Conclusion

Biotechnological production problems such as solvent toxicity have traditionally been addressed by random mutagenesis and process optimization. Development of *C. acetobutylicum* strain should be done to obtain a more solvent tolerant strain.

A more detailed investigation on the influence of fermentation parameters such as temperature, agitation velocity, pH and initial lactose concentration on butanol yield was beyond the scopes of this preliminary analysis. It would be, however, advisable to formulate a general kinetic model that could be used to improve cheese whey fermentation performance.

A deeper experimental analysis is, however, necessary to better ascertain the reasons of such a different behavior observed during fermentation experiments.

As a matter of fact, lactose solution, therefore, can be regarded as a poor fermentation substrate, as compared to cheese whey.

5. Acknowledgements

This work was supported by a grand from TWAS-CAS Fellowship for Visiting Scholars.

6. References

1. Alsaker KV, Spitzer TR, Papoutsakis ET. (2004). Transcriptional analysis of spo0A overexpression in *Clostridium acetobutylicum* sporulation and its effects on the cell's response to butanol stress. J Bacteriol 186:1959– 1971.
2. Atsumi S, Hanai T, Liao JC. (2008). Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature 451:86–90.
3. Ballongue J, Amine J, Mason E, Petitdemange H, Gay R. (1985). Induction of acetoacetate decarboxylase in *Clostridium acetobutylicum* . FEMS Microbiol Lett; 29:273– 7.
4. Bahl H, Andersch W, Gottschalk G. (1982). Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a two stage phosphate limited chemostat. Eur J Appl Microbiol Biotechnol 15:201–205.
5. Biebl, H. (1999). Comparative investigations of growth and solvent formation in *Clostridium saccharoperbutylacetonicum* DSM 2152 and *Clostridium acetobutylicum* DSM 792. Journal of Industrial Microbiology & Biotechnology (1999) 22, 115–120.
6. Bowles LK, Ellefson WL. (1985). Effects of butanol on Clostridium acetobutylicum. Appl Environ Microbiol 50:1165–1170.
7. Bryant DL, Blaschek HP. (1988). Buffering as a means for increasing growth and butanol production by *Clostridium acetobutylicum*. J Ind Microbiol 3:49
8. CAPMS. (2004). Annual Book of the Central Agency for Public Mobilization and Statistics, Cairo, Egypt, pp. 155-158.

9. Charles L. K. (2008). Whey to Ethanol: A Biofuel Role for Dairy Cooperatives, Agricultural Economist, USDA Rural Development Research Report 214.
10. Chen, H.C and R.R. Zall (1982). Continuous fermentation of whey into alcohol using an attached film expanded bed reactor process. Process Biochem., 17: 20-25
11. Compere AL, Griffith WL (1979). Evaluation of substrates for butanol production. Dev Ind Microbiol;20:50 9–17 .
12. Desai RP, Nielsen LK, Papoutsakis ET. (1999). Stoichiometric modeling of *Clostridium acetobutylicum* fermentations with non-linear constraints. J Biotechnol 71:191–205.
13. Dürre P. (2007). Biobutanol: An attractive biofuel. Biotechnol J, 2:1525– 1534.
14. Dürre P, Kuhn A, Gottwald M, Gottschalk G. (1987). Enzymatic investigations on butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 26:268–272.–55.
15. Ezeji TC, Qureshi N, Blaschek HP. (2004). Acetone–butanolethanol (ABE) production from concentrated substrate: Reduction in substrate inhibition by fed-batch technique and product inhibition by gas stripping. Appl Microbiol Biotechnol 63:653–658.
16. Fond O, Matta-Ammouri G, Petitde mange H, Engasser JM. The role of acids on the production of acetone and butanol by *Clostridium acetobutylicum* . Appl Microbiol Biotechnol 1985;22:195 – 20 0.
17. Francisco Diez-Gonzalez and James B. Russell (1996). The regulation of thiomethylgalactoside transport in *Clostridium acetobutylicum* P262 by inducer exclusion and inducer expulsion mechanisms. Federation of European Microbiological Societies (FEMS), Microbiology Letters, 136, 123- 127.
18. Hartmanis MGN, Gatenbeck S. (1984). Intermediary metabolism in Clostridium acetobutylicum: Levels of enzymes involved in the formation of acetate and butyrate. Appl Microbiol Biotechnol 47:1277–1283.
19. Kim BH, Bellows P, Datta R, Zeikus JG. (1984). Control of carbon and electron flow in *C. acetobutylicum* fermentations: Utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. Appl Environ Microbiol 48:764–770.
20. Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J. and Jung S. K. (2008). Fermentative Butanol Production by Clostridia. Biotechnology and bioengineering, 101, 2, 209-228
21. Mermelstein LD, Papoutsakis ET, Petersen DJ, Bennett GN. (1993). Metabolic engineering of Clostridium acetobutylicum ATCC 824 for increased solvent production by enhancement of acetone formation enzyme-activities using a synthetic acetone operon. Biotechnol Bioeng 42:1053–1060.
22. Moreira AR, Ulmer DC, Linden JC. 1981. Butanol toxicity in the butylic fermentation. Biotechnol Bioeng Sym 11:567–579.
23. .Qureshi N. and Maddox I. S. **(**1987). Continuous solvent production from whey permeate using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. Enzyme and Microbial Technology, 9, 11, 668-671.
24. Qureshi, N and I. S. Maddox (2005). Reduction butanol inhibition by perstraction: Utilization of Concentrated Lactose/Whey Permeate by *Clostridium cetobutylicum* to enhance butanol fermentation economics. Food and Bioproducts Processing, 83(C1): 43–52.
25. Qureshi, N., Paterson, A.H.J. and Maddox, I.S., (1988). Model for continuous production of solvents from whey permeate in a packed bed reactor using cells of Clostridium acetobutylicum immobilized by adsorption onto bonechar, Appl Microbiol Biotechnol, 29: 323 – 328.
26. Salman, Z., Mohammad, O., (2005). Ethanol production from crude whey by *Kluyveromyces marxianus*. Biochem Eng J, 27: 295–8.
27. Sansonetti, S., Curcio, S., Calabro, V., Iorio, G., (2009). Bio-ethanol production by fermentation of ricotta cheese whey as an effective alternative non-vegetable source. Biomass and Bioenergy, 33 (12) 1687-1692.
28. Terracciano JS, Kashket ER (1986). Intracellular conditions required for initiation of solvent production by Clostridium acetobutylicum. Appl Environ Microbiol 52: 86 – 91.
29. Woods DR. (1995). The genetic engineering of microbial solvent production. Trends Biotechnol 13:259–264.
30. Zhao Y, Tomas CA, Rudolph FB, Papoutsakis ET, Bennett GN. (2005). Intracellular butyryl phosphate and acetyl phosphate concentrations in Clostridium acetobutylicum and their implications for solvent formation. Appl Environ Microbiol 71:530–537.

4/9/2010