ABSTRACT

Title of Document: BUTANOL PRODUCTION FROM GLYCEROL BY *Clostridium pasteurianum* IN DEFINED CULTURE MEDIA- A PHENOTYPIC APPROACH.

David Leonardo Ramos Sanchez, Master of Science, 2009

Directed By: Associate Professor Nam Sun Wang
Department of Chemical and Biomolecular Engineering

ABSTRACT

The fluctuations in oil prices have stimulated the production of renewable biofuels, in particular the production of bioethanol and biodiesel. The production of biodiesel has expanded almost six fold in the past years. The ten wt% of the biodiesel process results in crude glycerol. Once a valuable product, nowadays glycerol is considered a waste and a surplus material. Its current low price makes it an attractive substrate for a fermentation process.

Molecular genetics have unveiled new insights about solvent production in *Clostridia*. It has been recognized that endospore development and solvent formation share a regulatory mechanism. The solvent production, particularly the butanol fermentation of glycerol by *Clostridium pasteurianum* was studied. Taking advantage of the characteristics of the sporulation phenotype, the study of the butanol fermentation

was approached. A relation between spore formation and butanol production was found in *C. pasteurianum* by applying molecular genetics concepts.

BUTANOL PRODUCTION FROM GLYCEROL BY *Clostridium pasteurianum* IN DEFINED CULTURE MEDIA- A PHENOTYPIC APPROACH

By

David Leonardo Ramos Sanchez

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2009

Advisory Committee:
Professor Nam Sun Wang, Chair
Ganesh Sriram
John Fisher

© Copyright by
David Leonardo Ramos Sanchez
2009

Dedication

To Jesus Christ who never abandoned me and answered my prayers. He is worthy to receive praise.

.

Acknowledgements

To Catalina for all her patience, support and love showed during all this process.

To my mother and sister and family members who encouraged me during the hard times.

To my professors and friends who gave me wise advise.

To the Fulbright commission, LASPAU, Sucromiles S.A, and Colciencias for sponsoring my studies..

Table of Contents

Dedication ii
Acknowledgements iii
Table of Contents iv
List of Tables vi
List of Figures vii
Chapter 1: Introduction and Overview 1
1.1. Problem description and motivation 1
2. Chapter 2: Background: glycerol, *Clostridium pasteurianum* and ABE fermentation process 4
2.1. Glycerol from Biodiesel: A premier 4
2.1.1. Biodiesel Feedstocks 4
2.1.2. Transesterification reaction 5
2.2. Lessons from the ABE process 6
2.2.1. Brief history of the industrial process 6
2.2.2. Previous Work Reviewed 9
2.3. Clostridium pasteurianum and the sporulation process 10
2.3.1. Class Clostridia and relevant aspects to *C. pasteurianum* 10
3. Chapter 3: Materials and Methods 12
3.1. Strain utilized 12
3.2. Growth Medium and fermentation conditions 12
3.3. Glycerol Quantification 16
3.3.1. Spectophotometric method by Biodioli 16
3.3.2. Description of Bondioli Method 17
3.3.3. Glycerol Quantification using AOCS method 19
3.4. Fermentation Products 21
3.4.1. Quantitative analysis 22
3.4.2. Internal standard method 22
3.4.3. Gas chromatograph method 24
4. Chapter 4: Media Development 26
4.1. Introduction 26
4.2. Defined media Formulation 27
4.2.1. Materials and Methods 31
4.2.2. Results 34
4.2.3. Discussion 37
5. Chapter 5: Fermentation Parameters 39
5.1. Introduction 39
5.2. Kinetic Fermentation Parameters 40
5.2.1. Specific growth rate 40
5.3. Determining Yield coefficients 43
5.3.1. Growth Yield 44
5.4. Product Formation Kinetics 47

5.4.1. Type of microbial product 47
6. Chapter 6: Sporulation and Solvent Production........ 51
6.1. Introduction........ 51
6.2. Clostridial Sporulation........ 52
6.3. Methods and rationale behind the experiments 54
6.3.1. Selection of heat resistant spores........ 54
6.3.2. Experimental procedures 55
6.4. Results........ 56
6.4.1. Selection of heat resistant cells........ 59
6.4.2. Heat resistant individuals and the carbon source........ 60
6.4.3. Number of heat resistant cells and solvent production........ 61
6.5. Discussion........ 62
Chapter 7: Study of the effects of hydrosulfite during the growth and the effects in the solvent production........ 65
7.1. Introduction........ 65
7.1.1.Selecting the reducing Agent........ 65
7.1.2.Dissimilative Sulfate Reduction 67
7.2.Methods........ 69
7.2.1. Experimental methods 70
7.4.Discussion........ 76
8. Chapter 8: Conclusions........ 78
9. Chapter 9: Future Work 80
Bibliography 81

List of Tables

Table 2-1 ABE fermentation parameters at industrial scale. 8
Table 2-2 Relevant results in Biebl's work (Biebl 2001) 10
Table 4-1 Macronutrients and micronutrients required for most organisms. 28
Table 4-2 Media formulations 31
Table 4-3 Results comparing positive and negative controls. 37
Table 4-4 Statistical analysis 37
Table 5-1Specific growth rate at different glycerol concentrations............................ 42
Table 5-2 Reported specific growth rate for other glycerol fermentations................. 42
Table 5-3 Estimating doubling time 43
Table 5-4 Growth yields from anaerobic growth of heterotrophs (Hernandez and Johnson, 1967 cited in Atkison 1995)... 46
Table 7-1 different reducing agents available for biological media 66
Table 7-2 Oxidation state of some sulfur compounds 67
Table 7-3 Redox potencial of some sulfur compounds 68
Table 7-0-4 Results from fermentation with $6x10^{-5}$ M sulfite at t_0............................ 72
Table 7-5 Statistical analysis 72
Table 7-6 Effect of sulfite during exponential growth... 74
Table 7-7 Results (sum of ethanol and butanol) using an intermediate concentration of hydrosulfite 75
Table 7-8 Statistical analysis adding $6x10^{-4}$ M hydrosulfite during exponential growth.. 75

List of Figures

.Figure 3-1 Dry biomass calibration curve (n=14) 14
Figure 3-2 Bondiolli calibration curve.. 18
Figure 3-3 Sample of a chromatograph 25
Figure 4-1 Using replica plating to test auxotrophy 33
Figure 4-2 Box plot showing optical density with and without growth factors.......... 36
The net specific growth rate can be estimated from the slope on a semi logarithm plot of ln X versus time. Following this procedure one can calculate the different growth rates at different concentrations (Figure 5-1)... 41
Figure 5-2 Growth curve and specific growth of Clostridium pasteurianum in defined medium with 6 wt% glycerol.. 41
Figure 5-4 Results for one, three, six, nine percent of glycerol................................... 45
Figure 5-7 Figure Patterns of growth and product formation in batch fermentations (Gaden 1959, Shuler and Kargi 2006) (a) Growth associated, (b) mix-growth associated (c) no growth associated... 48
Figure 5-8 Glycerol consumption, growth and product formation............................. 49
Figure 6-1 Growth, glycerol consumption and product formation in batch mode with 9% glycerol in defined media. Result of at least three fermentations. 58
Figure 6-2 Fermentation broth sample plated without heat selection (left) and after heat selection (right) 59
Figure 6-3 Glycerol consumption and CFU after heat shock (bars) --- Exponential growth of number of CFU as function of the time... 60
Figure 6-4 Correlation between number of CFU heat resistant cells and butanol production at two different initial glycerol concentrations 6wt% (left) and 9wt% (right). 62
Figure 7-5 Growth curves of both cultures with and without hydrosulfite................. 73
Figure 7-6 Effect of sulfite addition compared against control without addition....... 75
Figure 7-7 Sulfite reduction by sulfite reductase.. 76
.

List of Illustrations

Ilustration 4-1 Replica plating 35
Illustration 6-1 Cartoon of the different stages during the sporulation process. Adapted from Stephenson (Stephenson, 2005)... 53
Illustration 6-2 Morphology differences of *Clostridium pasteurianum* during growth 57
.

Chapter 1: Introduction and overview

1.1. Problem description and motivation

The increase in oil prices has stimulated the production of renewable biofuels, in particular the production of ethanol and biodiesel. The application of the biorefinery concept (i.e obtaining added value products from bioprocess products, by-products and /or waste streams) is gaining importance. This research focuses on the utilization of one of the biodiesel byproducts: glycerol.

More precisely, the research focuses on the anaerobic fermentation of glycerol into butanol, relevant physiological aspects of *Clostridium pasteurianum* that are related to the solvent production.

According to the data collected by the Worldwatch Institute, the production of biodiesel has expanded almost six fold between 2001 and 2006 (F.O. Licht 2006 cited in Worldwatch 2007). The total reported biodiesel production for 2006 was 6153 million liters. During that year, Germany, the United States, and France shared a total percentage of 40.6%, 13.8%, and 10.2%, respectively (F.O. Licht 2006 cited in Worldwatch 2007). As explained by Johnson and Taconi, and Yazdani and Gonzalez (Johnson and Taconi 2007, Yazdani and Gonzalez 2007) , the 10 wt% of the biodiesel process results is crude glycerol (i.e. for each 100 kg of biodiesel from the transesterification of vegetable oils or animal fats; 10 kg of crude glycerol is obtained). Glycerol is a byproduct that was considered a value added chemical, but is now considered a waste (Yazdani and Gonzalez 2007) because of its surplus nowadays. The current low prices of glycerol (US$0.02-0.05 /lb (Johnson and

Taconi 2007, Yazdani and Gonzalez 2007) makes it an attractive substrate for a fermentation process.

Among the different uses of glycerol, the research focuses on anaerobic fermentation of glycerol into butanol as the main product. The selection of anaerobic fermentation came with the fact that, at the industrial scale, it has lower operational costs than anaerobic fermentation. (Yazdani and Gonzalez 2007) Among the different alternatives for fermentation products, the decision in favor of butanol was issued for the following reasons: First, butanol was already produced at industrial scale by *Clostridium acetobutylicum* in the first half of the twentieth-century (Jones 1986). The acetone butanol process (ABE process) was performed by a batch fermentation that used molasses (sometimes other carbon sources like maize) as a substrate at an initial concentration of 6.5% wt of fermentable sugars. Batches were controlled around 31˚C and CO_2 was bubbled to facilitate mixing. Total solvent production in the best cases was around 18 and 22 g/Liter in an acetone-butanol-ethanol distribution equal to 6:3:1 (Jones 1986).

Secondly, if one wants to apply the bio-refinery principles, butanol is a great alternative because it could be produced in a biodiesel plant. The crude glycerol could be fermented in the same manufacturing facility. This could result in a reduction of substrate transportation costs.

Third, there is a need to find alternative sources of fuels. Whether it is because of the exhaustion of fossil fuels or because of an increase in oil prices, the recent demand for alternative biofuels can be met by the production of butanol from glycerol.

In fourth place, butanol as fuel has better properties than ethanol. Not only butanol is less volatile (117.17 °C versus 78.3°C) but it also has greater energy content. Ethanol heat of combustion is 328 kcal/mol while butanol heat of combustion is 639 kcal/mol. (Monik 1968) This is at least 48% more kilo calories per mole of butanol .

2. Chapter 2: Background: glycerol, *Clostridium pasteurianum* and ABE fermentation process

2.1. Glycerol from Biodiesel: A premier

A transesterification reaction of an oil or fat with an alcohol (in a more accurately sense, with a monohydric alcohol) is necessary to obtain a fatty ester (methyl or ethyl ester). This mixture of fatty esters is known as biodiesel. Methanol is preferred because it is the least expensive alcohol (Knothe 2008) and also because there are process concerns : it is more difficult to separate the glycerol from the biodiesel when using ethanol (Worldwatch 2007). A simplified way to write the reaction reads:

100 Kg of oil + 10 Kg of methanol → 100 Kg of Biodiesel + 10 Kg of glycerol (Gerpen 2004)

Roughly speaking, 10%wt of glycerol is produce per each 100 Kg of Biodiesel produced. (read more details of the reaction below)

2.1.1. Biodiesel Feedstocks

According to the feedstock, biofuels can be classified into carbohydrate-derived biofuels and lipid-derived biofuels (Worldwatch 2007). In the first group ethanol from sugar cane, corn, wheat and/or starches and butanol (from the ABE process) can be found. In this group ethyl tertiary butyl ether (ETBE) was also incorporated by the Worldwatch's report. ETBE is produced by combing ethanol with isobutylene and

it has a advantage over ethanol because it does not raise the vapour pressure of gasoline blends (Worldwatch 2007).

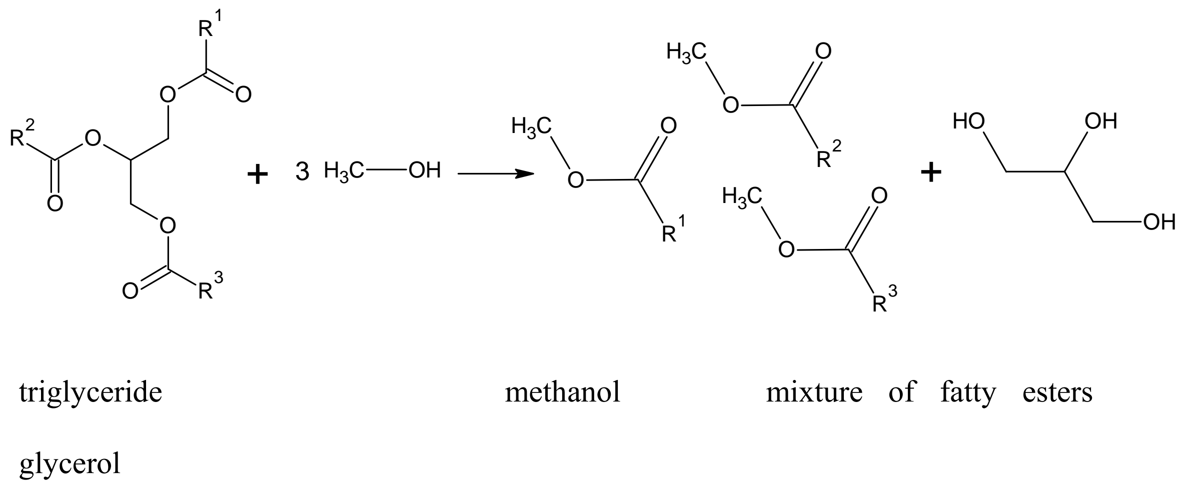
In the lipid-derived biofuels group one can find straight vegetable oil (SVO) and biodiesel. Soybean is the larger oilseed cultivated worldwide followed by rapeseed and cottonseed (USDA 2006 cited in Worldwatch 2007). But rapeseed (primary planted in Europe) is the main feedstock for biodiesel. In addition to soybean and rapeseed palm (cultivated in Asia and south America), peanut (with crops in India, China and U.S), sunflower (cultivated in Europe) and waste vegetable oil are some the sources for oil (Worldwatch 2007).

Among the nonedible options jatropha (which is grown in India) has the most promising potential (Worldwatch 2007). Dorado (Dorado 2008) presents some other nonedible crops such as bahapilu, castor oil plant, cottonseed oil, cuphea, rubber seed oil (commonly known as Para' rubber tree) and tonk bean oil. Another alternative source for lipids is algae (Worldwatch 2007, Lee and others et al. 2007)

2.1.2. Transesterification reaction

A general chemical reaction between a trygliceride and methanol is shown in equation 2-1.

It is important to understand that biodiesel as product has a greater range in characteristics due to the different possible feedstock (recall that bioethanol is a specific molecule in contrast to biodiesel which is a mixture of fatty esters) (Worldwatch 2007)



triglyceride methanol mixture of fatty esters glycerol

Equation 2-1 Transesterification reaction *(Gerpen 2004)*

2.2. Lessons from the ABE process

The motivation of this research is the need to find renewable sources of energy and it is intended to support all the efforts that are going in that direction. Eventually this fermentation is going to be scaled up. Biobutanol production was an existing industrial process before the second half of the 20th century. It is worth to review the achievements of that technology.

2.2.1.Brief history of the industrial process

Butanol and acetone were produced by fermentation at industrial scale in the first half of the twentieth century. The first plant in southern England started the production of solvents in 1916. One of his founders, Chaim Weizman issued British Patent 4845 (Weizman 1915 in the United States U.S. Patent 1,315,585) describing the process

and the isolation of the microorganism (Biebl 2000). He was not only the first president of Israel, but his patent was the first one that covered a biological process.

World War I and World War II stimulated the manufacturing of this products, but the synthetic process of butanol made the fermentation process not economically viable and around 1960 all the manufacturing facilities in America and Europe were abandoned. The last factory in the western hemisphere was closed in South Africa in 1983 (Biebl, 2000, Jones, 1986).

The following table summarizes the main characteristics of the once industrial process:

Microorganism	Reference
Clostridium acetobutylicum	(Chiao 2007, Biebl 2000, Jones 1986)
Clostridium beijerinckii	(Biebl 2000)
Feedstock - Initial Concentration	
Corn mash - 8% wt/vol	(Chiao 2007)
Starch feed stocks, such as corn, cassava, potato, and sweet potato - 8-10% wt/vol	(Biebl 2000)
Beet molasses - 5.0 to 7.5%,wt/vol.	(Jones 1986)
Yield	
25 to 26% based on dry-weight corn equivalents	(Jones 1986)
35-37% of starch	(Chiao 2007)

Fermentation time	
30-60 hours	(Biebl 2000, Jones 1986, Watson 1999)
Products/ Solvents Ratios	
Acetone:Butanol:Ethanol/ 3:6:1, and $CO_2:H_2$ / 60:40	(Chiao 2007, Biebl 2000)
Type of fermentation / capacity	
Batch fermentation / 90 m^3-750 m^3	(Biebl 2000 Jones 1986)
Cycle of continuous fermentation / 1000 m^3 -2000 m^3	(Durre 2008, Chiao 2007)

Table 2-1 ABE fermentation parameters at industrial scale.

Before choosing the initial concentration of substrate, it is important to realize that the final concentration of butanol is not likely to be higher than 13g/L (Qureshi and others 2001) due to solvent toxicity. Qureshi published a report about a hyperbutanol *Clostridium beijerinkii* mutant that achieved a final concentration of 21 g/L g butanol (Qhreshi and others 2001). Biebl (Biebl 2001) using glycerol as a carbon source, reported a maximum concentration of 17g /L and beyond that concentration range, butanol becomes a toxic product and inhibition of cells is observed (Biebl 2001).

The chemical reaction equations for butanol and ethanol fermentation from glycerol can be written (Biebl 2001):

$$2C_3H_5(OH)_3 \rightarrow C_4H_9OH + 2CO_2 + 2H_2 + H_2O \qquad \textit{Equation 2-2}$$

Glycerol Butanol

$$C_3H_5(OH)_3 \rightarrow C_2H_5OH + CO_2 + H_2 \qquad \textit{Equation 2-3}$$

Glycerol Ethanol

From butanol equation and the maximum non toxic concentration, one can estimate the maximum initial concentration of the substrate. If the maximum concentration expected is 20 g butanol/L then:

$$\frac{2g\text{Butanol}}{100mL}\times\frac{2\times 92.09g.Glycerol}{74.12g\text{Butanol}}=0.049\frac{g.Glycerol}{mL}\approx 5\%Glycerol \quad \textit{Equation 2-4}$$

This assumes that all the glycerol is converted into butanol. Nevertheless this is not a real scenario because there are other metabolic products and some carbon source is required for biomass formation (Eleftherios 1983). The above calculation tells that the initial glycerol concentration should be close to the five percent. Thus the initial glycerol concentrations studied through this research were six and nine weight percent.

During the second half of the twentieth century, the AB fermentation was discarded mainly because the costs of raw material, which made up about 60% of the overall cost (Jones 1986). Thus surplus glycerol and fluctuating oil prices are factors that have made this research relevant.

2.2.2. Previous Work Reviewed

The work of two researchers Hano Biebl (Biebl 2001) and Birgit Dabrock (Dabrock and others 1992) who also studied the glycerol fermentation by *Clostridium pasteurianum* have been explored. The highest final butanol concentration reported by Biebl was 17 g/L trough out a feed batch fermentation starting at 50g/l. The following table summarizes the operation parameters of his work (Biebl 2001):

Parameter	Value
Type of fermentation	Batch
Initial glycerol concentration	50g/L
Temperature	35 C
Value of pH control	6
Growth rate	0.37 h^{-1}
Fermentation time	21-22 h
Working volume	500 mL
Butanol (mmol/L)	145 (as reported)
Butanol (g/L)	10.73 (calculated)

Table 2-2 Relevant results in Biebl's work (Biebl 2001)

Biebl has used yeast extract as growth factor in a concentration equal to one gram per liter. In contrast to Biebl's work (Biebl 2001), the experiments in this research have been done in defined media instead of complex media. It is worth to mention that Biebl reported that when using 25µg/l of biotin instead of yeast extract, cells took three times more the fermentation time when compared to a culture with the complex media. In addition to that the fermentation run until hour 72.

2.3. *Clostridium pasteurianum and the sporulation process*

2.3.1. Class Clostridia and relevant aspects to *C. pasteurianum*

The genus Clostridium was named in 1880 by Prazmowski (Jan et al. in chapter 2 Nigel 1989). There are four characteristics that helped in the classification of this type

of bacteria, namely: (1) the ability to form spores (2) the anaerobic energy metabolism (i.e. obligate anaerobes) (3) the possession of a gram positive cell wall and (4) the inability to carry out a dissimilative reduction of sulfate.

Class *Clostridium* is gram positive. *Clostridium acetubutylicum* stain gram-positive in growing cultures (i.e. violet) but stain as gram negative during the stationary phase and when forming spores (Biebl 2000, Jan et al. in chapter 2 Nigel 1989)

Clostridium pasteurianum has been classified as a saccharolytic, nitrogen fixer bacteria. With respect to the type of fermentation it is said to be butyrate proteolytic producer. *C. pasteurianum* shares this group with *C. butyricum, C. acetobutylicum* among others. (Staley 2007, Paredes and others 2005). The last two species are recognized to produce solvents.

Both gram positive bacteria *Bacillus subtilis* and Clostridia have the capability to form endospores, however the factors that activate the sporulation process in each of them are not the same (Paredes and others 2005, Ravagnani and others 2000). In *B. subtilis* sporulation initiates when the cells experience nitrogen and carbon starvation (Ravagnani and others 2000). In contrast, clostridium sp. seems to need sufficient source of substrate. Inactivation of gene *Spo0A* has shown to regulate sporulation in *C. beijerinckii* and according to Paredes and others (Paredes and others 2005), apparently it does so in all clostridial. Previous clostridia studies have served as reference to try to understand the sporulation process in *Clostridium pasteurianum*, even though *Clostridium pasteurianum* genome has not been sequenced up to date.

3. Chapter 3: Materials and Methods

3.1. Strain utilized

The microorganism used throughout the experiments was *Clostridium pasteurianum* ATTC® 6013 ™ a type strain identical to *Clostridium pasteurianum* DSM 525. Freeze-dried cultures were activated in Reinforced Clostridial Medium (R.C.M.) (Difco Manual 1985) in closed cap tubes. Cultures were preserved at 4˚C using modified R.C.M. as follows (amounts per liter of deionized water): casitone, 10 g.; Bacto peptone, 10 g; yeast extract, 3 g; NaCl, 5 g; soluble starch, 1 g; $NaCH_3OO$, 3 g; agar 0.5 g; cysteine hydrochloride 0.5 g; dextrose, 2.5 g; glycerol, 2.5 g. The reason for thickening the fluid media by incorporating agar (0.05-0.1 wt%) is to reduce diffusion of oxygen into the media (Willis 1979).

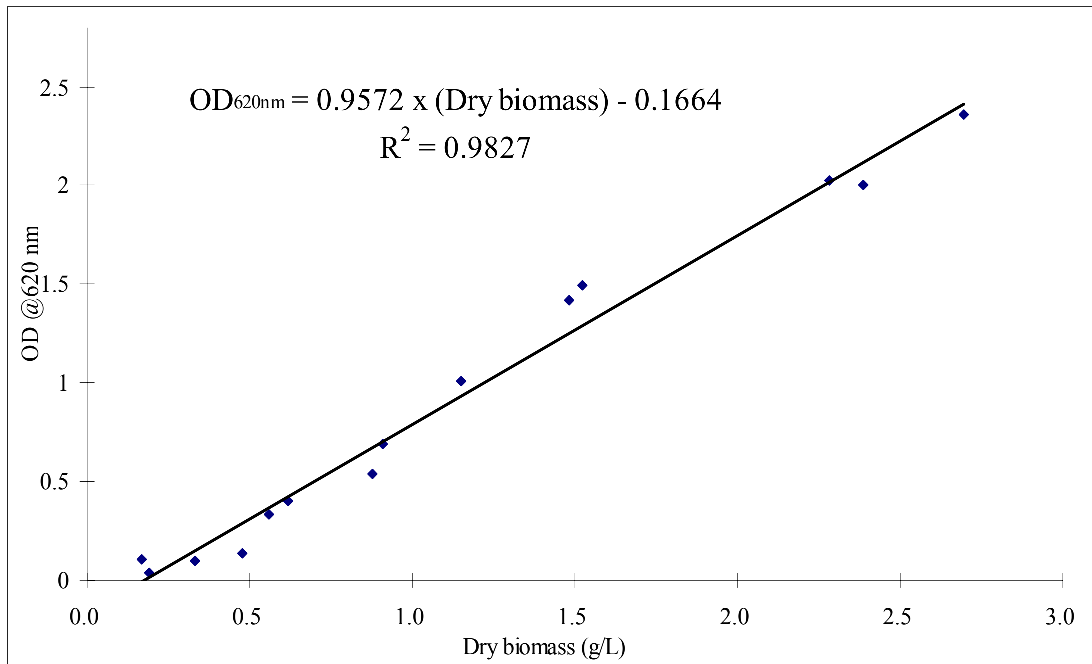
3.2. Growth Medium and fermentation conditions

Cultures from the refrigerator were activated in modified R.C.M. in closed cap tubes at 37˚C. After 24 hours, the corresponding amount equal to 10 %v/v was used to inoculate the fermentors. During the inoculation nitrogen was pumped to keep anaerobic conditions.

Defined media contained per liter: Glycerol 90 g or as indicated; $MgCl_2$, g; $NaMoO_4$·2H2O, 0.01 g; KH_2PO_4, 10.9 g.; K_2HPO_4, 2.84 g; $(NH_4)_2SO_4$,08 g; NH4Cl 0.66 g; $MnCl_2$•$4H_2O$ 0.016 g; $CaCl_2$·2H2O, 0.02 g; $FeSO_4$•$7H_2O$ 0.018g; biotin 0.004 g; p-aminobenzoic acid 0.004 g.

Batch mode was used to run the fermentations. 300 ml and 150 ml cultures were grown anaerobically in a closed Erlenmeyer with check valve (Scienceware bel-art Valve, PP, Check 1/8''-1/4'') that acted as a pressure relief valve. A tube with a valve in the outside end was introduced. Cole Parmer stock 1 way-male lock connector was attached to the sampling tube using autoclavable tubing. That allowed the researcher to pump nitrogen and to take samples without introducing air into the broth. Nitrogen was pumped after each sample was inoculated and also after sampling. This configuration was not found previously in the literature fo anaerobic gas producing bacteria.

Cell growth was measured by culture turbidity (OD_{620}) in spectronic 20 genesys spectrophotometer. Cell dry weight was measured after centrifuging 50 mL of broth at 5000 rpm (3836 x g) for 20 minutes in a Beckman Avanti J-251 centrifuge. Supernatant was saved for glycerol and product analysis and the cells were washed twice before drying them at 80ºC. Biomass was weighed directly after scratching the inside pellet. A calibration curve was constructed to relate the optical density with the dry biomass. (figure 3-1)



.Figure 3-1 Dry biomass calibration curve (n=14)

Biomass was then related using the following equation

$$\text{Dry Biomass } (g/L) = \frac{OD_{620nm} + 0.1664}{0.9827} \qquad \textit{Equation 3-1}$$

Replica Plating

Nutritional mutants can be detected by the technique of replica plating. (Sneyder and Champness, 2007, Brock, 1991) In order to screen mutant cells in a solid culture media, it is possible to use negative selection by spreading a countable number of desirable cells on a non selective nutrient agar, and incubate the plates at the specific conditions to allow the formation of colonies.

A sterile Whatman filter number 2 (12.5 cm diameter), or a sterile piece of cloth can be used to cover a wooden block to imprint the colonies from the master plate (Step 1). Each plate containing selective media can be inverted over the imprinted cloth and then pressed it down, so the colonies can be transferred to the selective culture media

(Step 2). After the incubation, it is possible to compare the selective media plates with the master plate to identify the mutant colonies, this is, those that do not reappear on the selective media plates (Step 3). A cartoon of the replica plating technique can be observed in Illustration 3-1

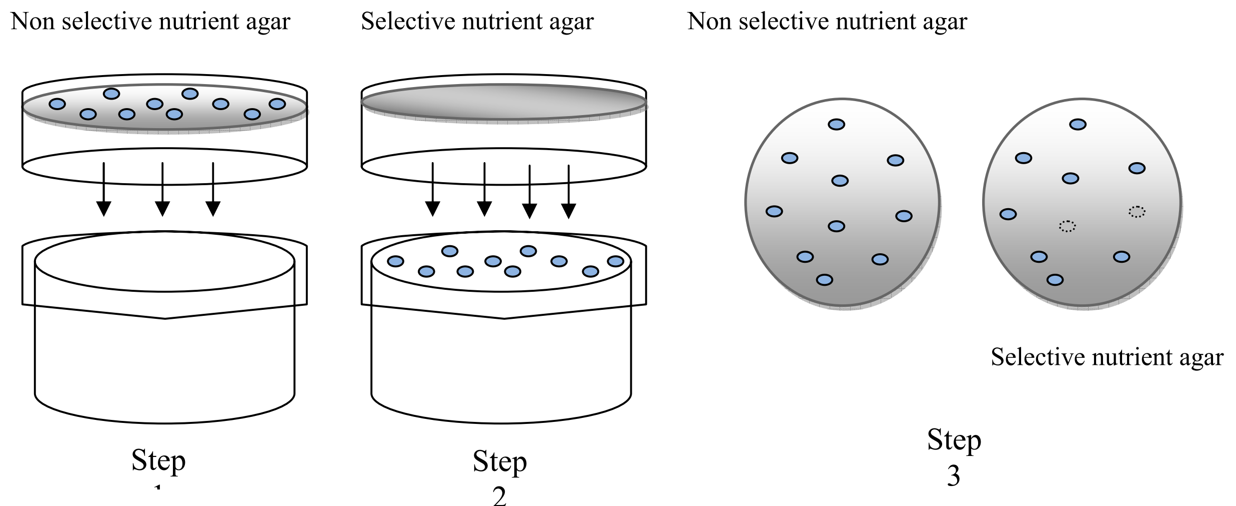


Illustration 3-1 Replica Plating Method

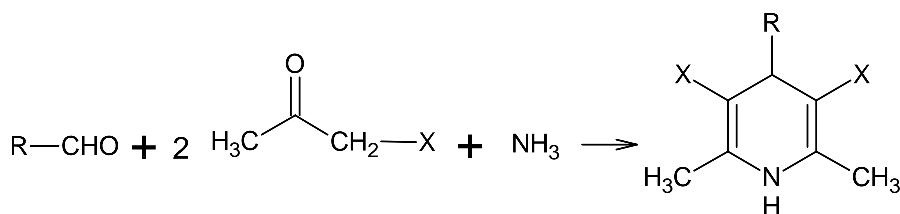
During the present study, replica plating technique was used with a different purpose other than nutritional mutant selection. It was used to end up with an even number of clones in reinforce clostridium media plates, so that different media composition could be tested without addition of liquid broth within the inoculums. Colonies were swap with sterile cotton tipped applicator, and screw cap tubes with defined media (30mL of working volume) were inoculated with the same population of each plate.

3.3. Glycerol Quantification

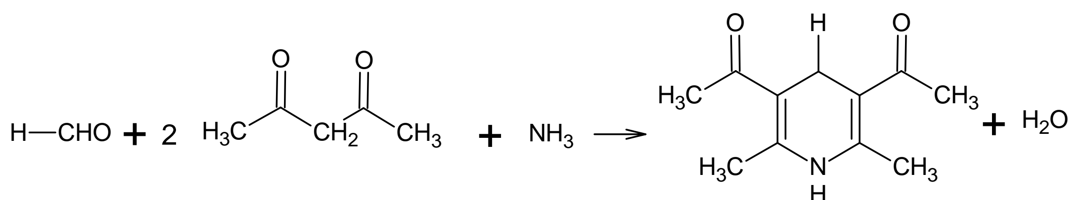
Different methods for the determination of glycerol were searched (Bondioli 2005, Biebl 2001, Matzouridou 2008, Forsberg 1987, AOCS Ea 6-51). HPLC analysis and enzymatic analysis were not tried. Two methods were tested, the spectrophotometric proposed by Bondioli and AOCS Ea 6-51 method.

3.3.1. Spectophotometric method by Biodioli

The first method explored (Bondioli2005) was designed to measure trace amounts of glycerol in biodiesel. It works in the range 3µg/L to 30 µg/L. However Matzouridou et al. (Matzouridou 2008) have used it to measure glycerol in fermentation samples. The core of the method involves series of reactions between glycerol and periodate to yield formaldehyde. Subsequently, the Hantzsch's reaction proceeds to completion according to equation 3-2



Equation 3-2 General scheme of Hantzsch Reaction (Love 1964)
For the particular reaction under study



Equation 3-3 Formaldehyde and 2-4 Pentanedione reaction

The product can be measured at 410 nm in a spectrophotometer.

3.3.2. Description of Bondioli Method

- Prepared stock solutions (stable over time according to authors)

Acetic acid 1.6 M (9.6g/100 ml)

Ammonium acetate 4.0 M (30.8g/100 ml)

- 2-4 Pentanedione solution 0.2 M (or acetylacetone solution 0.2 M):

In 5 mL of acetic acid solution and 5 mL of Ammonium acetate solution dissolve 200µl (0.2 mL) of 2-4 pentanedione ($CH_3COCH_2COH_3$), As emphasized by Bondioli (Bondioli 2005) this solution must be prepared daily.

- Sodium periodate ($NaIO_4$) solution 10 mM:

Weigh approximately 21 mg of sodium meta periodate. Then add 5 mL of acetic acid stock solution. After $NaIO_4$ is completely dissolved add 5 mL of ammonium acetate solution. It is recommended to prepare this solution daily (Bondioli 2005).

- Working solvent solution

Mix equal volumes if distilled water and 95% ethanol.

- Glycerol reference stock solution (3mg glycerol/mL glycerol ref. sol.)

  Weigh approx 150 mg of glycerol in a 50 mL flask. Dissolve using the working solvent solution up to complete the 50 mL.

- Glycerol reference working solution (0.03 mg glycerol/mL glycerol ref. sol.)

Transfer 1 mL of glycerol reference stock solution to a 100 mL calibrated flask.

The calibration curve was prepared as follows:

Transfer to a 10 mL tube different amounts of the glycerol reference solution. Dilute with the working solvent in order to obtain a final volume of 2 mL

Add 1.2 mL of a periodate solution and shake for 30 s.

Add 1.2 mL of the acetylacetone solution and put in water at 70C for one minute.

After reaction time cool immediately immersing in a beaker with tap water at 20-25C.

Read the samples at 410nm in a spectrophotometer.

Glycerol fermentation samples were diluted in 50%v/v ethanol water solution as suggested by Matzouridou (Matzouridou 2008) Even though the linearity of the calibration curve was attained (figure 3-2) when the glycerol concentration was compared with broth samples of known concentrations, results did not corresponded to the expected concentration values (data not reported).

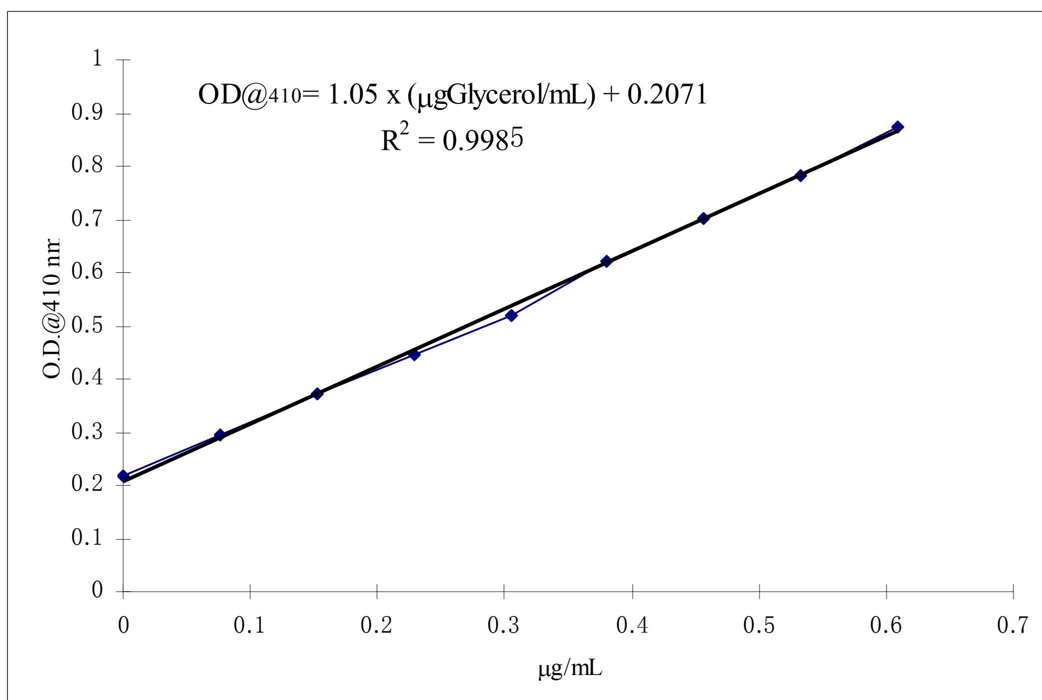


Figure 3-2 Bondiolli calibration curve

3.3.3.Glycerol Quantification using AOCS method

Glycerol was measured according to the method AOCS Ea 6-51. It is recommended to determine glycerol and other polyalcohols containing three or more adjacent hydroxyl groups. As explain by Sodium Periodate Oxidation Method (i.e. Ea6-51) “Trimethylene glycol and other and other polyalcohols in which the hydroxyl groups are adjacent do not react at room temperature”.

Glycerol concentration was quantified by the sodium periodate oxidation method. The reaction of glycerol with sodium periodate occurs in an acid solution, forming aldehydes and formic acid. The latter product is a measure of the glycerol in the sample.

$$C_3H_5(OH)_3 + 2HIO_4 \rightarrow 2HCHO + HCOOH + 2HIO_3 + H_2O$$

Equation 3-4

The following materials were used to perform the analysis:

- Sodium periodate solution
- Distilled water
- Sulfuric acid 0.1N
- Sodium hydroxide solution 0.125N
- Sodium hydroxide solution 0.05N
- Sulfuric acid solution 0.2N
- Ethylene glycol solution
- Bromthymol blue indicator solution 0.1% in distilled water

The samples were centrifuged to remove the cells from the fermented broth. Clear fermented broth was weighed in clean and dry volumetric flasks, containing between 0.15 and 0.25 grams of glycerol, according to the table 1 from the AOCS official method Ea 6-51. This procedure was scaled down in order to use half amounts of chemical reactants.

All the samples were washed with distilled water to dilute them, and then, five drops of bromthymol blue indicator was added to each sample. After the samples were acidified, with 0.2N sulfuric acid, to a definite yellow-green color. Finally, the samples were neutralized with 0.05N sodium hydroxide solution, to the indicator end point, sharp yellow green to blue transition was noted

A blank was prepared, containing distilled water, but no glycerol, and carried through simultaneously with the samples.

The periodate reaction occurred by adding 25ml of sodium periodate solution while stirring. The flasks were covered with a watch glass and left standing for 30 minutes at room temperature in the dark. At the end of this period of time, 5mL of 50% ethylene glycol solution were added to each sample and allowed to stand for another 20 minutes.

The samples and the blank were titrated with sodium hydroxide solution 0.125N, using a pH meter to determine the end point, pH 6.5 ± 0.1 for the blank, and 8.1 ± 0.1 for the sample.

The final volume used to neutralize the sample was recorded to quantify the glycerol percentage in the fermented broth.

The following equation was used to calculate de final glycerol concentration:

$$\text{Glycerol (wt\%)} = \frac{(S\text{-}B) \times N \times 9.209}{W}$$

Where

S = volume in mL of sodium hydroxide solution to titrate sample

B = volume in mL of sodium hydroxide solution to titrate blank

N = normality of sodium hydroxide

W = mass of sample in grams

3.4. Fermentation Products

Various methods for analyzing fermentation methods were reviewed (Biebl 2001, Biebl 1991, Heyndrickx and others 1991 ,Yong 1991, Forsberg and others 1987). However, it was necessary to develop an analytical method in order to use the existing columns and gas chromatograph. The concepts and criteria for the final selection of the method are summarized in the following section. A summary of the method can be found at the end of the chapter.

The choice of the carrier gas depends on the selected detector; for FID detectors hydrogen, helium or nitrogen are valid options. Manufacturer (HP 1989) says that helium is an acceptable carrier gas. Hydrogen is the recommended carrier gas and nitrogen is the least desirable option. Thus for safety reasons He ultrapure grade was selected.

3.4.1. Quantitative analysis

As the components of the sample eluted, the detector produces a signal. The voltage output of the detector is proportional to the concentration of the component present (Braithwaite 1996). In consequence, there is a correlation between the relative areas in the chromatogram and the relative amounts of each compound in the sample to be analyzed . For a FID detector, not all the compounds ionize in a hydrogen flame to form the same type of number of ions, this is different compounds can produce varying signals for the same concentration. In consequence, response factors may be determined experimentally and have to be obtained for each gas chromatograph machine (Braithwaite 1996, Gilbert 2006).

3.4.2. Internal standard method

There are least four quantitative methods that can be used, namely normalizing peak areas, internal standards, external standards and standard addition (Braithwaite 1996). The internal standard method was selected because this method involves ratio peak areas rather than absolute values, thus it does not depend on the injection of a precisely known amount of sample (Braithwaite 1996). To carry out this method it is necessary to add a known amount of a reference substance (i.e. the internal standard) . Subsequently the response factor of the analysis is calculated relative to the amount to the internal standard (IS)

The procedure to determine the response factors (DRF) involves analyzing known amounts of each component plus a determined amount of the internal standard. Then the response factor for each compound is calculated.

$$DRF_i = \frac{PeakArea_i}{PeakAreaI.S.} \times \frac{KnownAmountI.S.}{KnownAmount_i}$$

Equation 3-5

Then using the equation 3-6 and the information from the chromatograph, one can calculate the amount of a particular unknown compound in the sample of interest.

$$UnknownAmount_i = \frac{PeakArea_i}{PeakAreaI.S.} \times \frac{KnownAmountI.S.}{DRF_i} \qquad \text{Equation 3-6}$$

Unknown ethanol, butanol, butyric acid, acetic acid, and 1,3-propanediol were determined by injecting acidified and centrifuged samples into a HP 5890 gas chromatograph equipped with a flame ionization detector. Each sample was prepared by adding 10 µL of 6N HCl and 100µL of the internal standard to a 900µL of cell-free sample. (Kim 1991)

The DB-5 (USP 627) fused silica capillary column was 15 m in length with a internal diameter of 0.324 mm with 0.25 µm phase film. The analysis of the products was carried out under the following conditions: Injection temperature 163˚C, detector temperature 220˚C, stepwise temperature program from 80˚C to 160˚C (3 min initially, 30˚C/min increment) followed by a second temperature increase from 160˚C to 300˚C (1 min initially then a 30˚C/min heating rate). Helium gas the carrier gas and benzyl alcohol as internal standard, were used.

The programmed temperature gas chromatography (PTGC) is recommended when the sample contains components with different boiling points (McNair 1997). This is the case of the sample. Normal boiling points of the main fermentation products read

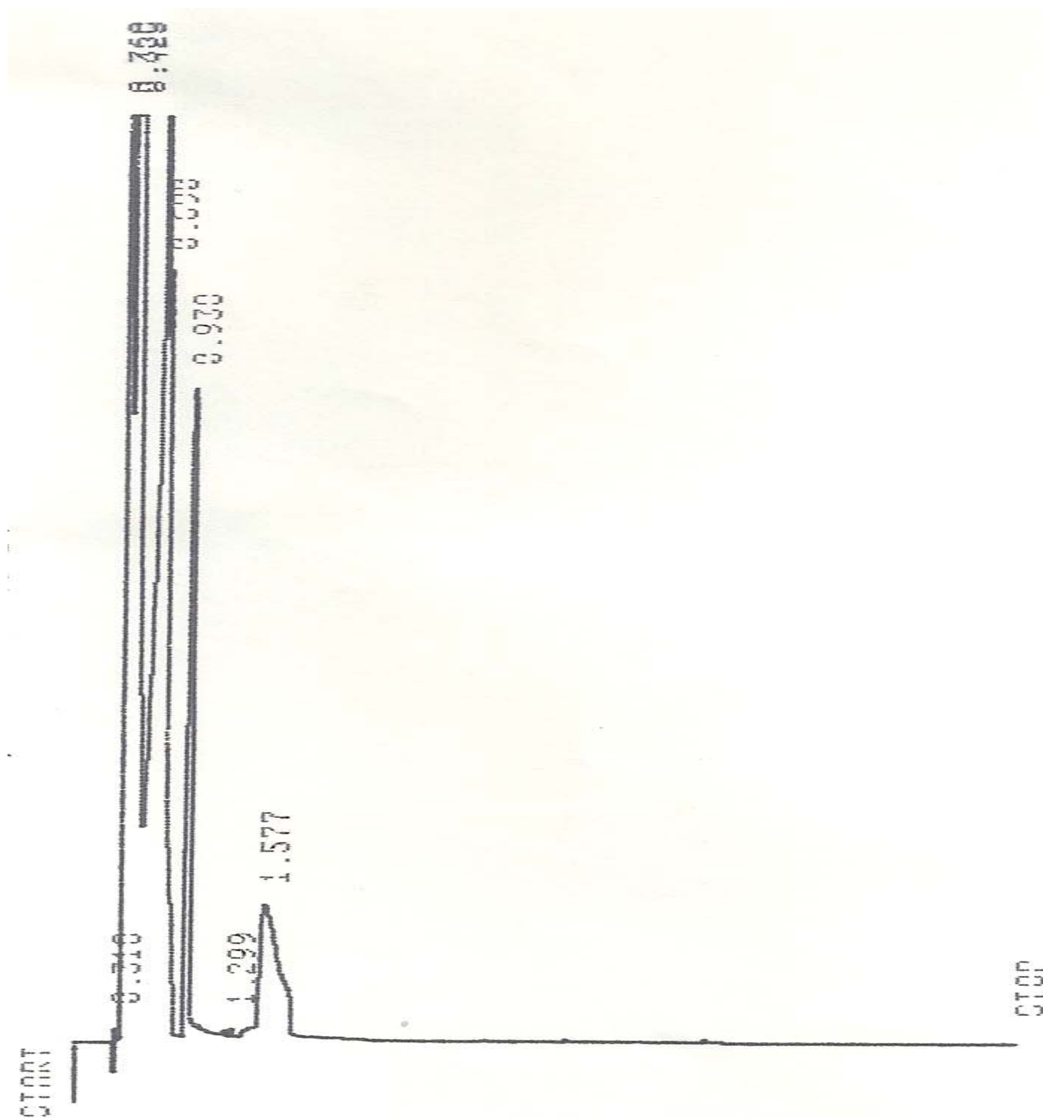
as follows: ethanol 78.4 ºC, butanol 117ºC, butiric acid 163.5 ºC, acetic acid 118.1 ºC, glycerol 290 ºC, and 1-3 propandiol 214 ºC. (Monick 1968)

There is no need to have the column temperature higher than the boiling point of some of the compounds, as explained by McMair (Macmair et al. 1997). The samples must be in the vapor phase not in the liquid phase, thus the column temperature must be kept above the dew point of the sample (Macmair et al. 1997)

3.4.3.Gas chromatograph method

Ethanol, butanol, butyric acid, acetic acid, and 1,3-propanediol were determined by injecting acidified and centrifuged samples into a HP 5890 gas chromatograph equipped with a flame ionization detector. Each sample was prepared by adding 10 µL of 6N HCl and 100µL of the internal standard to a 900µL of cell-free sample.

The DB-5 (USP 627) fused silica capillary column was 15 m in length with a internal diameter of 0.324 mm with 0.25 µm phase film. The analysis of the products was carried out in a Hewlett Packard 5890 series II chromatograph under the following conditions: Injection temperature 163˚C, detector temperature 220˚C, stepwise temperature program from 80˚C to 160˚C (3 min initially, 30˚C/min increment) followed by a second temperature increase from 160˚C to 300˚C (1 min initially then a 30˚C/min heating rate). Helium gas the carrier gas and benzyl alcohol 1wt% as internal standard, were used.



	RT	Area	DFR
Ethanol	0.31	12060	0.47
Butanol	0.36	1009715	0.93
n-butyric acid	0.425	3553422	0.66
Acetic acid	0.69	1655298	0.12
1-3-propanediol	0.93	466881	0.16
Internal standard	1.577	433428	-

Figure 3-3 Sample of a chromatograph

4. Chapter 4: Media Development

4.1. Introduction

There are two major types of culture media: chemically defined media and complex media. Defined media use compounds (organic or inorganic) with known chemical composition, therefore the exact composition of the growth media is known (Shuler and Kargi 2002, Brock 1991). In contrast complex media (sometimes called undefined media) contain natural occurring compounds, whose chemical composition is not accurately known (Shuler and Kargi 2002). Some examples of complex media nutrients are yeast extract, peptone, casein and beef extract. Growing cultures with complex media usually results in higher cell yields (Shuler and Kargi 2002). However an important disadvantage of using complex is media is the loss of control of the media specification, resulting in less reproducibility of fermentation runs (Shuler and Kargi 2002, Brock, 1991). Media optimization and the investigation of the role played by each of the compounds in the media can be studied more precisely with chemical defined media. Additionally purification of the product is often easier and cheaper when defined media was chosen (Shuler and Kargi 2002).

In order to scale up a process, reproducibility and control over the fermentation are desired. With this is mind it was decided to find a defined media composition using glycerol as the solely carbon source. Different media formulations reported in the literature were compared. Most of them used glucose as the carbon source and yielded no growth when they were tried. Thus a complementary medium in which cells were able to grow is proposed. This medium contains salts and biotin and p-

aminobenzoic acid (p-aba) as growth factors. *Clostridium pasteurianum* cells were tested for autotrophic growth with and without one or both of these two nutrients. A different approach using the replica plating technique was used to minimize the addition of complex nutrients to the inoculum to those formulations without the biotin and p-aba.

4.2. Defined media Formulation

It has been said that the choice to grow the cultures in defined media was made in order to have more control over the cells growth and to reduce the variability that growing cells with complex media yields (Shuler and Kargi 2002, Brock 1991). Additionally in order to calculate the substrate yield coefficient (Ys), is required to grow cells in minimal media.

Different chemically defined and complex formulations reported specifically for *Clostridium pasteurianum* where tested (Biebl 2001, Mallete 1974, Seargent 1968). In contrast with researchers mentioned, in the present study glycerol was used as the solely carbon. Although Dabrock and Biebl (Biebl 2002, Dabrock 1992) used glycerol as the carbon source, they added yeast extract to their formulation.

Chemically defined nutrients are classified in two major groups namely macronutrients and micronutrients (Shuler and Kargi 2002, Brock 1991). The name of these two categories is related to the amounts that are required for the microorganism in order to grow. It is said (Shuler and Kargi 2002) that macronutrients are needed in concentrations larger that 104- M and micronutrients (also called trace elements) in concentrations less than 104- M. The nutrients can be

found in different naturally or chemically synthesized compounds (i.e. the same nutrient can be found in more than one chemically structure).

MACRONUTRIENTS	
Name	Example of possible source
Carbon source	Sucrose $C_{12}H_{22}O_{11}$, glucose $C_6H_{12}O_6$,,glycerol $C_3H_5(OH)_3$
Nitrogen	NH_4 ,N_2 ,NH_4Cl , $(NH_4)_2SO_4$
Oxygen	Oxygen gas (O_2)
Phosphorus (PO_4^{3-})	KH_2PO_4 ,K_2HPO_4 ,NH_2PO_4
Sulfur	$(NH_4)_2SO_4$, biotin
Potassium (K^+)	KH_2PO_4, K_2HPO_4
Magnesium	$MgSO_4$•$7H_2O$, $MgCl_2$
MICRONUTRIENTS	
Name	Example of possible source
Cobalt	Present in corrinoid compounds such as vitamin B_{12}
Calcium	$CaCO_3$, $CaCl_2$•$2H_2O$
Iron	Fe•Na•EDTA, $FeSO_4$•$7H_2O$
Manganese	$MnSO_4$•H_2O, $MnCl_2$
Sodium	NaCl, Na_2MoO_4•$2H_2O$
Molybdenum	Na_2MoO_4•$2H_2O$
Copper	$CuCl_2$, $CuSO_4$
Zinc	$ZnSO_4$, $ZnCl_2$
Nickel	$NiCl_2$

Table 4-1 Macronutrients and micronutrients required for most organisms.

The preceding table (Table 4-1) summarizes the main macro and micronutrients required by most organisms (Shuler and Kargi 2002, Brock 1991)

It is worth to mention that literature identifies calcium as a component within the endospores and it is used to stabilize cell walls of some microbes (Staley et al. 2007). These two aspects are desirable for the purpose of the current research.

During the literature research the different elements of every formulation were ordered by nutrient source for a better comparison.

Biotin and p-amenobenzoic acid (p-aba) are the two growth factors that were added to the defined media in accordance to previous work (Heyndrickx, 1986). Biebl was able to grow *C. pasteurianum* using only biotin (Biebl 2001) and he reported that *C.*

acetobutylicum requires biotin and p-aba (Biebl 2000). It is said that biotin acts a prosthetic group for enzymes that act in carboxylation reactions and p-aba is a precursor of folic acid, a coenzyme involved in one-carbon unit transfer (Staley et al. 2007):

A set of experiments were designed to test auxotropic growth, (not the specific growth rate) (μ) of these two growth factors in *C. pasteurianum*.

Nutrient	Source	I. (g/L)	II. (g/L)	III. (g/L)	IV. (g/L)	V. (g/L)	VI. (g/L)	VII. (g/L)	VIII. (g/L)
Carbon	Glucose			10			20	20	
source	Sucrose	10							20
	Glycerol				20	50			
Mg	$MgSO_4•7H_2O$	0.25		0.1	0.251	0.2	0.1	0.1	
	$MgCl_2•6H_2O$	0.1							0.2
Sodium	NaCl	0.1	0.1				0.1	0.1	
(Na)	$Na_2HPO_4•12H_2O$			2.2					2.84
	$Na_2MoO_4•2H_2O$	0.012	0.01	0.01			0.01	0.01	0.01
	$Na_2S_2O_4•2H_2O$	0.035							
	$NaHCO_3$				6				
	$NaC_6H_{11}O_7$	20							
Phosphate	KH_2PO_4	0.95	1.4	5.97		0.5	1.4	0.5	10.9
& K^+	K_2HPO_4	5.75	7.8		1.74	0.5	15.6	0.5	
Potassium	KCl				0.596				
Calcium	$CaCO_3$	0.1						10	
(Ca)	$CaCl_2•2H_2O$			0.01		0.02			
Nitrogen	$(NH_4)_2SO_4$	1		6		3	0.8	0.8	0.08
	NH_4Cl	0.8			0.66				0.66
Iron	$FeSO_4$	0.0056		0.015		0.005	0.01	0.01	
	$FeCl_3$	0.025							$5x10^{-3}$
	Fe•Na•EDTA				0.069				
Mn	$MnSO_4•4H_2O$			0.015					
	$MnCl_2•4H_2O$	0.002	0.01						
Growth	Biotin	$1x10^{6-}$	$4x10^{6-}$	$0.2x10^{-3}$	$0.24x10^{-3}$		$5x10^{6-}$	0.001	0.02
Factors	p-aminobenzoic acid	$5x10^{6-}$	$4x10^{6-}$	0.002	0.004		$5x10^{6-}$	0.001	0.02
	Cysteine•HCl				0.5				
Complex	Yeast Extract				0.5	1			

I. Bender growth culture media (Bender, R., 1973)

II. Mc Cready growth culture media (Mc Cready, R. G., 1975)

III. Heyndrickx growth culture media (Heyndrickx, M., 1986)

IV. Dabrock growth culture media. Used 1mg of resazurin as indicator (Dabrock, B., 1992)

V. Biebl growth culture media (Biebl, 2001)

VI. Sargeant growth culture media (Sargeant, K. 1968)

VII. Carnahan growth culture media (Sargeant, K. 1968)

VIII. Mallette growth culture media (Mallette, M. 1974)

Table 4-2 Media formulations

4.2.1.Materials and Methods

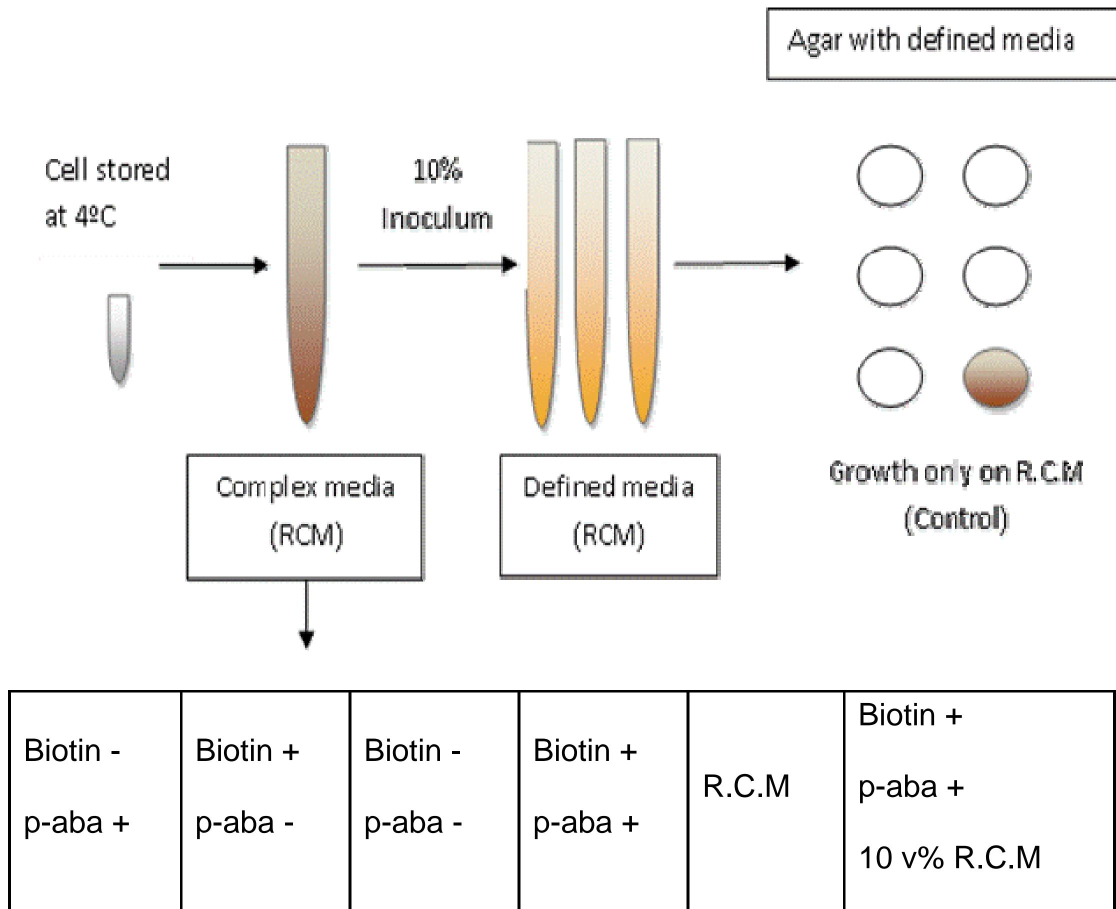
The selected media chemically media formulation contained (g/L): Glycerol 90 g. or as indicated; $MgCl_2$, g; NaMoO4·$2H_2O$, 0.01 g; KH_2PO_4, 10.9 g; K_2HPO_4, 2.84 g; $(NH_4)_2SO_4$,08 g; NH_4Cl 0.66 g; $MnCl_2$•4H2O 0.016 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $FeSO_4 \cdot 7H_2O$ 0.018g; biotin 0.004 g; p-aminobenzoic acid 0.004 g.

When the amounts per gram were identified, each nutrient amount was kept constant. This is, even though a different source for that particular nutrient was used correction to the weight were made. For example Bender (Bender 1973) used 0.25 g/L of $MgSO_4 \cdot 7H_2O$ as magnesium source. In order to use the same amount of Mg^{2+} within $MgCl_2$ some calculations were required because of the different molecules attached to magnesium in $MgSO_4 \cdot 7H_2O$ (M.W. 246.47 g/mol) and $MgCl_2$ (M.W. 95.21 g/mol) . Recall also that water is not present in anhydrous $MgCl_2$.

Defined media (read above) was prepared with and without biotin and p-aba . The positive controls were medium with both biotin and p-aba, medium without biotin and p-aba but 0.1% of yeast extract and Reinforced Clostridium media. Petri dishes containing 12.5 g/l of agar where prepared for each of the already mentioned formulations. Cells from a culture in defined media in the stationary phase were used to plate the Petri dishes in addition to colonies from plates grown in RCM plates. Growth was not identified in none of the Petri dishes except for the one with RCM. Because it was expected to see growth in the Petri dishes with p-aba another set of experiments were planned. This time using 10% of inoculum of media grown in liquid RCM was used to inoculate closed cap tubes. After 45 hours, growth was identified by an increase in the optical density of the samples. It was suspected that the 10% of the complex media contained enough nutrients to make the cells grow.

It was desired to minimize the fact that, when inoculating from a complex media, it is not possible to tell in trace of complex nutrients are been supplied within the inoculum. For example, Sargeant in his defined media formulation reported 50 μg/L of p-aminobenzoic acid (Sargeant 1968), which is a very small amount.

It was necessary then to find a way to inoculate without using liquid inoculum. Although one could argue that inoculating a single colony from a plate could yield similar results, it is important to recall that spontaneous mutations occur in bacteria due to replication errors with a frequency of $1x10^{-6}$ (Snyder and Champness 2007). Even though the probability is low, collecting a bigger number of cells would minimize the chance of selecting one mutant that could yield to wrong conclusions.



Biotin - p-aba +	Biotin + p-aba -	Biotin - p-aba -	Biotin + p-aba +	R.C.M	Biotin + p-aba + 10 v% R.C.M

Figure 4-1 Using replica plating to test auxotrophy

One of the advantages of bacteria is that bacteria are haploid. They have only one copy or allele of each gene. In addition to that, they multiply by asexual reproduction. It has the implication that the progeny are genetically identical to their parent. In other words bacteria produce clones. (Snyder and Champness 2007). With this knowledge in mind , it was decided to use the replica plating technique in order to end up with a even number of clones to inoculate the different media formulations with and without the vitamins under study

The cells were test for autotrophy for either or both of the vitamins (i.e. biotin and p-aba). Microbial growth was determined by measuring the increase in the optical density (OD 620) after 26 hours.

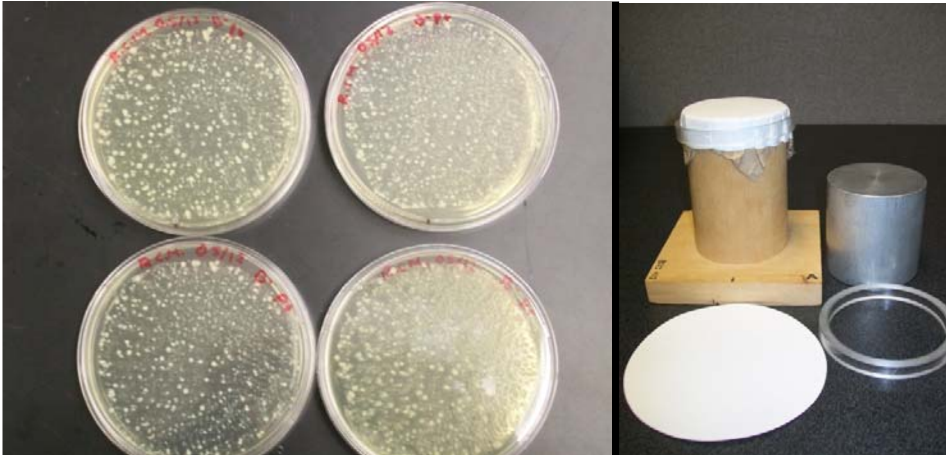
The different combinations were based on the same concentration of the defined media with a initial glycerol concentration of three percent. The six .distinct cultures media were as follows (the last two formulations served as controls):

Biotin - p-aba +	Biotin + p-aba -	Biotin - p-aba -	Biotin + p-aba +	R.C.M	Biotin + p-aba + 10 v% R.C.M

When a growth factor was not supplemented, the required amount of water was added, so the final concentration of all the other nutrients was conserved. Three weight percent of glycerol initial concentration was used.

4.2.2.Results

Replica plating technique was completed using non selective R.C.M. agar. Plates were inoculated in an anaerobic chamber at 37ºC for 48 hours. Colonies were counted, and the CFU number were compared among plates. The average number of individual colonies was 266 ± 29.



Ilustration 4-1 Replica plating

At least three different cultures of each combination of growth factors and the two positive controls were inoculated with cotton tipped applicator. Colonies in each plate served as inoculum for each one of the different media formulations complemented with the growth factors. A sample of the broth after inoculation was taken for each culture and it served as the blank.

Three percent of glycerol concentration as the solely carbon source and chemically defined media yielded growth in all the cultures. Foam formation and an increase in the broth turbidity evidenced growth.

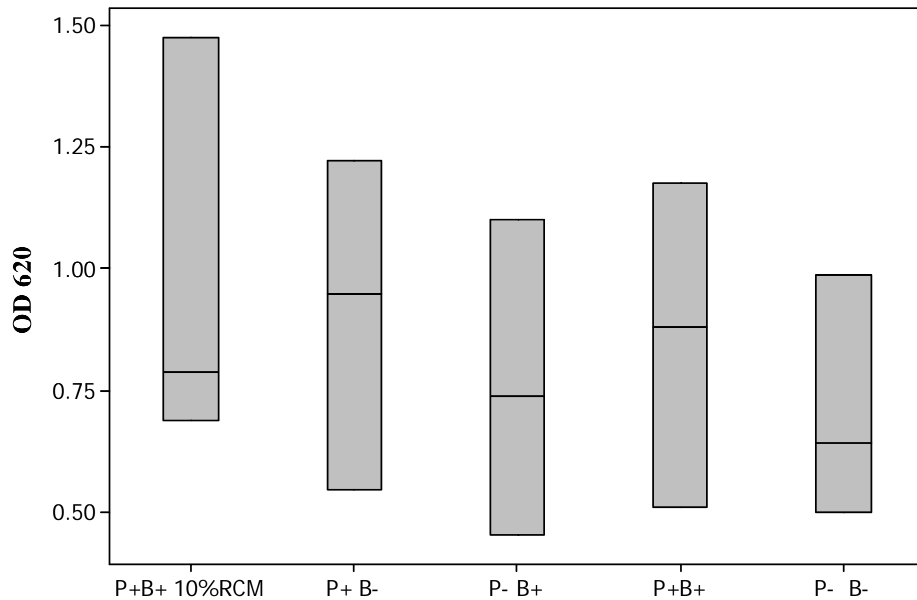


Figure 4-2 Box plot showing optical density with and without growth factors.

One can observe that the O.D was not zero for any of the samples. In fact an increase in the turbidity of the broth indicated cell growth. Just looking at the media without both biotin and p-aminobenzoic acid, one can tell cells can grow without those to organic compounds.

Results comparing both P+B+ and P-B- were subject to t-Test. Statistical analysis is summarized in the following tables

	Optical Density	
	Mean	Standard Deviation
Biotin + p-aba + (n=3)	0.86	0.33
Biotin - p-aba - (n=3)	0.71	0.25

Table 4-3 Results comparing positive and negative controls.

Statistical analysis results	
Degrees of freedom (υ)	4
Variance (s^2)	0.0872
Critical value of $t_{4, 0.05}$	2.776
t Test value	0.61

Table 4-4 Statistical analysis

The statistical analysis yields to no significance difference according the t-Test parameters ($t_{4, 0.05}$)

4.2.3.Discussion

Clostridium pasteurianum was able to grow in chemically defined media with glycerol as the solely carbon source without supplementing copper, zinc, nickel and cobalt. Future work could test if addition of these elements could have a positive effect on the growth and on solvent production.

Clostridium pasteurianum was able to grow without biotin a p-aminobenzoic acid. Thus it can be said it is not an auxotroph for this particular pair of nutrients. The

criteria to test for autotrophy test were the ability of Clostridium pasteurianum to growth without those growth factors. This experiment did not measure the specific growth rate (μ) with and without biotin a p-aminobenzoic acid.

The use of the replica plating technique as a source of an even number of clones was proven to work. The described methodology has the advantage that it minimizes noise that could be included when using liquid broth to inoculate. This approach can be especially useful to test the effect of different nutrients. It is not necessary practical to use when one wants to run a fermentation once the media composition has been determined.

The ability to identify the effect of each nutrient is especially important in the scale up process. Industrial glycerol may content impurities like salts or organic materials depending on each biodiesel manufacturing facility (Mantzouridou 2008). It means that in order to use industrial glycerol either one decides either to purify it (and add additional cost to the process) or to adjust the media formulation based on the chemical analysis of the raw material. For example if the industrial glycerol has already sodium salts (Mantzouridou 2008), the amount to be added to the culture media can de reduced in order to keep the same concentration that was used during the laboratory fermentations.

5. Chapter 5: Fermentation Parameters

5.1. Introduction

Before the drop in prices of glycerol, it was not considered as a reasonable substrate to produce commodities because its relative high cost (Biebl, 2001). With the increase in the production of biodiesel, by product glycerol became an attractive alternative as raw material. (Yasdani, 2007). From the several alternatives where the by-product glycerol can be used , the production of solvent was chosen during to course of this research. It is known that the description of the cellular processes cannot be omitted prior to the design of a fermentation process (Nielsen 2006). That is why it was considered relevant to study the fermentation parameters of the glycerol fermentation.

The concepts described here are general for microbial growth. However, we have described those relevant parameters (and the way how they can be calculated) for the purpose of this study, which is: anaerobic bacteria fermentation. Thus aerobic growth or fungi or mammal cell kinetics was not considered here.

Mathematical models that describe the fermentation process can be as simple as empirical correlation or can involve complex models considering the major metabolic reactions (Nielsen 2006). The fermentation parameters that were calculated correspond to those according to the unstructured model approach. This is, cells were lumped all together into one component assuming fixed cell composition (Shuler and Kargi 2006).

Kinetics of the stationary phase has brought our attention, because in the phase of the growth curve is when solventogenic clostridia produces butanol. (Paredes and others 2005, Wilkinson and others 1995)

5.2. *Kinetic Fermentation Parameters*

5.2.1. Specific growth rate

Microbial growth is modeled as an autocatalytic reaction (Shuler and Kargi 2002). The rate of growth is proportional to the cell concentration (Shuler and Kargi 2002). The net specific growth rate is defined as

$$\mu_{net} = \frac{1}{X}\left(\frac{dX}{dt}\right) \qquad \textit{Equation 5-1}$$

Where X is c ell mass concentration. The μ net specific growth rate can be expressed as the difference of the specific growth rate (μg) and the rate of loss of mass due to cell death or endogenous metabolism k_d (h^{-1}), as follows:

$$\mu_{net} = \mu_g - k_d \qquad \textit{Equation 5-2}$$

During the exponential growth phase k_d can be neglected and it is said the component of the cell grow at the same rate (i.e. balanced growth) (Shuler and Kargi 2002). Growth parameters are intrinsic of the particular microorganism – substrate system (Atkinson 1993). Here, *Clostridium pasteurianum*-glycerol, thus they have to be calculated. That is one reason why glycerol was studied as the solely carbon source, so we can define our system in the mentioned terms.

Since growth is independent of the nutrient concentration at constant temperature, one can write a first order equation:

$$\left(\frac{dX}{dt}\right) = \mu_{net} X \qquad \textit{Equation 5-3}$$

Integration from an arbitrary cell concentration Xo and to after the lag phase, we have :

$$\int_{Xo}^{X} \frac{dX}{X} = \mu_{net} \int_{to}^{t} dt \qquad \text{Equation 5-4}$$

$$\ln \frac{X}{Xo} = \mu_{net} t \qquad \textit{Equation 5-5}$$

The net specific growth rate can be estimated from the slope on a semi logarithm plot of ln X versus time. Following this procedure one can calculate the different growth rates at different concentrations (Figure 5-1)

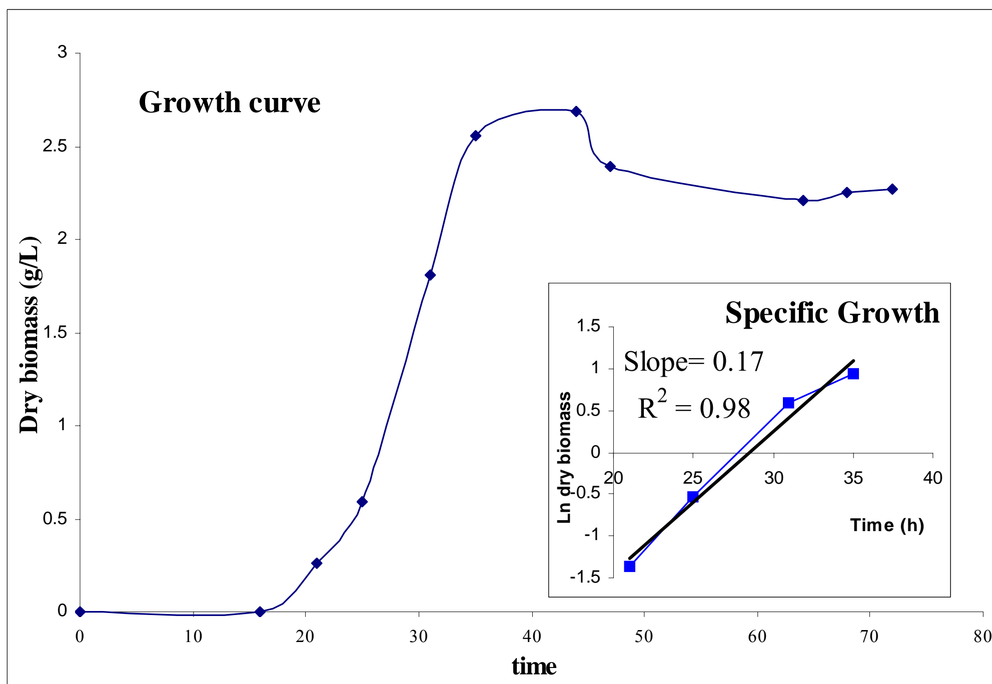


Figure 5-2 Growth curve and specific growth of *Clostridium pasteurianum* in defined medium with 6 wt% glycerol

For convenience, O.D. measurements can be used to determine the cell concentration. Then a correlation between the O.D. and the dry cell mass can be used

The following table summarizes the specific growth values using different glycerol concentrations

Initial Glycerol Concentration	**Specific Growth (h^{1-})**
3 % (n=2)	0.253 ± 0.056
6 % (n=2)	0.143 ± 0.04
9% (n=3)	0.075 ± 0.03

Table 5-1Specific growth rate at different glycerol concentrations

It can be seen that specific growth rate follows the following pattern:

μ Glycerol 3% >μ Glycerol 6% >μ Glycerol 9%

Some experimental specific growth values for glycerol metabolizing bacteria has been reported (see table below) (Atkinson 1996).

Microorganism	**μ (h^{-1})**
Aeromonas punctata	0.05-0.35
B. megaterium D440	0.10-0.50
B. megaterium M	0.05-0.15

Table 5-2 Reported specific growth rate for other glycerol fermentations

Thus *Clostridium pasteurianum* specific growth on glycerol in defined media is in the same order of magnitude when compared with *B. megaterium* D440.

The doubling time, called τd, can be calculated with the following relation solving for t (recall that t equals τd)

$$\tau_d = \ln\frac{2Xo}{X}\left(\frac{1}{\mu_{net}}\right) = \frac{\ln 2}{\mu_{net}} = \frac{0.693}{\mu_{net}}$$ *Equation 5-6*

Using the previous equation, one can estimate the doubling time for the fore mentioned concentrations.

Initial Glycerol Concentration	**Specific Growth (h)**
3 %	2.7
6 %	4.8
9%	9.2

Table 5-3 Estimating doubling time

5.3. Determining Yield coefficients

A biological reaction can be described in a pseudochemical reaction as follows:

Substrates + Nitrogen source→ Biomass + Extracellular Products+ Water + Carbon dioxide

However to describe growth kinetics it is useful to use some stoichiometrically related parameters in order to calculate material balances (Shuler and Kargi 2006,

Atkison 1993). They can be written for both product formation and cell growth if stoichiometrically coefficients can be estimated (Atkison 1993). For example for an anaerobic fermentation, like the one under the current study can be written as an stoichiometric balance equation as suggested previously (Papoutsakis 1984):

$CH_mO_l + aNH_3 = Y_{s/x}CHpOnNq + zCHOsNt + bH_20 + cCO_2$

Where $CHmO_l$, $Y_{x/s}CHpOnNq$ and $Y_{x/s}CHpOnNq$ correspond to organic substrate, cell biomass and extracellular products respectively. Then in a defined medium m,l,a,s,t are known. $Y_{s/x}$ and $Y_{p/x}$ are also known as yield coefficients (Shuler and Kargi 2006,Ratledge 2006, Atkison 1993). These parameters are defined on the amount of consumption of another material. Thus $Y_{s/x}$ is called growth yield.

For batch cultures we can measure the apparent or observed yield growth, which is not always constant (Shuler and Kargi 2006).

5.3.1.Growth Yield

The determination of the molar growth yield was performed according to the method designed by Stouthamer. Some considerations should be followed in order to calculate the growth yield (Stouthamer 1969):

- Chemically defined media should be used to grow the cells. If complex media is used, the carbon source should be labeled in order to follow its consumption.
- It is necessary to measure the grow yield at different substrate concentrations. The plot of growth versus yield should be linear.

The glycerol growth yield was measured at one, three, six, nine twelve and eighteen percent of initial glycerol concentration. When the maximum optical density was reached, the weight of the microorganism was determined as describe in Materials

and Methods. Results for one, three, six, nine percent of glycerol are plotted in Figure 5-4. .

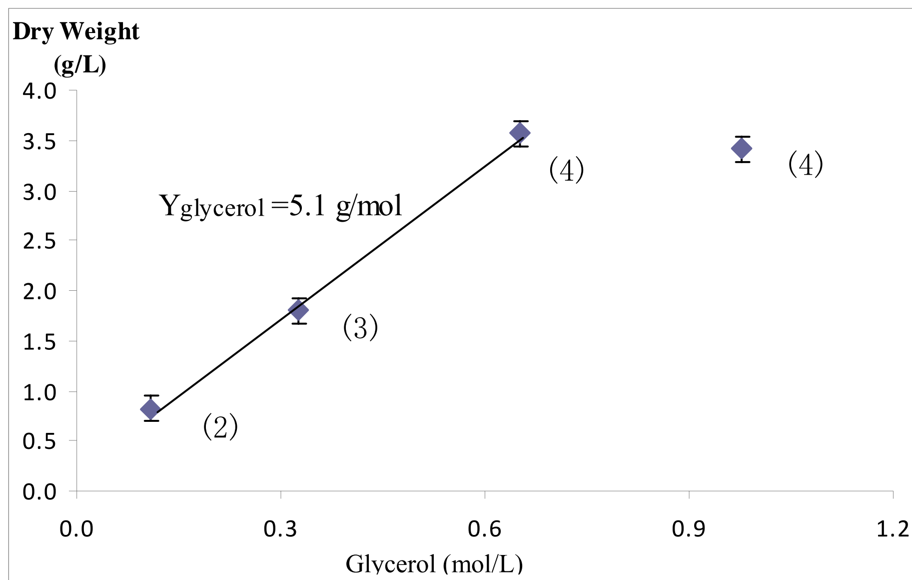


Figure 5-3 Results for one, three, six, nine percent of glycerol

Molar growth yield of *Clostridium pasteurianum* according to method of Stouthamer (Stouthamer 1969) Standard deviation from the mean are represented by the heights of the vertical bars. Number of estimations is shown in parentheses.

The calculated growth yield was 5.1 g/mol glycerol using glycerol as the solely carbon source. From the experiments that were performed, one can see that the linear relationship is conserved until 0.6 mol of Glycerol per liter. Data suggests that at 0.9 mol/L of glycerol the dry biomass keeps around the same value as it does at 0.6 mol/L of glycerol,

Previous growth yield values for *Clostridium pasteurianum* in glycerol where not found. The following table summarized data reported by Johnson (Johnson 1967 cited in Atkison 1995 p 139)

Microorganism	Ys (g /mol glycerol)	Source
Propionibacterium pentosaceum	20	Bauchop 1960 cited inAtkins 1983
Propionibacterium freudenreichii	26.3	Stouthamer 1976 cited in Atkiins 1983

Table 5-4 Growth yields from anaerobic growth of heterotrophs (Hernandez and Johnson, 1967 cited in Atkison 1995)

The ATP yield coefficient represents the amount of biomass synthesized per mole of ATP generated and it has been observed to be nearly constant at 10 to 11 g dry weight/mol ATP for growth under anaerobic conditions. For heterotrophic growth tha accepted value is 10.5 g dry cells/mol ATP and 6.5g dry cells /mol ATP for autotrophic growth (Shuler and Kargi 2002).

Additionally yield growth (Yx/s) and ATP yield coefficients are related with the amount of mole of ATP produced per gram of substrate produced N^{ATP} (Shuler and Kargi 2002, Atkinson 2005):

$Y_{x/s} = Y_{x/ATP}\ x\ N^{ATP}$ *Equation 5-7*

If fermentation pathways are known, the amount of ATP from the degradation of the substrate can be calculated . For example it is known that the glycolysis pathway yields 2 ATP/mol of glucose. For that particular case of glycolysis the N^{ATP} can be calculated as follows:

$$N^{ATP} = \frac{2 \text{ moles ATP}}{180 \text{ g glucose}} = 0.011 \frac{\text{mol ATP}}{\text{g glucose}}$$ *Equation 5-8*

Following an analog procedure and using the same assumptions one can estimate the the product $Y_{x/ATP}$ x N^{ATP} for Clostridium pasteurianum using glycerol as the carbon source should be close to 5.1 g/mol. However a better understanding of the metabolic pathways in Clostridium pasteurianum is need to estimate N^{ATP}.

5.4. Product Formation Kinetics

When the study of the butanol fermentation from glycerol by *Clostridium pasteurianum* started, it was not clear what pattern of microbial product it follows. Experiment results and understanding of clostridia life cycle has allowed classifying the butanol production as a non growth associated fermentation. The following section explores the product formation and its relation to the microbial growth.

5.4.1. Type of microbial product

Microbial products can be classified using the classical Gaden classification (Gaden 1959). There are three categories of fermentation products according to this classical approach namely: growth associated, non growth associated and mix-growth associated

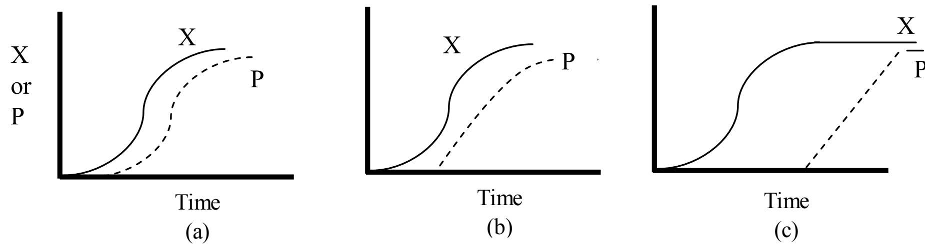


Figure 5-4 Figure Patterns of growth and product formation in batch fermentations (Gaden 1959, Shuler and Kargi 2006) (a) Growth associated, (b) mix-growth associated (c) no growth associated

Growth associated products are formed concurrently with microbial growth. Mixed growth associated products are produced during the slow growth and stationary phases. Finally no growth associated products occur during the stationary phase when the growth rate is zero (Shuler and Kargi 2006)

The stationary phase starts at the end of the deceleration phase. Some characteristics of this phase are (Shuler and Kargi 2002):

- Net growth rate is zero (no cell division)
- Cell lysis may occur and viable cell mass drop.
- Endogenous metabolism may be active.

For the particular case of solventogenic clostridia during the stationary phase one can encounter the following events (Paredes and others 2005, Wilkinson and others 1995) :

- Solvent formation
- Sporulation
- Autolysis

- Granulose accumulation.

Experiments were conducted to study the particular behavior of the glycerol fermentation.

Figure shows the course of batch glycerol fermentation by *Clostridium pasteurianum* (results of at least three fermentations).

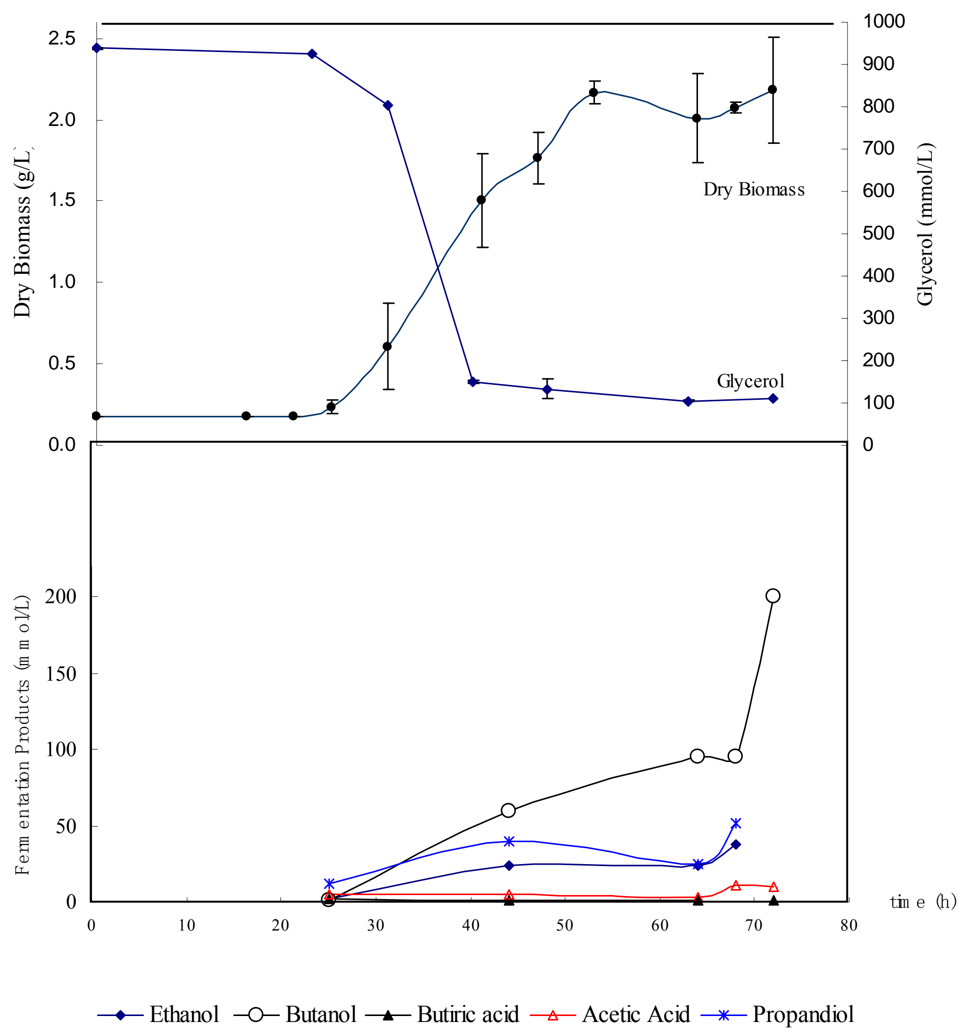


Figure 5-5 Glycerol consumption, growth and product formation

It can be observed that production of most of the butanol takes place during the

stationary phase. It has been said than two decades it has been recognized that endospore development and the solvent formation share a regulatory mechanism (Lee and others 2008). Clostridia abandon growth (i.e. its vegetative phase) to initiate the sporulation process (Stephenson 2005). Thus growth and sporulation do not occur simultaneously. After putting all these concepts together (for more evidence read below) one can classify the butanol production as no growth associated.

Shuler and Kargi (Shuler and Kargi 2002) have proposed an equation to describe the loss of cell mass due to cell lysis

$$\frac{dX}{dt} = -k_d X \qquad \textit{Equation 5-9}$$

If integration is carried over from the cell mass at the beginning of the stationary phase

$$\int_{Xso}^{X} \frac{dX}{X} = -k_d \int_{tso}^{t} dt \qquad \textit{Equation 5-10}$$

$$X = X_{so} e^{-kdt} \qquad \textit{Equation 5-11}$$

where Xso is the cell mass concentration at the beginning of the stationary phase.

$$ln \frac{X}{Xso} = -k_d t \qquad \textit{Equation 5-12}$$

6. Chapter 6: Sporulation and Solvent Production

6.1. Introduction

For more than two decades it has been recognized that endospore development and the solvent formation share a regulatory mechanism (Lee and others 2008). That is why it is relevant to study the sporulation process in the glycerol into butanol fermentation.

The ability to from spores has been identified as a pleitropic phenotype in solventogenic clostridia (Paredes and others 2005, Wilkinson and others 1995). This is solvent formation, sporulation and granulose accumulation share a regulatory mechanism. When sporulation was inhibited in mutants strains, the ability to produce solvents and to accumulate a glycogen-like polymer was also repressed (Wilkinson and others 1995). Previous researchers have shown that Spo0A gene controls the switch from the acidogenesis phase to the solventogenesis phase in *Clostridium acetobutylicum* (Wilkinson and others 1995, Ravagnani and others 2000). What it significant to the current research is that the Spo0A gene was also found in *Clostridium pasteurianum* ATCC 6013 (Wilkinson and others 1995, Brown 1994). That suggests that a similar process might happen in *Clostridium pasteurianum.* All these information has revealed the importance to study the sporulation process and the correlation with solvent production in *Clostridium pasteurianum.* An experiment was designed to try to verify if that relationship exist.

6.2. Clostridial Sporulation

The ability to form endospores is one of the four characteristics that was used to classify the genus Clostridium (Nigel 1989) (read above). Bacteria have the ability to adapt and sense environmental changes. Cells respond to by altering the pattern of gene expression in a coordinated manner (Stephenson 2005). Clostridia and bacilli apparently produce spores in order to survive to an unfavorable environment, this is when conditions become disadvantageous for growth (Paredes and others 2005, Stephenson 2005). In other words, cells will abandon cell division (the vegetative phase) to start the sporulation process (Stephenson 2005).

The process of sporulation starts when the vegetative cell (stage 0) instead of complete DNA replication, forms a pair of partially replicated chromosomes in an axial chromatin filament, a continuous structure spanning the length of the cell (stage I). The nest stage includes an asymmetric division that produces two compartments of unequal size, a larger mother cell and a smaller cell, the prespore (stage II). The prespore becomes engulfed by the mother cell, forming an independent protoplast. When the stage III is completed, the synthesis of a layer of peptidoglycan will cover the prespore (stage IV), and the subsequent deposition of protective layers of spore coat protein defines the stage V, meanwhile the prespore is dehydrated. Then the spore acquires full resistance properties during the spore maturation (stage VI). Finally, the mother cell lyses to release the endospore (stage VII). It will survive extreme environmental conditions, lying dormant and awaiting the restoration of

favorable growth conditions. (Paredes and others, 2005, Stephenson, 2005, Snyder and Chapness and Chapness 2007)

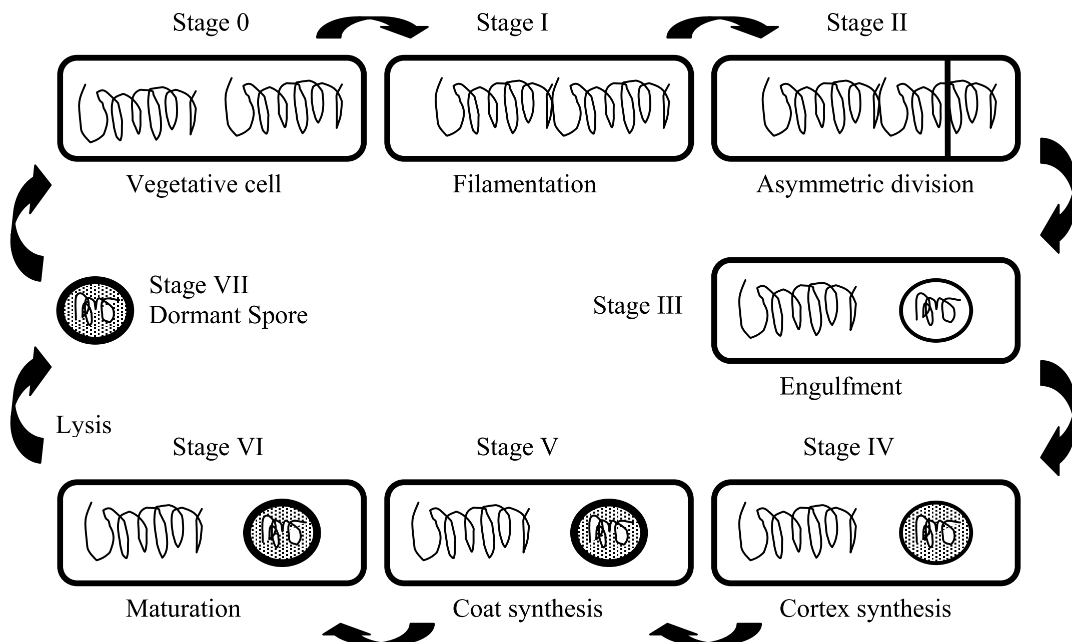


Illustration 6-1 Cartoon of the different stages during the sporulation process. Adapted from Stephenson (Stephenson, 2005)

These clostridial endospores survive to extreme conditions and are resistant to heat, oxygen, alcohol (Nigel 1989, Paredes and others 2005). They lie metabolically dormant awaiting for favorable growth conditions to return to the vegetative phase (Stephenson 2005).

Initiation of the sporulation process has been studied extensively for *Bacillus subtilis*. (Paredes and others 2005, Stephenson 2005, Snyder and Chapness and Chapness 2007) but it is not completely understood for clostridial species (Paredes

and others 2005, Nigel 1989). In fact, it is suggested that the signals that trigger the sporulation in *Clostridium* are different for those in *B. subtillis*. (Paredes and others 2005). One difference is that it seems that clostridial sporulation require a condition of sufficient carbon source (Paredes and others 2005), while in B. subtillis starvation is one of the factors that triggers the sporulation. (Ragavani 2000, Snyder and Chapness 2007). *B. subtilis* cells enter the stationary phase when starved and develops some adaptive responses including sporulation, competence, antibiotic and extracellular degradative enzymes production (Paredes and others 2005, Snyder and Chapness, 2007). In solventogenic clostridia, stationary phase events include solvent production, autolysis, granulose accumulation and sporulation (Paredes and others 2005, Wilkinson and others 1995).

The activate Spo0A protein has been shown to regulate the sporulation process apparently in all clostridia (Paredes and others 2005)

6.3. Methods and rationale behind the experiments

6.3.1. Selection of heat resistant spores

Classical genetics study individuals that differ from the wild type by a certain observable attribute or phenotype. A phenotype of an organism is an observable property of that microorganism (Snyder and Chapness 2007). What phenotype is useful for genetic experiments varies depending on the matter under study and on the microorganism. If a selective condition is applied to the environment, only those individuals resistant to the selective condition (or those who posses that phenotype) will survive (Snyder and Chapness 2007).

Eventhough the scope of the research does not include finding mutants defective in sporulation or solvent production, these concepts can be applied to select a members of population with one characteristic. The phenotype that has been chosen is the formation of spores. Clostridia spores have the attribute of being resistant to heat (Nigel 1989, Paredes and others 2005).

Then if heat is applied to a population it is expected that only those members of the population in the spore stage are going to be able to survive. However, doing this during the course of the fermentation is not possible to tell if the a requirement for survival requires that the cell has reach the stage VII of the sporulation process (read above). The goal of the experiment is to test if there is a correlation between the number of heat resistant individuals (assuming they are in some stage of the sporulation process) and the solvent production, particularly the production of butanol.

The selective condition was the same described by previous scientists (Ragavani 2000) trying to identify Spo- mutants. One difference with respect to the cited methods, is that cells were sampled directly from the fermentation broth rather than colonies from agar plates.

6.3.2. Experimental procedures

Clostridium pasteurianum ATCC 6013 cells were grown in defined media as described previoulsly using (read above) six and nine percent of initial glycerol concentration. Non selective Reinforced clostridium Media solid agar with glucose instead of glycerol was used to plate the cells. Gram stain technique was followed according to previous reference (SeeLey 1991). For growth curves, optical density

was measured in a Spectronic 20 Genesys spectrophotometer at 620 nm. The light path length was 1 cm.

Cell free samples were subjected to analyss of glycerol and butanol. Glycerol concentration was measured according to AOCS official Method Ea 6-51. Fermentation products were measured as described in material and methods (read above)

The heat selection procedure reads as follows:

- One mL of sterile sample was poured into a ependorf tube.
- Tubes were incubated at 80°C for 15 min.
- Samples were serially diluted in peptone water (50g/L peptone and 25 g/L NaCl) and then plated in duplicate or triplicate on RCM agar.
- Plates inside an anaerobic chamber were incubated at 37°C.
- Colonies were counted after 48 hours.

It was noted that it is important to count the plates after 48 hours. If a longer incubation period is chosen, the formation of liquid products as butanol could spoil the plates.

6.4. Results

Batch fermentations were started with both 6% and 9% of glycerol initial concentration. When broth samples were subjected to Gram stain, an interesting pattern was found. *Clostridium pasteurianum* stain Gram-positive in growing cultures (i.e. violet) but stain as gram negative during the stationary phase and when forming

spores. This is consistent to what has been reported previously (Biebl 2000, Jan et al. in chapter 2 Nigel 1989) for *Clostridium acetubutylicum*

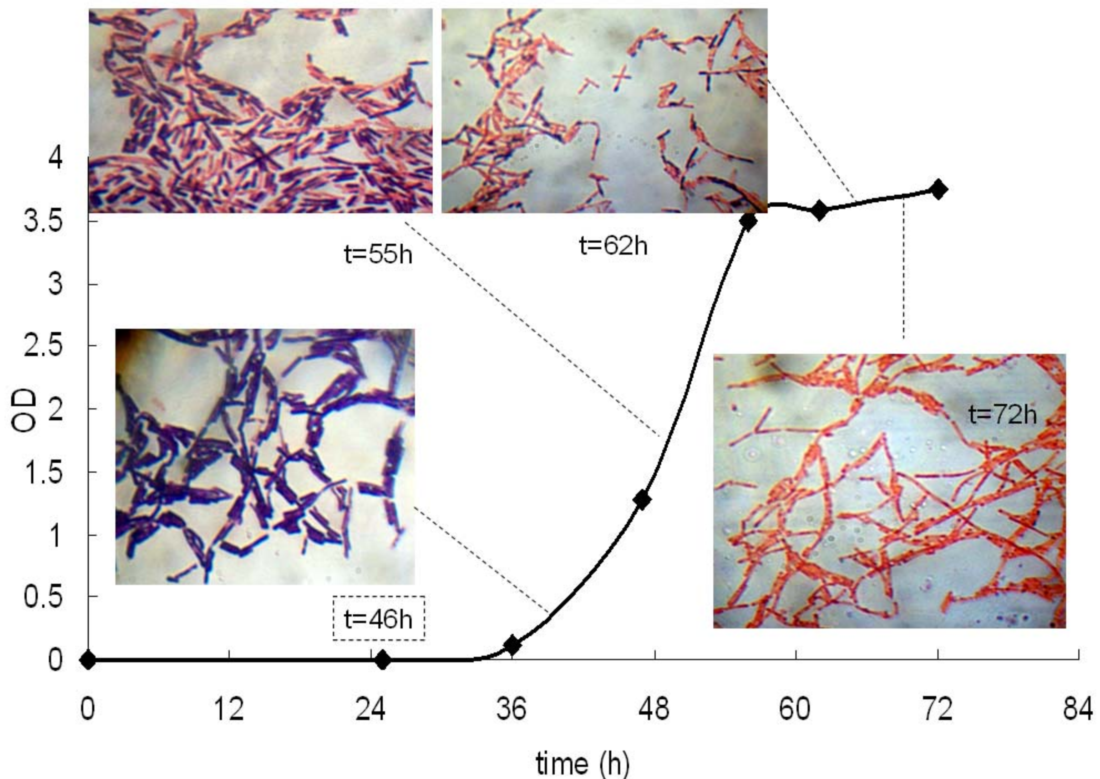


Illustration 6-2 Morphology differences of *Clostridium pasteurianum* during growth

Figure 6-1 shows the course average results of the at least 3 fermentations at nine percent of the carbon source. Biomass concentration was measured by optical density (OD_{620}) and the relationship with the dry biomass was used to plot the results (read above chapter about materials and methods).

The highest butanol concentration achieved was 21 g/L after 72 hours during one batch using nine percent of initial glycerol concentration. Previously reported highest

value was 17g/L. (Biebl 2001) This previous work analyzed the products after 24 hours and started with six percent of glycerol.

Regarding the relationship between the so called stationary phase events, it can be seen that most of the butanol is produced when the exponential growth cease (i.e., during the exponential phase). This observation is supported with what has been reported for solventogenic bacteria (Paredes and others, 2005).

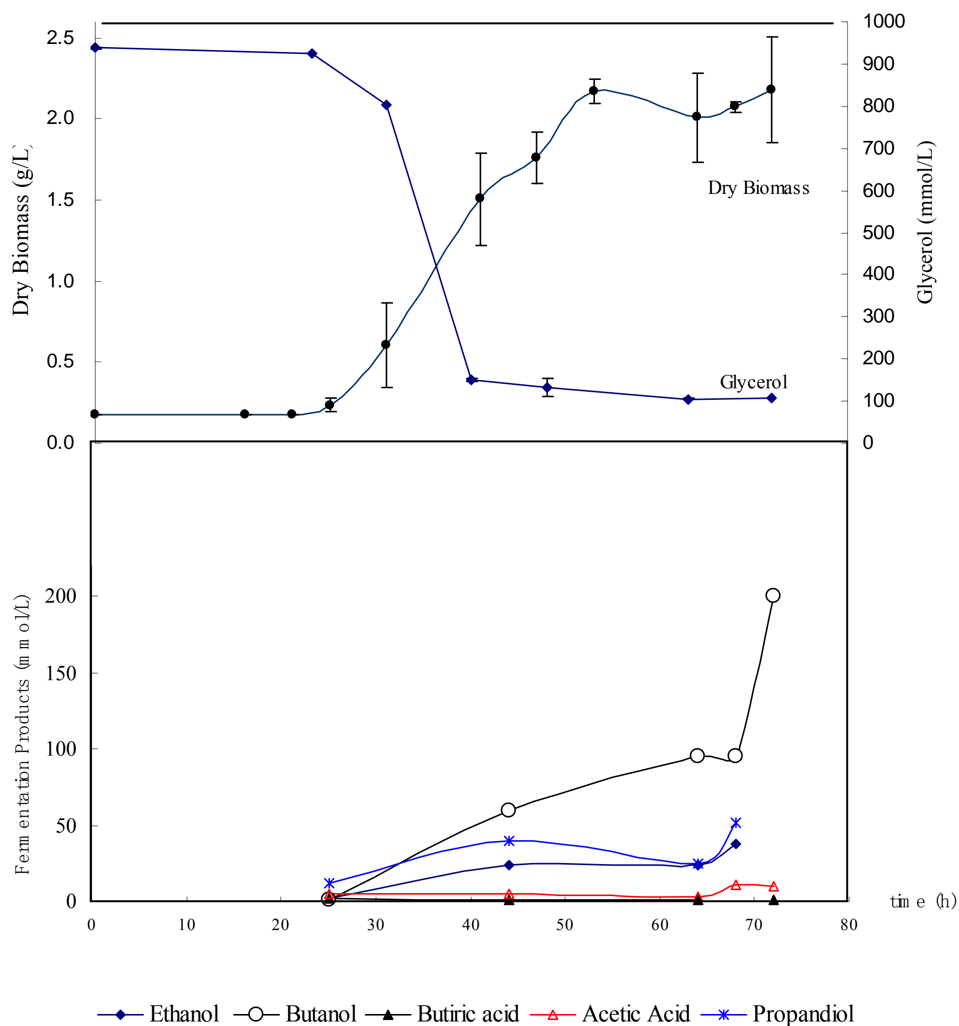


Figure 6-1 Growth, glycerol consumption and product formation in batch mode with 9% glycerol in defined media. Result of at least three fermentations.

However it was desired to explore if a closer relationship exists with the formation of spores, rather than just examining the growth curve.

6.4.1. Selection of heat resistant cells

It was first verified that the proposed methodology had a selective effect (this is, incubation at 80°C during 15 minutes). Samples were plated with and without the heat treatment. Plates for the same serial dilution without heat selection yielded too many colonies to be counted. In many cases no single colonies were found but a layer of cells. Plates within cells after heat treatment showed less CFU when they were plated. A representative picture is shown in Figure 6-2

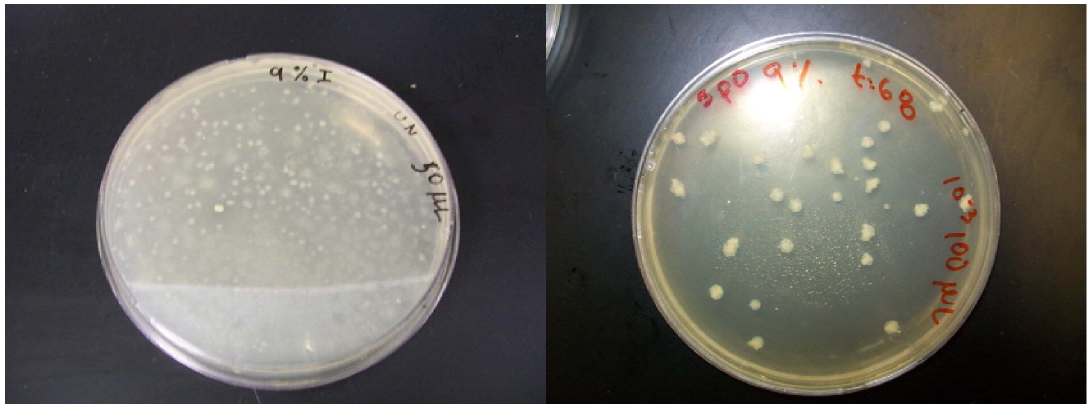


Figure 6-2 Fermentation broth sample plated without heat selection (left) and after heat selection (right)

This suggests that just individuals who were in some stage of the sporulation process were able to survive. However it is not possible to tell in what stage of the sporulation process these cells were, when the heat was applied to them.

6.4.2.Heat resistant individuals and the carbon source

Not only solvent production was measured to the sample but also the residual glycerol present in the broth.

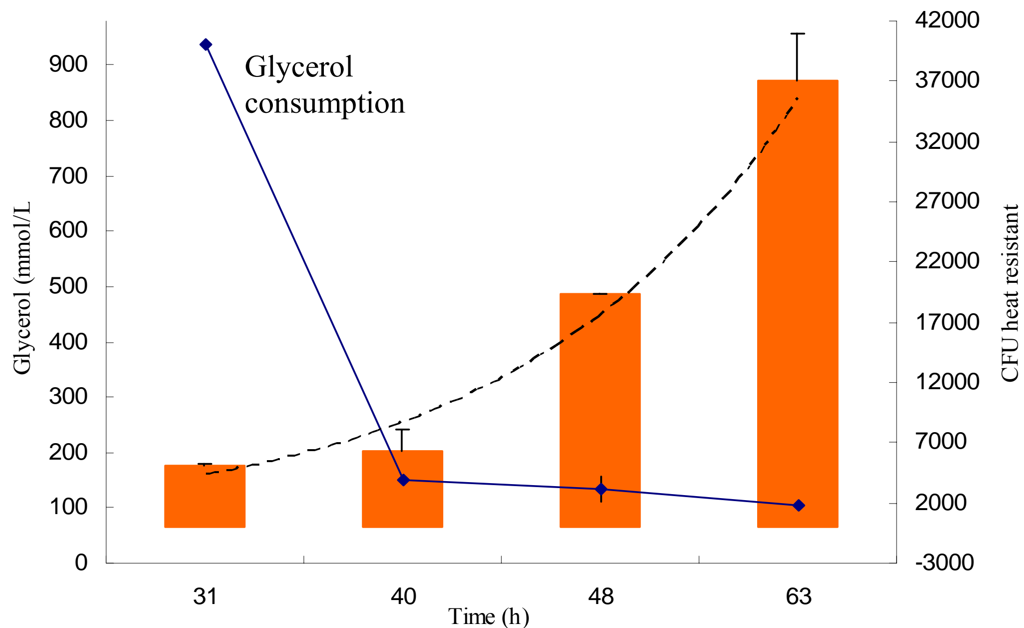


Figure 6-3 Glycerol consumption and CFU after heat shock (bars) --- Exponential growth of number of CFU as function of the time

It is interesting to see that even before the depletion of glycerol some heat resistant cells can be found. It can be observed that glycerol after 40 hours was close the 100 mmol/L (1wt% approximately). In addition, one can see that the glycerol concentration keeps constant even though the number of spore is growing. These observations are in agreement with has been found during clostridia sporulation regarding the levels of carbon source. It has been said that sporulation requires a condition of sufficient carbon source (Paredes and others 2005). One can ask an interesting question: What is the source of energy (i.e. ATP) during the sporulation

process? Same question is valid for *B. subtilis* sporulation; recall that in that case starvation of the carbon source is achieved before the sporulation process starts.

The best mathematical function (best curve fitting) that describe the increasing number of spores over the time was a exponential curve (R2=0.94). For this particular experiment starting with a glycerol concentration of 9wt%, the data showed the following mathematical function

$$CFU_{\text{heat resistant}} = 2117x\ e^{0.705\,t}$$

When the sporulation process in clostridia was studied, descriptions to the fate of a individual cell were mentioned. No information in relation to the behavior of all the population in a bulk sense was described. However exponential functions (rather than lineal models) have been used to describe the behavior of population growth (Shuler and Kargi, 2006, Staley 2007).

6.4.3.Number of heat resistant cells and solvent production

The number of colonies after heat selection was counted at different stages during the fermentation batch. Part of the sample was plated and the remaining one was subject to measurement of the butanol concentration. This was done for at least four runs both 6% and 9% initial glycerol concentration. A semi log plot, ln CFU heat resistant cells vs. butanol concentration was constructed and results are shown in figure 6-4.

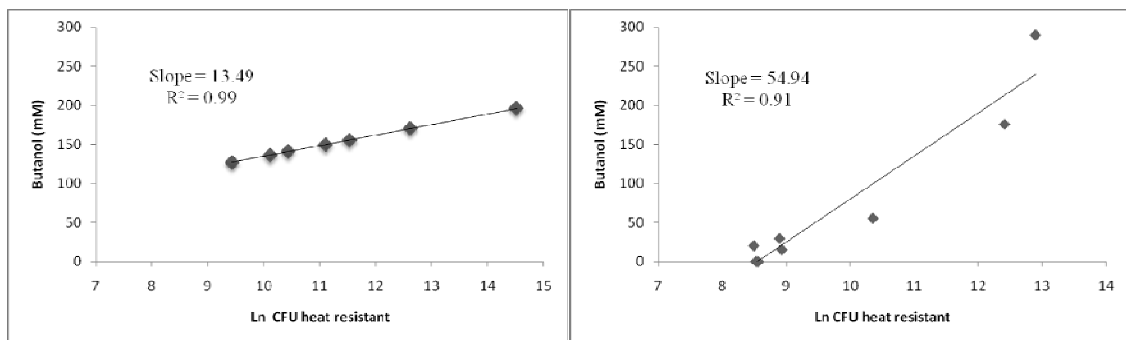


Figure 6-4 Correlation between number of CFU heat resistant cells and butanol production at two different initial glycerol concentrations 6wt% (left) and 9wt% (right).

The results suggest that there is a correlation between number of cells and the butanol production. Furthermore it can be noted that a linear regression can be obtained when the logarithm of the CFU is plotted rather than the actual number. The trend was observed working with two different glycerol concentrations and results of at least 5 different cultures. It can also be observed that the slope of the line is different for each of the concentrations.

6.5. Discussion

Environmental parameters have been studied to identify what triggers the solvent formation, with emphasis in the fermentation products rather than the sporulation process (Paredes and others 2005). Although both approaches might be equivalent, the rationale throughout the current research took other pathway: study the sporulation process trying to favor the solvent production.

The following information (from literature and from the current experiments) has suggested that sporulation and solvent production (and possibly granulose accumulation) share a master regulatory system in *Clostridium pasteurianum*:

- *Clostridium pasteurianum* spolurates. That was seen in the microscope and when selection by heat was applied to cells within the broth.
- Spo0A gene was reported in the literature for *Clostridium pasteurianum* ATCC6013 (Wilkinson and others 1995, Brown 1994)
- One aspect of the pleiotropic phenotype related to the sporulation process is the accumulation of a glycogen like polymer. The ability to produce α-glucan was reported for *Clostridium pasteurianum* (McCready, 1976)
- The greater number of spores was found in the stationary phase and most of the butanol as well. It was said the in solventogenic clostridia stationary phase events include solvent formation, sporulation and granulose accumulation. (Paredes and others 2005)
- An increasing number of spores was correlated with the production of butanol.

By definition the spores are in a dormant stage (Stephenson 2005). If the end of the sporulation process is attained (stage VII) it is very likely to think that any formation of products can be achieved after that event. If the sporulation controls the solvent production that might be a limiting factor. It has been said that solvent toxicity ceases the cellular metabolism (Lee and others 2008) but no relation with the sporulation process was mentioned. Metabolic engineering has focused on producing

more solvent tolerant strain of clostridia (Lee and others 2008). Then it is important to determine what stops the solvent production, namely the solvent toxicity or the end of the sporulation process.

One interesting question that can be raised is if the sporulation cascade of events can be stopped once it has started. The answer to this question would have implications in the selection of the fermentation mode, namely batch fermentation or continues fermentation. It has been explained how growth and sporulation are separate process (Stephenson 2005) , thus the operator of the fermentor can favor one of the other event. That is what is happening over time in the batch fermentation (and was evidenced through the experiments). Conditions in the environment change as the fermentation runs. Those changes include more cell concentration, less carbon source available and extracellular products secrete into the broth. However it is possible to investigate a two stage continues fermentation arranged in a way where one fermentor favors the growth and the second one posses characteristic that can trigger the sporulation (those still to be determined).

Chapter 7: Study of the effects of hydrosulfite during the growth and the effects in the solvent production.

7.1. Introduction

Both gram positive bacteria *Bacillus subtilis* and Clostridium spp. have the capability to form endospores, however the factors that activate the sporulation process in each of them are not the same (Paredes and others 2005, Ravagnani and others 2000). In *B. subtilis* sporulation initiates when the cells experience nitrogen and carbon starvation (Ravagnani and others 2000). In contrast, clostridium sp. seems to need sufficient source of substrate. Some of the following factors are suggested (Paredes and others 2005) to trigger the stationary phase in solventogenic clostridia. (This is the phase when sporulation is initiated): Low internal pH, the addition of butyrate, high levels of carbon source and ATP, and, or increased levels of intracellular reduction energy. Stress in the form low pH can accelerate the solventogenesis but might not be absolutely required. An expression stress gene is induced in response to stress by butanol, oxygen and heat. (Paredes and others 2005).

From the previous factors, the one related to the internal reduction energy was decided to be explored. It was then tested effect of growing the cells in a higher reduction environment using a reducing agent.

7.1.1.Selecting the reducing Agent

The tendency of a substance to donate electrons (i.e act as a reducing agent) or accept electrons (i.e. act as a oxidizing agent is expressed as the reduction potential. (Eo). It is measured in reference to a standard substance H2. By convention, reduction

potential are expressed for half reactions written with the oxidant on the left, this is as reductions (Brock 1991)

Oxidant + e- Reduced product

The oxidation reduction pair with greatest Eo negative values have greatest tendency to donate electrons.

Electron acceptor compounds can be organic and inorganic (Brock 1991).

The following is a list of some of the reducing agents that are utilized to grow anaerobic microorganism with its respective concentration (Willis 1979):

Reducing Agent	**Concentration**	**Comments**
Thioglycollic acid	0.01-0.2%	It is inhibitory to the growth of some clostridia
Ascorbic acid	0.10%	Inhibitory to some non-sporing anaerobic bacilli
Cysteine hydrochloride	Up to 0.05%	Higher concentrations may inhibit growth
Sodium Sulphide (Na_2S)	0.03%	Inorganic compound

Table 7-1 different reducing agents available for biological media

From the previous compounds sodium sulfide is the only inorganic compound and it looks like to be suitable for industrial purposes (recall butanol is a commodity product and it is not a common practice to add to the media a chemical with a higher added value like vitamin C).

7.1.2. Dissimilative Sulfate Reduction

Sulfur is a nutrient that is absolutely required for growth because its part of both amino acids cysteine and methionine and is part of vitamins such biotin and thiamine (Stanley 2007, Brock 1991). More precisely, inorganic $SO4^{-2}$ is reduced as a source of sulphur and the end product of such reductions are sulfhydryl groups (-SH). When it is used as a nutrient source it is said it was assimilated by the microorganism, but when it is used as electron acceptor for energy metabolism it is said to be dissimilative. Assimilative metabolism and dissimilative metabolism are not the same and have different purposes. The former end up as part of the cell composition at it is identified as organic sulfur (R-SH). The later utilizes sulphate as an electron acceptor for energy generation (Brock 1991).

Previous work (Harrison 1984 cited in Nigel Minton 1989) has identified an inducible dissimilatory type sulfite reductase in *Clostridium pasteurianum.* Then it is possible that *Clostridium pasteurianum* might have a sulfite dissimilative metabolism. It is important to notice that there is more than one form of inorganic sulfur compound where one can find sulfur with different oxidation states.

Compound	**Formula**	**Oxidation state**
Organic S	R-SH	-2
Sulfide	H_2S	-2
Thiosulfate	$(S_2O_3)^{2-}$	2
Sulfite	$(SO_3)^{2-}$	4
Metabisulfite	$(S_2O_5)^{2-}$	2
Dithionite (hydrosulfite)	$(S_2O_4)^{2-}$	3

Table 7-2 Oxidation state of some sulfur compounds

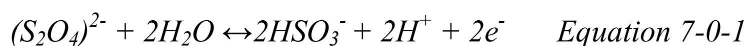
However in terms of reduction potential what one should look at is the E_0 values. The oxidation-reduction pair with greatest E_0 negative values have greatest tendency to donate electrons. The following table puts together some of these values for sulfur compounds.

Redox Pair	E_0' (v)	Source
SO_4^{2-}/HSO_3^-	-0.52	Brock (1991)
$(S_2O_4)^{2-}/HSO_3^-$	-0.386	Mayhew (1978)
$(S_2O_3)^{2-}$/HS- $+HSO_3^-$	-0.36	Brock (1991)
SO_4^{2-}/HS^-	-0.217	Brock (1991)
HSO_3^-/ HS^-	-0.11	Brock (1991)

Table 7-3 Redox potencial of some sulfur compounds

From the above list, the redox pairs with highest reduction potential are SO_4^{-2}/HSO_3^- and $(S_2O_4)^{-2}/HSO_3^-$. However anaerobic reduction of sulphate $(S_2O_4)^{-2}$ is describe as the redox pair of SO_4^{-2}/HS^- instead, with the associate reduction power of -0.217 V (Brock 1991). In addition to that, under aerobic conditions, sulfate ($SO4^{-2}$) is the stable form of sulfur, but hydrogen sulfide (H_2S) is the stable form under anaerobic conditions (Liamleam 2007). Then it was decided to select dithionite in the form of sodium hydrosulfite, $Na_2S_2O_4$ (CAS 7775-14-6) as the reducing agent.

In biochemical research the reduction reaction reads as follows (Mayhew 1978):



$(S_2O_4)^{2-} + 2H_2O \leftrightarrow 2HSO_3^- + 2H^+ + 2e^-$ *Equation 7-0-1*

The experiment aims to test if the addition of hydrosulfite has an effect in the sporulation process and as a consequence in the solvent production. As noted by other investigators working with solventogenic clostridia, the pleiotropic character of the sporulation process (Wilkinson and others 1995) has allowed correlating both events.

Using defined media without hydrosulfite as control (μ_1) a statistical analysis was carried on. The null hypothesis that $Ho=\mu_1=\mu_2$, having the alternative hypothesis $Ho=\mu 1 \neq \mu 2$.

If the null hypothesis is rejected, then we will accept the hypothesis that the addition of hydrosulfite has an effect in the sporulation process reflected in an increase of butanol production.

7.2.Methods

It was decided to study if the is a significant difference between the two treatments (this is, with and without sulfite addition). The statistical procedure called t Test or student's test was chosen for this purpose. The reason why it was chosen was that the fore mentioned test, allows to test the differences in two groups.

The test examines the following ratio (Glantz S.A. 1992):

$$t = \frac{\text{diference in sample means}}{\text{standard error of difference of sample means}}$$ *Equation 7-2*

To be able to conclude that the two samples were unlikely taken from the same population, this ratio should be large. In other words that the treatment produced an effect.

The t-Test definition for samples of different size reads as follows:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\left(s^2 / n_1 \right) + \left(s^2 / n_2 \right)}} \qquad \textit{Equation 7-0-3}$$

In this case the degrees of freedom are calculated as follows: $\upsilon = n_1 + n_2 - 2$.

And the pooled estimate of variance according to:

$$s^2 = \frac{(n_1 - 1) SD_1^2 + (n_2 - 1) SD_2^2}{n_1 + n_2 - 2} \qquad \textit{Equation 7-4}$$

Where SD stands for standard deviation of each of the populations under study.

Two Sample Hypotheses (Testing for difference between two means). It is worth to mention that the same equation is valid if the number of samples is the same for both groups (Stanton 1992).

7.2.1. Experimental methods

Clostridium pasteurianum was grown in 300 ml and 150 ml batches in media containing the following amounts (g/L): Glycerol 90 g; $MgCl_2$ g; $NaMoO_4 \cdot 2H_2O$, 0.01 g; KH_2PO_4, 10.9 g; K_2HPO_4, 2.84 g; $(NH_4)_2SO_4$, 08 g; NH4Cl 0.66 g; MnCl2•4H2O 0.016 g; FeSO4•7H2O 0.018g; biotin 0.001 g; p-aminobenzoic acid

0.001 g; Ten volume percent of cultures grew in modified reinforce clostridium media was used as inoculum. Cells were harvested after 72 hours.

Tree levels of sodium hydrosulfite ($Na_2S_2O_4$) were selected having as reference those concentrations that were reported previously in a study were Na_2SO_4 and Na_2SO_3 were used instead. (McCready, 1975)

The selected concentrations were: $6x10^{-5}$ M, $6x10^{-4}$ M and $6x10^{-3}$ M.

The addition of $6x10^{-3}$ M. $6x10^{-5}$ M was made after autoclaving all the other nutrients and before inoculation. Additions $6x10^{-5}$ M, $6x10^{-4}$ M and $6x10^{-3}$ M of hydrosulfite during the exponential growth were made weighing the corresponding amount and dissolving it with 5 ml of sample of the current fermentation. A common source of inoculum and media was used for both groups; this is the control and the batches with the hydrosulfite addition.

7.3 Results

When hydrosulfite was added from the beginning of the fermentation, no growth was detected using $6x10^{-3}$M of reducing agent. Samples were then recovered from the control batches (i.e. media without hydrosulfite) and from the $6x10^{-5}$ M fermentors. Specific growth (μ) was then calculated as describe previously (read above).

From the preceding results it was noted that concentrations at the level of $6x10^{-3}$M (two replicas) showed a inhibitory effect on the growth under the conditions described (i.e. minimum media and glycerol initial concentration of 9 wt%).

Specific growth of control groups (6 samples) and those within $6x10^{-5}$ M of sodium hydrosulfite were compared statistically at a significance level of 0.05. The analysis showed that significance difference.

	Specific growth (h^{-1})	
	Mean	Standard Deviation
$6x10^{-5}$ M Sulfite at t_0 (n=2)	$2.7x10^{-4}$	$1.34x10^{-6}$
Control (i.e. without sulfite) (n=6)	$2.9x10^{-4}$	$6.69x10^{-5}$

Table 7-0-4 Results from fermentation with $6x10^{-5}$ M sulfite at t_0

Statistical analysis results	
Degrees of freedom (υ)	6
Variance (s^2)	$3.7x10^{-9}$
Critical value of $t_{6, 0.05}$	2.447
t Test value	-0.379

Table 7-5 Statistical analysis

This mean statistically that the two groups compared are not different (Ho: μ1=μ2). Knowing that $6x10^{-5}$ M sulfite has not an inhibitory effect in the growth, them it was proposed to test the hypothesis that adding this concentration during the exponential growth would trigger the sporulation. The success criterion in this new set of experiments would be to see the stationary phase earlier in comparison with the control batch and or a change in the specific growth. The rationale behind this is the following: the sporulation process is associated with the stationary phase and not with

the growth phase (Wilkinson and others 1995). In other words, Clostridium cells do not sporulate while they are growing.

Data from 12 runs (6 per each group) is summarized. Hydrosulfite addition was done during the exponential growth at the 44 and 48 hour.

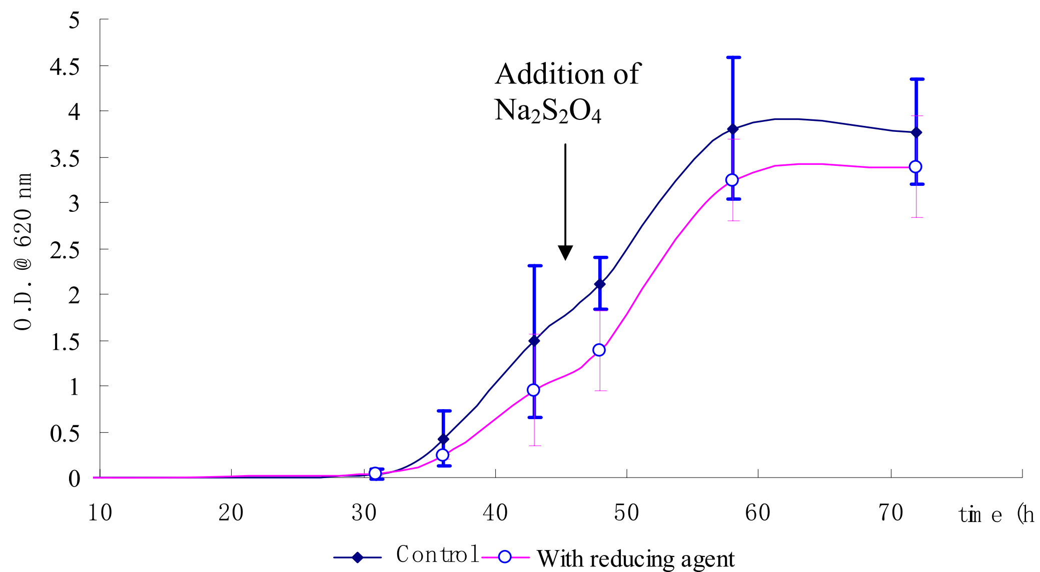


Figure 7-1 Growth curves of both cultures with and without hydrosulfite

It can be seen that the standard deviation bars overlap on the growth curve. The lag phase did not started at an earlier time. The Statistical analysis comparing the specific growth with six batches per group, confirmed that there is no statistical difference between the two groups at the level of 0.05

	Specific growth (h^{1-})	
	Mean	Standard Deviation
$6x10^{-5}$ M Hydrosulfite at t_{44-48} (n=6)	$3.4x10^{-4}$	$9.49x10^{-5}$
Control (i.e. without sulfite) (n=6)	$2.9x10^{-4}$	$6.69x10^{-5}$

Statistical analysis results	
Degrees of freedom (υ)	10
Variance (s^2)	6.7
Critical value of $t_{6, 0.05}$	2.228
t Test value	1.017

Table 7-6 Effect of sulfite during exponential growth

The fact that cells didn't grow using $6x10^{-3}$M sulfite but was no significance difference was found at $6x10^{-5}$ M level, suggested that an intermediate concentration should be used. Then 4 runs adding $6x10^{-4}$M sulfite during the exponential growth $6x10^{-4}$M hydrosulfite were set. For this experiment the amount production of solvents (i.e. butanol plus ethanol) was measured and compared against batches without sulfite addition. Concentrations after 72 hours are summarized in the following graph.

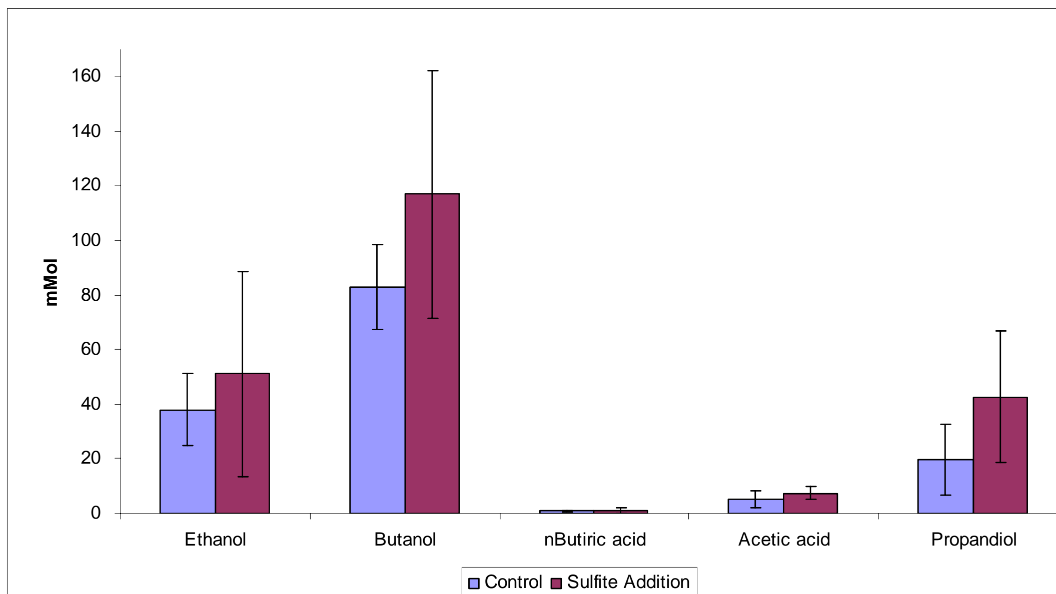


Figure 7-2 Effect of sulfite addition compared against control without addition

Product formation of both set of fermentation without addition (control) and with addition of hydrosulfite at the level of $6x10^{-5}$ M.

	Solvent Production (mM)	
	Mean	Standard Deviation
$6x10^{-4}$ M Hydrosulfite at $t_{44\text{-}48}$ (n=4)	168.1	45.33
Control (i.e. without sulfite) (n=3)	120.9	15.38

Table 7-7 Results (sum of ethanol and butanol) using an intermediate concentration of hydrosulfit*e*

Statistical analysis results	
Degrees of freedom (υ)	5
Variance (s^2)	1327
Critical value of $t_{5,\,0.05}$	2.776
t Test value	1.697

Table 7-8 Statistical analysis adding $6x10^{-4}$ M hydrosulfite during exponential growth

The variance of the experiments according to s^2 value and the standard deviation , yielded the t-Test value lower than the critical value. As a consequence the hypothesis that the addition of hydrosulfite increases the solvent production is not statistical representative under the noted conditions.

7.4.Discussion

The assimilatory sulphite reductase reaction is described as follows (Staley 2007)

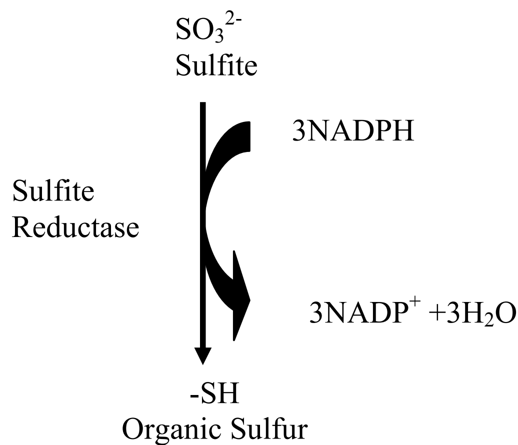


Figure 7-3 Sulfite reduction by sulfite reductase

Even though a dissimilatory sulphite reductase was reported for *Clostridium pasteurianum* (Harrison 1984), this study showed the ability of the cells to reduce SO_3^{2-} and not hydrosulfite $S_2O_4^{2-}$. Also the inducible effect was achieved adding 10mM of cysteine to fermentation media.

Many organisms use sulphate as sulphur source but the ability to utilize sulphate as an electron acceptor for energy generation process is restricted to a special group of anaerobic bacteria (Brock 1991)
The clostridia lack a cytochrome system and a mechanism for electron-transport phosphorylation and hence they obtain ATP only by substrate level phosphorylation (Brock 1991). Thus it is not sufficient to harvest cells in a more reduced environment if the cells can not activate the dissimilatory metabolism.

A previous study about morphological modifications of cells of *Clostridium pasteurianum* grown on sulphate (SO_4^{2-}) and sulphite (SO_3^{2-}) in defined media using glucose as a carbon source (McCready 1975) was revised (recall that in the actual study $S_2O_4^{2-}$ was used and glycerol served as carbon source). These authors showed that granules (amylopectin) were found in cells grown on sulfate but barely in those cells grown on sulfite (McCready 1975). These results looks like to have an agreement with the pleiotropic phenotype of the *Spo0A* gen in clostridium. Namely sporulation solvent production and granulose accumulation.

What triggers the sporulation in clostridial is still unknown (Paredes and others 2005). DNA microarrays systems to monitor gene expression under the sporulation events have been used (Paredes and others 2005, Staley 2007) and it is believed that will give more insight of the genes that involved in the sporulation cascade.

8. Chapter 8: Conclusions

Glycerol as the solely carbon source in chemically defined media was shown to produce butanol and ethanol. It opens the possibility to use it a fermentation substrate to produce biofuels.

When the ABE fermentation was the main pathway to produce industrial butanol (first decade of 20th century), molecular genetics concepts were not available. A genomic view of clostridia sporulation has unveiled new insights about the solvent production. Regarding clostridia fermentation, it has been recognized that endospore development and the solvent formation share a regulatory mechanism. Researchers have shown that the *Spo0A* gene controls the switch from the acid phase to the solvent phase. The homologous reported Spo0A gene in *Clostridium pasteurianum* suggested that it might have a similar role these bacteria. The experiments performed here have shown that a relationship between number of spores and butanol can be constructed. In addition to that, a experimental procedure was introduced to select spores during a batch fermentation. That might serve as reference to study kinetics parameters based on the number of spores during the course of the fermentation . Experimental procedure might be used with other endoespore forming bacteria.

It has been said that solvent toxicity ceases the cellular metabolism (Lee and others 2008). However, an interesting question can be asked regarding the physiology of the sporulation process. If the final fate of the dormant spore limits the solvent production, new strategies should be used in order to increase the solvent production.

The rationale and the experimental procedure to use the replica plating technique as a source of an even number of clones were introduced. The advantage of this methodology over using a liquid inoculum or a simple colony was discussed.
An advantageous design for a flask fermentor was described. It was mentioned that is especially useful for anaerobic gas producing bacteria.

During one of the batch fermentations 21 g/L of butanol were produced after 72 hours and using a nine percent of glycerol as the initial concentration.. Previous highest yield was 17g/L (Biebl 2001) . Biebl's fermentation yielded 17g/L in less than 30 hours, which means his process has a higher productivity. The feasibility of an industrial would not depend only on the solvent concentration. Some other factors that could affect the industrial process are: cost of the glycerol, productivity of the strain and cost of the downstream process. The last one of relevant importance, taking in account that when the solvent production ceases (around 2 wt% butanol) there is still more than 90wt% of aqueous solution that needs to be removed.

9. Chapter 9: Future Work

In order to scale up the process and integrated system (i.e. fermentation and recovery of products) should be studied. Some authors have suggested in situ recovery (Lee and others 2008). The *Clostridium pasteurianum* product distribution is different from the AB fermentation, thus its own recovery process should be designed.

It is also important to run fermentations using raw or industrial glycerol. Impurities from the biodiesel might or might not affect the glycerol fermentation. If impurities have a negative effect, an extra purification process should be added.

It is considered of great importance to study what is the limiting factor in the solvent production: the solvent toxicity or the dormant endoespore. Also it is possible to try to measure solvent production kinetics in relation to the number of spores.

There are still many alternatives to build up experiments that try to identify what triggers the beginning if the sporulation process. Stress in form of oxygen and low pH are alternatives that were not explored.

Mutants that can block each of the stages of the sporulation process can be found. Measuring the ability of this mutant to produce solvents might reveal in what stage of the sporulation process the majority of the solvent is produce. Future studies can then determine if locking the sporulation process at some stage (i.e. not letting the cell to end up in the dormant spore) could increase the solvent production.

Bibliography

Andrade, J.C., Vasconcelos, I. 2003. Continuous cultures of *Clostridium acetobutylicum*: culture stability and low-grade glycerol utilization. Biotech Lett, 25:121-125.

Ashworth M.R.F. 1979. Analytical Methods for Glycerol. Academic Press Inc., edition. Edited and with_final chapter by A.A. Newman

Atkison, B., Ferda, M. 1993. Biochemical Engineering and Biotechnology Handbook. Second Edition, Stokton press. Chapter 4

Bender, R. and Gottschalk, G. 1973. Purification and properties of D-Gluconate Dehydratase from *Clostridium pasteurianum*. Journal of Biochemistry, 40, 309-321

Biebl, H. 2001. Fermentation of glycerol by *Clostridium pasteurianum* - batch and continuous studies. Journal of Industrial Microbiology and Biotechnology, 27:18-26.

Biebl, H., Robinson R.K., Batt, C.A., Patel, P.D. 2000. Encyclopedia of Food Microbiology. Academic Press, 445-450.

Braithwaite, A., and Smith, F. J., 1996, Chromatographic methods. Fifth edition. Blackie Academic and Professional. New York.

Brock T.D., and Madigan, M.T. 1991. Biology of Microorganisms, Sixth Edition, Prentice Hall U.S.A.

Castle J.E. Carnahan J. E. Some requirements of biological nitrogen fixation. Journal of Bacteriology, 75:121-124, 1958.

Dabrock, B., Bahl, K., and Gottschalk. 1992. Parameters affecting solvent production by *Clostridium pasteurianum.* Applied and Environmental Microbiology, 1233-1239.

Difco Manual Dehydrated culture media and reagents for Microbiology. 1985. Tenth Edition Difco Laboratories.

Dorado M.P. 2008. Biofuels refining and performance. Raw materials to produce low-cost biodiesel, McGraw Hill, 107-139.

Durre, P. 2008. Fermentative butanol production - bulk chemical and biofuels. Annals of the New York Academy of Sciences, 125: 353-362.

Enke C.G. 2001. The art and science of chemical analysis. John Wiley and Sons, Inc.

Foreig Agricultural Service. Councelor and attached reports official statistics, estimates for March 2004/2005. March 2006.

Forsberg, C. W. 1987. Production of 1,3-Propanediol from Glycerol by Clostridium *acetobutylicum* and other *Clostridium* species. Applied and Environmental Miicrobiology, 53, 4, 639-643.

Gaden E. 1959 Fermentation process kinetics J of Biochemical and Microbiological Technology and Engineering, 1, 629-635 1959

Gerpen, V.J., Shanks, B., Pruszko, R., Clements D., Knothe, G. 2004. Biodiesel Production Technology. National Renewable Energy Laboratory.

Gilbert, J. C., and Martin, S. F., 2006, Experimental Organic Chemistry: A miniscale and microscale approach. Fourth edition. Thomson Brooks, Belmont, USA.

Glantz, S.A., 1992. Primer of Bio-statistics. McGraw Hill Third Edition, United States.

Harrison, G., Curle, C., and Laishley, E. J., 1984. Purification and characterization of an inducible dissimilatory type sulfite reductase from *Clostridium pasteurianum*. Archives of Microbiology. 138: 72 -78.

Heyndrickx, M., Vansteenbeeck, P. DE VOS, and De Ley, J. 1986. Hydrogen gas production from continuous fermentation of Glucose in a minimal medium with *Clostridium butyricum* LMG 1213t1. Applied Microbiology, 8, 239-244

Hewlett Packard, 1989. Hp 5890 SERIES II Site Prep and Installation Manual. Hewllet Packard, June 1989.

Jens, N., 2006. Microbial process kinetics in Basic Biotechnology. Cambridge Press Third Edition.

Johnson, DT., Johnson and Taconi, K. A., 2007. The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. Environmental Progress, 26:4,338.348.

Jones, D.T., Woods, D.R., 1986.Acetone-butanol fermentation revisited. Microbiology Review, 50:4, 484-524

Kim, Y-J. 1991. Modeling and application of aqueous 2-phase system in a-b-e fermentation process. Advasior Weigand W.A. Thesis Universty of Maryland.

Knothe, G. 2008. Biofuels refining and performance. Chapter: Fuel and Physical Properties of Biodiesel Components. McGraw Hill, 149-164.

Lars, G., 2003. Biochemistry and physiology of anaerobic bacteria. Springer, New York, 123.

Lee and others, S.Y., Park, J.H., Jang, S.H., Nirlsen, L.K., Kim J., and Jung K.S. 2008. Fermentative Butanol Production by Clostridia. *Biotechnology and bioengineering*. 101:2, 209-227

Levett, P.N. 1991. Anaerobic microbiology. a practical approach. Resazurin solution protocol p.7. Oxford University Press. The practical Approach Series

Liamleama, W. and Annachhatre, A. P., 2007. Electron donors for biological sulfate reduction Biothecnology Advances , 452-463

Licht, F.O. World ethanol and biofuels report. April, 2006, in Biofuels for Transport, global potential and implications for sustainable energy and agriculture. Worldwatch Institute, 2007, Earthscan, Sterling, VA.

Love B, Snader KM, 1964 Hantzsch reaction .I. Oxidative Dealkylation of Certain Dihydropyridines ,24,11, 1914-1916

Mallette, M. F., Reece, P., and Dawes, E. A., 1974. Culture of Clostridium pasteurianum in define medium and growth as a function of sulfate concentration. Applied Microbiology, 28, 6, 999-1003.

Mantzouridou F. Naziri E. and Tsimidou M. 2008 Industrial glycerol as a Supplementary Carbon Source in the Production of β-carotene by Blakeslea trispora. Journal of Agricultural and Food Chemistry. 56, 2668-2675

Mayehew, S. G., 1978. Redox potential of dithionite and so2- from equilibrium reactions with flavodoxins, methyl viologen and hydrogen plus hydrogenase. European Journal of Biochemistry, 85, 535-547

Mc Cready, R.G., Laishley, E. J., and Krouse, H. R., 1975. Stable isotope fractionation by *Clostridium pasteurianum*. Canadian Journal of Microbiology, 21, 235-244

McCready, R.G. L., Costerton, J.W., and Laishley, E. J., 1975. Morphological modifications of cells of *Clostridium pasteurianum* caused by growth on sulfite. Canadian Journal Microbiology, 22: 269-275

McNair, H.M., Miller, J.M. Basic gas chromatography. 1997. John Wiley and Sons.

Minton, N. P., and Clarke, D. J., 1989. Clostridia. Biotechnology Handbooks. Plenum Press, New York.

Monick J. A. Alcohol .1968 Their Chemistry, properties and manufacture. Reinhold book corporation. New york , USA.

Papoutsakis, E. T., 1984, Equations and Calculations for Fermentations of Butyric Acid Bacteria. Biotechnology and Bioengineering, 26, 174-187.

Paredes and others, J. C., Alsaker, K. V., and Papoutsakis, T.E. 2005. A comparative genomic view of clostridial sporulation and physiology. Nature Reviews Microbiology 3, 969-978.

Petersen, G. Werpy, T., 2004. Top value added chemicals from biomass. Results of screening for potencial candidates from sugars and synthesis gas.

Qures, N., Blaschek, H. P., 2001, Recent advances in ABE fermentation: hyper-butanol producing *Clostridium beirerinckii* BA101. Journal of Industrial Microbiology & Biotechnology 27, 287-291

Ravagnani and others, A., Wilkinson and others, S. R., 2000. Spo0A directly controls the switch from acid to solvent production in solvent-forming clostridia**.** *Molecular Microbiology* 37, 1172-1185.

Sargeant, K., Ford, J. W. S., and Longyear, V. M. C. 1968. Production of *Clostridium pasteurianum* in a defined medium. Applied Microbiology, 16, 296-300

Seeley, H., Vandermark, P. J., and Lee and others, J., 1991, A laboratory manual of microbiology. Fourth edition. W. H. Freeman and Company.

Shuler and Kargi, M. L., and Kargi, F., 2006. Bioprocess Engineering, basic concepts. Second Edition, Prentice Hall of India.

Stouthamer H.A., 1969. Determination and significance of molar growth yields. In: Methods in Microbiology. Vol 1, J.R. Norris and D.W. Ribbons (eds), p.634. New York Academic Press.

Sun, Z. Chiao, J. History of the acetone-butanol-ethanol fermentation industry in China: Development of continuous production technology. Journal of Microbiology and Biotechnology, 13,12–14

Taconi K.A. Johnson D.T. The glycerin glut: options for value-added conversion of crude glycerol resulting from biodiesel production. Environ Prog, 26(4):338{348, December 2007.

Yazdani, S.S. ,Gonzalez, R., 2007. Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Curr Opin Biotech, 18:213-219, 2007. p 214 from www.thejacobsen.com.

Werpy, T., and Petersen, G., 2004. Top Value Added Chemicals from Biomass: Results of Screening for Potential Candidates from Sugars and Synthesis Gas. National Renewable Energy Laboratory

Willis, A., 1979. Anaerobic Bacteriology: Clinical and Laboratory Practice Third Edition Butterworths, London.

Wilkinson and others S.R., Young D.I.,Morris J.G., and Young Michael.1995. Molecular genetics and the initiation of solventogenesis in *Clostridium beijerinckii* (formerly *Clostridium acetobutylicum*) NCIMB 8052. *FEMS Microbiology Reviews* 17 (3): 275-285.

Worldwatch Institute. Biofuels for transport: global potencial and implications for sustainable energy and agriculture. Earthscan, 2007. Figure 1.2 p.4 Table 1.2 p 7.

Worldwatch Institute, 2007. Biofuels for transport **:** global potential **and** implications for sustainable energy and agriculture , Earthscan.

Young- Jick, K., 1991.Modeling and Application of Aqueous 2-Phase System in A-B-E Fermentation Process.