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TESIS DOCTORAL:

BIOTECHNOLOGICAL VALORISATION OF CRUDE GLYCEROL FROM BIO-DIESEL MANUFACTURING PROCESS: PRODUCTION OF 1,3-PROPANEDIOL BY *CLOSTRIDIUM BUTYRICUM*

Presentada por Marina Manuela Loureiro Pinto para optar al grado de Doctor por la Universidad de Valladolid

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MARINA LOUREIRO PINTO ha realizado bajo su dirección el trabajo "BIOTECHNOLOGICAL VALORISATION OF CRUDE GLYCEROL FROM BIO-DIESEL MANUFACTURING PROCESS: PRODUCTION OF 1,3-PROPANEDIOL BY *CLOSTRIDIUM BUTYRICUM"*, en el departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Escuela de Ingenierías Industriales de la Universidad de Valladolid. Considerando que dicho trabajo reúne los requisitos para ser presentado como Tesis Doctoral expresan su conformidad con dicha presentación.

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Reunido el tribunal que ha juzgado la Tesis Doctoral: "BIOTECHNOLOGICAL VALORISATION OF CRUDE GLYCEROL FROM BIO-DIESEL MANUFACTURING PROCESS: PRODUCTION OF 1,3-PROPANEDIOL BY *CLOSTRIDIUM BUTYRICUM"* presentada por la Ingeniera Química Marina Loureiro Pinto, y en cumplimiento de lo establecido por el Real Decreto 99/2011 de 28 de enero de 2011

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CONTENTS

Nomenclature

B	constant of the Contois kinetic model (g/g)
HAc	acetic acid concentration (g/L)
HBut	butyric acid concentration (g/L)
1,3 PD	1,3-propanediol concentration (g/L)
S	glycerol concentration (g/L)
$S_{P/HAc}$	selectivity (g 1,3propanediol per g acetic acid)
$S_{P/HBut}$	selectivity (g 1,3propanediol per g butyric acid)
X	biomass concentration (g/L)
$Y_{PD/S}$	yield factor (g of 1,3-propanediol formed per g of glycerol consumed)
$Y_{HAc/S}$	yield factor (g of acetic acid formed per g of glycerol consumed)
$Y_{HBut/S}$	yield factor (g of butyric acid formed per g of glycerol consumed)
$Y_{PD/X}$	yield factor (g of 1,3-propanediol formed per g of biomass produced)
μ	specific growth rate (h^{-1})
μ_{max}	maximal specific growth rate (h^{-1})
Qp	volumetric productivity (g of product per L per h); Subscripts 0, r and max indicate the initial, remaining and maximum quantity, respectively, of the components, in the kinetics performed.

Abstract

1,3-Propanediol can be biologically produced from glycerol by several microorganisms, namely clostridia. Different strategies have been tried to optimize this conversion and set up a practicable process. In this work, the production of 1,3-propanediol from crude glycerol for the biodiesel industry was investigated, testing different process strategies using *Clostridium butyricum* DSM 10703.

In order to optimize the operation conditions that maximize the production of 1,3-propanediol, the influence of operation variables (temperature, pH and initial glycerol concentration) in 1,3-propanediol biosynthesis was studied. Furthermore, different culture techniques (batch, fed-batch and chemostat cultures) were tested with pure glycerol (Sigma 99% (wt/wt)) as substrate for further comparison with crude glycerol experiments.

A first set of experimental runs, based on a factorial design of experiments, were conducted in a batch system with pure glycerol to obtain the operating conditions that maximize both 1,3-PD concentration and yield factor. The inhibitory effect of the initial substrate concentration on fermentation to 1,3-PD was also studied for pure glycerol.

In all fermentations, 1,3-propanediol was the main product and acetic and butyric acids were by-products of the reactions. The maximum concentration of 1,3-propanediol (40.0 g/L) and the highest yield factor ($Y_{PD/S}$= 0.57 g/g) were obtained at 37 °C, pH 6.5 and 70 g/L glycerol. Moreover, the maximum tolerance of *C. butyricum* at the inhibitory effect of the substrate was 100g/L. A kinetic Contois type-model successfully simulates the batch fermentation of glycerol by *Clostridium butyricum* DSM 10702.

In fed-batch fermentations, 37.3 g/L of 1,3-propanediol was formed with a corresponding production yield of 0.53 g of 1,3-propanediol formed per g of glycerol consumed. Furthermore, a total amount of 49 g of 1,3-propanediol was achieved. The total amount of by-product concentration was 11.7 g/L.

In continuous cultures, the maximum 1,3-propanediol obtained was 23.51g/L at a low dilution rate (D=0.05) and 70 g/L of glycerol in the fed stream. Lower values of by-products were produced, 3.93g/L of butyrate and 0.86g/L of acetate. The continuous

strategy does not provide, as expected, the highest amounts of 1,3-propanediol, but a lower production of by-products and high productivity were obtained.

Assays with biodiesel crude glycerol were carried out under the operation conditions that maximize 1.3-propanediol concentration and yield. The concentration of 1,3-propanediol reached a maximum of 35.9 g/L, slightly lower than the concentration reached using pure glycerol. The yield factor was 0.51 g of 1,3-PD/g glycerol, also below the value reached in the fermentation of pure glycerol. The main by-product was butyric acid (7.9 g/L) followed by acetic acid (2.7 g/L). In this case, butyric acid production was higher than when pure glycerol was used, while the acetic acid concentration was quite similar for both substrates.

The maximum initial crude glycerol concentration tolerated by *C. butyricum*, while maintaining a high production of 1,3-PD, was 70g/L, lower than the 100g/L for pure glycerol experiments. Batch fermentation of glycerol by *Clostridium butyricum* DSM 10702 was also successfully simulated by the kinetic Contois type-model.

In fed-batch, using crude glycerol as substrate, the maximum 1,3-propanediol concentration was 36.1 g/L, close to the 37.3g/L reached in fed-batch pure glycerol experiments. Acetic and butyric acid concentrations reached, respectively, 2.5g/L and 9.0 g/L.

Continuous cultures provided 27.5 g/L of 1,3-propanediol at a dilution rate of 0.05 h^{-1} and 70g/L of glycerol in the fed stream. Nevertheless, at D=0.15 h^{-1}, a high 1,3-PD concentration was achieved (≈ 22 g/L), with a lower production of butyrate and acetate but higher productivity.

A pretreatment process for biodiesel-based crude glycerol was evaluated to eliminate a number of impurities generated during the transesterification process and reduce the concentrations of growth inhibitors, such as salts and pigment. Two different pretreatments were tested based on ion exchange resins. Serum bottle batch fermentations by *C. butyricum* DSM 10703 were run, at different initial substrate concentrations, to select the most effective pretreatment in terms of 1,3-propanediol concentration, yield, productivity and high substrate tolerance.

The production of 1,3-propanediol by *C. butyricum* DSM 10702, using pretreated glycerol, was feasible in the two pretreatments tested.

The serum bottle results showed that the presence of salts between 4 and 5 wt% in crude glycerol do not originate *C. butyricum* DSM 10702 inhibition. For the two different fermentation modes tested (batch and fed-batch systems), the proposed pretreatment improves the results reached in fermentations carried out with untreated crude glycerol.

An immobilization technique was evaluated, and the use of sodium alginate gel as the immobilization material on *C. butyricum* cell growth and 1,3-propanediol production from crude glycerol was studied. The concentration and yield of 1,3-propanediol under batch and continuous cultures was also studied and compared with the results of free cell culture.

The production of 1,3-propanediol by immobilized *C. butyricum* in alginate bio-beads demonstrated desirable characteristics, including repeated cultivations with a high biomass density.

The batch fermentation results with immobilized *C. butyricum* provided a higher concentration of 1,3-propanediol (13.77 g/L, an increase of 2.21 g/L compared to the free cells) and substantially higher biomass concentrations (2.3-fold higher than that of the free cell culture). Furthermore, the acetic acid route was affected, and this acid was not detected in the effluent.

In continuous fermentations, the results were similar to those obtained with free cell continuous fermentations of crude glycerol with the exception of acetic acid, which was considerably below the concentrations

Resumen

El objetivo del presente proyecto de tesis es la valorización de “glicerol crudo” (subproducto de la industria de obtención de biodiesel) mediante su transformación bioquímica en 1,3-propanodiol, para su posterior empleo en procesos de polimerización.

1,3-propanodiol (1.3-PD) es un monómero que se puede producir biológicamente a partir de glicerol por varios microorganismos, entre ellos los del género *Clostridium*, utilizando en el presente trabajo *Clopstridium butyricum.* Diferentes estrategias han tratado de optimizar esta conversión y establecer un proceso viable.

Se llevaron a cabo diferentes técnicas de cultivo (batch, fed-batch y continuo) utilizando inicialmente glicerol puro (Sigma 99% (w/w)) como sustrato para su posterior comparación con los experimentos de glicerol crudo. Se establecieron así mismo los métodos analíticos necesarios para caracterizar tanto los productos de fermentación como la concentración de microorganismos.

Ensayos con soluciones modelo de glicerol

Una primera serie de ensayos, basado en un diseño factorial de experimentos, se llevaron a cabo en un sistema discontinuo con glicerol puro para obtener las condiciones de funcionamiento que maximizaran tanto la concentración de 1,3-PD como el rendimiento. También se estudió el efecto inhibitorio de la concentración inicial de sustrato en la fermentación de 1,3-PD para glicerol puro.

En todas las fermentaciones, 1,3-propanodiol fue el producto principal y los ácidos acético y butírico subproductos de la reacción. La concentración máxima de 1,3-propanodiol (40,0 g/L) y el factor de rendimiento más alto (Y_{PD}/S = 0,57 g / g) se obtuvieron a 37 º C, pH 6,5 y 70 g/L de glicerol. Por otra parte, la tolerancia máxima de *C. butyricum* sin efecto inhibidor por sustrato fue 100 g/L. Un modelo cinético tipo Contois simuló con éxito la fermentación de glicerol por el *C. butyricum* DSM 10702.

Se realizaron fermentaciones en fed-batch, aplicando distintas formas de alimentación, con la finalidad de maximizar la producción de 1,3-propanodiol. Se experimentó alimentando bien de forma continuada o en forma cíclica, a diferentes flujos y cargas.

La estrategia de alimentación que proporcionó los mejores resultados fue en continuo, donde se obtuvo 37,3 g/L de 1,3-propanodiol con un rendimiento correspondiente de 0,53 g de 1,3-propanodiol formado por g de glicerol consumido. Asimismo, se logró un total de 49 g de 1,3-propanodiol. La concentración total de subproductos fue de 11,7 g/L.

Se operó en continuo a partir de los ensayos planificados, modificando las variables de proceso: velocidad de dilución (a 3 niveles: 0,05; 0,1; 0,2 h^{-1}) y concentración inicial de sustrato (30; 50 y 70 g/L), con objeto de alcanzar elevada productividad y selectividad en 1,3-propanodiol.

En cultivos continuos, la máxima concentración de 1,3-propanodiol obtenida fue 23,51 g/L a una baja tasa de dilución (D = 0,05) y 70 g/L de glicerol en la corriente de alimentación. Se obtuvieron bajas concentraciones de subproductos, 3,93 g/L de ácido butírico y 0,86 g/L de ácido acético. La estrategia continua no proporcionó, como se esperaba, las mayores cantidades de 1,3-propanodiol, pero se obtuvo una menor producción de subproductos y una alta productividad.

Ensayos con soluciones de glicerol crudo

Se caracterizó la “glicerina cruda” procedente de la producción de biodiesel (factoria ACOR_Biodiesel, Valladolid), cuantificando tanto el contenido en glicerina como el de las impurezas presentes en dicha fracción. Los resultados reflejan que la glicerina “cruda” contiene un 88% (w/w) de glicerol, 5%(w/w) de sales de sodio, 4,3%(w/w) de agua y 2,11%(w/w) MONG.

Se realizaron ensayos utilizando esta fracción de glicerina, modificando diferentes variables de proceso, concentración inicial de substrato y modo de operación. Se compararon los resultados obtenidos en estas fermentaciones con los derivados de soluciones modelo de glicerina pura.

Se llevaron a cabo ensayos con glicerol crudo bajo las condiciones de operación que maximizan la concentración de 1,3-propanodiol y el rendimiento. La concentración de 1,3-propanodiol alcanzó un máximo de 35,9 g/ L, ligeramente menor que la concentración alcanzada utilizando glicerol puro. El rendimiento fue de 0,51 g de 1,3-PD/g de glicerol crudo, también por debajo del valor alcanzado en la fermentación con glicerina pura. El principal subproducto fue el ácido butírico (7,9 g/L) seguido de ácido acético (2,7 g/L). En este caso, la producción de ácido butírico fue mayor que cuando se utiliza glicerol puro, mientras que la concentración de ácido acético fue muy similar para ambos sustratos.

La máxima concentración inicial de glicerol crudo tolerado por *C. butyricum* fue 70 g/L, inferior a los 100g//L para experimentos con glicerol puro. La fermentación de glicerol crudo por *C. butyricum* también fue simulado con éxito por el modelo cinético tipo Contois.

En fermentaciones fed-batch utilizando glicerol crudo como sustrato, la concentración máxima de 1,3-propanodiol obtenida fue 36,1 g/L, muy cerca de la concentración de 1.3-PD alcanzada en el ensayo modelo respectivo. Las concentraciones de ácido butírico y acético alcanzaron, respectivamente, 2,5 g/L y 9,0 g/L.

Cultivos en continuo proporcionan 27,5 g/L de 1,3-propanodiol a una tasa de dilución de 0,05 h^{-1} y 70 g/L de glicerol en la corriente alimentada. Asimismo, para D = 0,15 h^{-1} fue posible obtener una alta concentración de 1,3-PD (≈ 22 g / L), con una menor producción de ácidos acético y butírico y una mayor productividad.

A pesar de las altas concentraciones de 1,3-propanodiol sintetizadas con glicerol crudo, se observan reducciones significativas en el rendimiento. Este hecho, estableció por tanto, la necesidad de eliminar la actividad inhibitoria de las impurezas presentes en el glicerol crudo.

Ensayos con soluciones de glicerol crudo pretratado

En este sentido, se investigaron tecnologías para el pre-tratamiento de la glicerina “cruda” que permitieran reducir y/o eliminar las impurezas que afectan el rendimiento en diol. Una primera selección condujo al empleo de resinas (adsorción/intercambio iónico) como el proceso más eficaz en la remoción de impurezas

(sales de sodio y pigmentos). La eficacia de los pretratamientos se determinó mediante tests preliminares a pequeña escala (serum bottles), estableciéndose como idóneo un pre-tratamiento compuesto por un set de 3 resinas en serie, que condujo a resultados muy similares a los obtenidos en los ensayos donde se utilizaba glicerol puro (99%) como substrato.

Ensayos con soluciones de glicerol crudo. Biorreactor con células inmovilizadas.

Se evaluó una técnica de inmovilización para *C. butyricum* mediante inclusión en gel de alginato sódico, para su posterior aplicación en proceso continuo. La inmovilización de organismos unicelulares ha generado gran interés debido a sus grandes ventajas técnicas y económicas respecto a la fermentación tradicional. Entre las principales ventajas que presentan los sistemas biotecnológicos que utilizan células inmovilizadas se encuentra la posibilidad de una mayor densidad celular, disminución de efectos tóxicos/inhibitorios por producto o substrato, un mejor control en sistemas continuos y la potencial recuperación de la biomasa para su posterior reutilización.

Se estudió la concentración y el rendimiento de 1,3-propanodiol bajo cultivos discontinuos y continuos y se comparan con los resultados del cultivo libre de células.

La producción de 1,3-propanodiol por células inmovilizadas de *C. butyricum* en alginato demostró características deseables, incluyendo cultivos repetidos con una alta densidad de biomasa**.**

Los resultados de la fermentación batch con *C. butyricum* inmovilizado aumentaron de forma substancial la concentración de biomasa (2,3 veces superior a los cultivos con células libres) y de 1,3-propanodiol (13,77 g/L, un incremento de 2,21 g/L en relación a cultivos con células libres). Además, la ruta de ácido acético se vio afectada, y este ácido no se detectó en el efluente.

En fermentaciones continuas, los resultados fueron similares a los obtenidos con células libres de fermentaciones continuas con glicerol crudo, con excepción de la concentración de ácido acético, que fue considerablemente más baja.

CHAPTER 1. INTRODUCTION

1. Background and interest of the subject

The greenhouse gas emissions responsible for climate change, the decreasing quantities of petroleum reserves and, most of all, the soaring crude oil costs, are encouraging the world to produce technical chemicals and alternative fuels from renewable resources by biological processes and green technologies. The need for a sustainable resource supply is driving rapid advances in plant biotechnology and microbial genetics.

At present, almost all the important bulk chemicals, except ethanol, are produced via the petrochemical route, even though biotechnology has the potential to produce many of these chemicals from renewable sources (Wilke D, 1999). Compared with petrochemical-based compounds, fermentation products have generally much lower production volumes (less than one million tons/year) but higher prices (Zeng and Biebl, 2002). Biological technologies, however, have the advantages of renewable feedstock, green emissions and the fact that only low temperature and pressure are required. To date, biotechnology has only established a solid position in producing fine chemicals such as organic acids, vitamins, and pharmaceutical compounds, but the growing efforts to optimize green technologies are gradually changing the scenario.

Oil is the fossil fuel that is most in danger of running out. Crude oil is a complex mixture that is between 50% and 95% hydrocarbon by weight. About 98% of carbon emissions come from fossil combustion. Reducing the use of fossil fuels would considerably reduce the amount of carbon dioxide produced, as well as reducing the level of pollutants. In fact, the estimated cost of controlling carbon emissions balances the higher cost of carbon-free and carbon reducing technologies. The abundant supply of biomass and the finite nature of crude oil have been recognized, and this has attracted interest in the sustainable production of chemical feedstock and fuels. Production of biofuel is already at a large magnitude, and is projected to greatly increase in the future.

Industrialized nations are dependent on petroleum and the rising price of crude oil has already had an adverse effect on the world's economy. For the consumer, the price of natural gas, gasoline and diesel has quadrupled over the last decade. Although

Europe and the USA are still the main consumers of fossil fuels, these developments are also set to benefit the rapidly growing economies of Asia.

In response to the petroleum crisis, the principles of green energy and sustainable living movements have been gaining in popularity. This fact has led to increasing interest in alternative fuel research such as fuel cell technology, hydrogen fuel, bioethanol and biodiesel. However, to date, only bioethanol and biodiesel have been seen as significant alternatives to fossil fuel.

In the market, new technology and energy efficiency measures become desirable for consumers seeking to decrease their energy costs. Recent strong fluctuations in the price of crude oil have prompted several large oil importing nations to seriously seek alternatives to relieve their dependency on oil imports.

The importance of biofuel goes beyond the petroleum crisis and environmental concerns. The use of feedstock and liquid fuels from renewable materials will open a door to the currently unexplored productivity in agriculture and forestry residues. The favorable impact on global climate, higher environmental and economic security, and functionally superior value-added products are other reasons of import favoring the biofuel option. However, the ecological challenge and political intention alone cannot change the actual situation, as economic restraints are still holding back a sustainability era.

The chemical industries have, until recently, considered biotechnology as an expensive high-tech tool that was not appropriate on a large scale. However, this perception is undergoing a gradual change, as reflected by the current development projects of major chemical companies to produce bulk chemicals such 1,3-propanediol via biotechnological routes.

In fact, today’s largest fermentation product, bioethanol, although subsidized, is proof of the bulk chemical manufacturing potential of biotechnology.

1.1. Biodiesel Industry

In December 2012, most representatives at the United Nations' Climate Change Conference in Doha, Qatar, pledged to reduce emissions of carbon dioxide further, and set new targets and limits to be implemented during the Kyoto Protocol Extension (2012-2020). Furthermore, over the last year, Brent oil prices reached 87.19 € per barrel (www.indexmundi.com). Trapped between the rising prices of crude oil, and their commitment to stopping global climate change by cutting the emission of greenhouse gases, many countries are now investing in the development and use of alternative and renewable energy sources.

Biofuels, bioethanol and biodiesel seem to be an immediate solution; they decrease dependency on fossil fuels and are environment friendly. The governments of many industrialized and developing countries are therefore creating and expanding policies and research programs to increase the production and use of biofuels.

The European Union, in order to reach the ambitious target of a 20% share of energy from renewable sources in the overall energy mix, plans to focus efforts on biofuels. In transport, which is almost exclusively dependent on oil, the European Commission hopes to target a 10% share of biofuels in overall fuel consumption by 2020. Biodiesel is a renewable fuel source. It can be produced from animal fats or vegetable oils. The possibility of using vegetable oils as fuel has been acknowledged since 1893, at the beginning of diesel engines. However, vegetable oil has a high viscosity for use in most existing diesel engines as a straight replacement fuel oil.

There are a number of ways to reduce vegetable oil viscosity. Dilution, microemulsification, pyrolysis and transesterification are techniques applied to reduce viscosity. In the biodiesel industry, the most common method used to reduce oil viscosity is transesterification. Transesterification, also called alcoholysis, is the chemical conversion of oil to its corresponding fatty ester. In the transesterification reaction, triglycerides (1) are treated with an alcohol (ethanol or methanol) (2) with a catalytic base to give ethyl esters of fatty acids (3) and glycerol (4), Fig. 1.

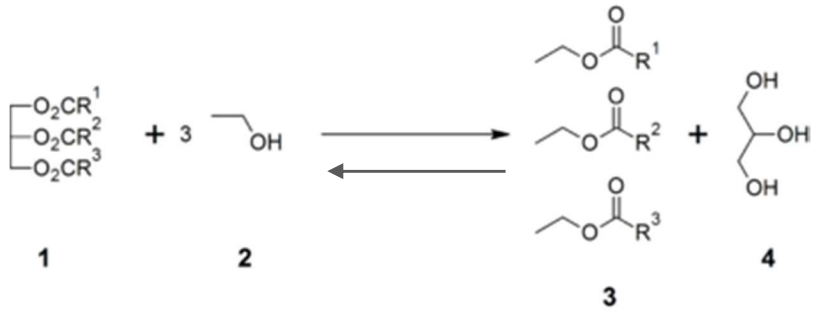

Fig. 1. Transesterification of triglycerides with alcohol

The catalyst is used to improve the reaction rate and yield. Because the reaction is reversible, an excess of alcohol is used to shift the equilibrium to the products side. The biodiesel reaction requires a catalyst such as sodium hydroxide (NaOH) or potassium hydroxide (KOH) to split the oil molecules, and an alcohol (methanol or ethanol) to combine with the separated esters. The main product is glycerol. This form of crude glycerin is often dark in appearance with a thick, syrup-like consistency. Biodiesel is, in fact, better than petro-diesel in terms of sulfur content, flash point, aromatic content and biodegradability (Bala, 2005).

Homogeneous catalysts are commonly applied in the transesterification of vegetable oils. Base homogenous catalysts such as NaOH and KOH are the most active under mild reaction conditions. Acid homogeneous catalysts are also used, but require a longer reaction time. However, homogeneous catalysts are difficult to recover and lead to downstream waste treatment, increasing the cost of the biodiesel production.

Heterogeneous catalysts are promising candidates for biodiesel production from vegetable oils. Different types of heterogeneous catalysts and their activity for transesterification are being studied (Yan et al. 2010). Unlike homogeneous ones, heterogeneous catalysts can be recycled and used several times with a better separation of the final product; they are also environmentally benign and can be used in a continuous process without the need for further purification steps, as well as being potentially cheap.

The use of biofuels still requires financial incentives for producers and consumers alike. Many countries thus support biofuel production through government tax credits and subsidies. The EU started with subsidies for bioethanol and biodiesel.

US producers receive tax credits of US$0.51 per gallon of bioethanol and US$1.00 per gallon of biodiesel. Germany currently encourages the use of biodiesel by levying lower taxes as compared with normal diesel. Indeed, Germany is currently the largest producer and consumer of biodiesel, exceeding 2.5billion liters annually (da Silva et al., 2009). Other EU countries have adopted the use of biodiesel, usually as an admixture to petroleum diesel. However, far more important than tariffs and subsidies, are usage targets to encourage the further production and use of biofuels. In the long term, the EU plans to set a target of 10% by 2020; Japan has set a target of 20% by 2030; and Canada 2% of biodiesel by 2012.

In Spain, the Renewable Energy Plan (REP) 2011-2020 establishes objectives in line with Directive 2009/28/EC of the European Parliament on the promotion of the use of energy from renewable sources. The REP is intended to reach, as indicated by the EU Directive, at least 20% of gross final energy consumption from the use of renewable sources by 2020.

According to data from the International Energy Agency (IEA), biofuels covered in 2010 2.08% of world oil supply. As a result of policy support for renewable energy under the Renewable Energy Plan 2005-2010, their growth in recent years has been remarkable, and so, in terms of primary energy consumption, they rose from 6.3% in 2004 to 11.3% in 2010 (Fig. 2).

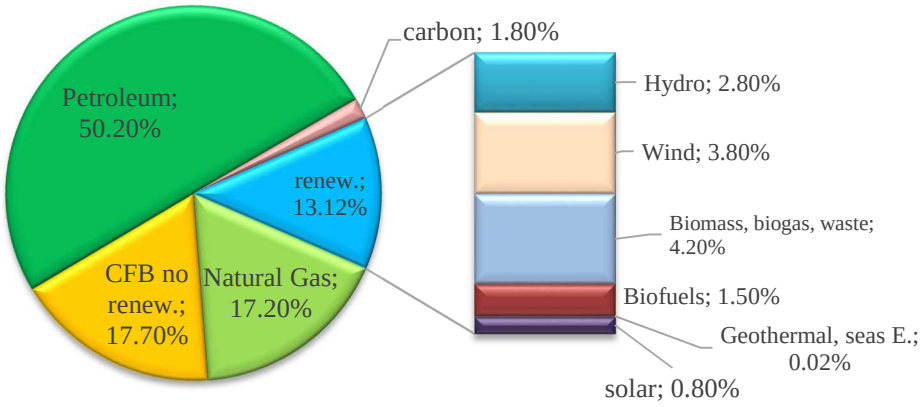

Fig.2 Final energy consumption in 2010 (REP 2011/2020).

The main markets for bioethanol are the US and Brazil, while the European Union has increased its use of biodiesel. In Spain, the installed production capacity at the end of 2010 exceeded 4 million tep, divided into 464 thousand tons of bioethanol (4 plants) and 4,318,400 tonnes of biodiesel (47 plants) (Per 2011/2020).

Regarding the technological perspective of the biofuels sector, it is undergoing a process of change that primarily affects the variety of materials suitable for use in production technologies. From this perspective, the implementation plan of the European Industrial Initiative on Bioenergy, established thermochemical and biochemical processes of converting raw materials as a priority technology

For biodiesel, a growth escalation is expected. Its use will continue an upward path over the decade, supported by the development of specifications for labeled blends. As for bioethanol consumption, this is expected to nearly double from 2011 to 2020. Its projected rise is partly motivated by the likely disappearance of the gasoline subsidies and the generalization of the specification of labeled gasoline mixtures.

Nevertheless, the growing demand for biofuel, in particular biodiesel, has raised new environmental and economic concerns about the viability of alternative fuels.

1.2. Glycerol, from major commodity to waste effluent

Glycerol, 1,2,3-propanediol, in its pure form, is a colorless, odorless, hygroscopic, viscous liquid belonging to the alcohol family of organic compounds with the molecular formula $OCH_2CHOHCH_2OH$. Figure 3 show the space-filling model of the glycerol molecule.

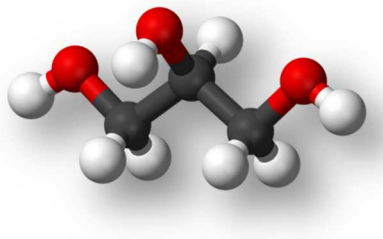

Fig.3 Space-filling model for glycerol molecule.

Glycerol contains one secondary and two primary alcohol groups per molecule, and it reacts with organic and inorganic acids to form aldehydes, esters, ethers and many derivatives. The presence of multiple alcohol groups facilitates the formation of polymers and coatings (polyesters, polyethers and alkyd resins). Glycerol acts as both a solvent and reactant, is water soluble, biocompatible, safe in topical formulations and approved for food and pharmaceutical applications.

In addition, non-toxic properties give glycerol thousands of uses. This organic compound is used in almost all industrial areas such, cosmetics, explosives, food, pharmaceuticals, polymer and printing industries (Fig. 4). It is a basic ingredient in the gums and resins used in the textile, automotive and building industries. It is also a component of mono and diglyceride emulsifiers, which are used as softening agents in baked goods, plasticizers in shortening, and stabilizers in ice cream. Glycerol also has varied uses in the pharmaceutical industries. Another recent important use is as a protective medium for freezing biological cells (red blood cells, eye corneas, and other living tissues).

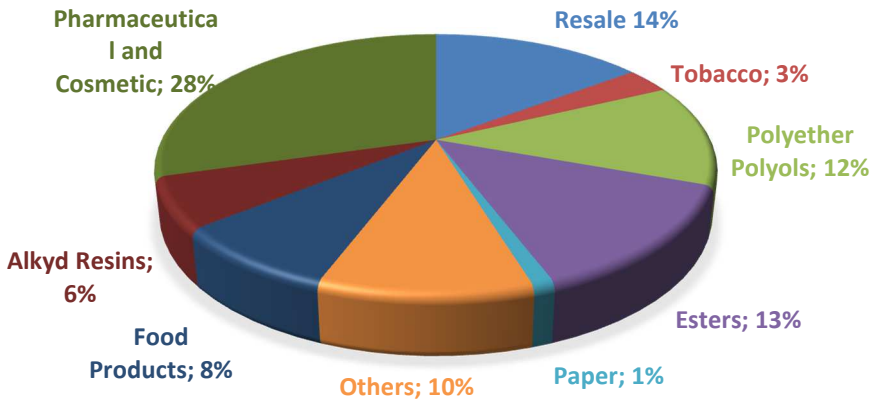

Fig. 4 Principal glycerin utilization in industry (Mota et al., 2009)

Glycerol can be produced either by microbial fermentation (Wang et al. 2001) or by chemical synthesis from petrochemical feedstock.

Microbial production of glycerol has been known for 150 years. In fact, glycerol was produced commercially by microbial fermentation during World War I. Glycerol production by microbial synthesis later declined, since it was unable to compete with

chemical synthesis from petrochemical feedstocks due to the low glycerol yields and the difficulty with the extraction and purification of glycerol from broth.

Nowadays, glycerol is mainly produced by the saponification of fats as a byproduct of soap-making (Fig. 5) and contributes significantly to the present glycerol production volume of about 600 000 tons annually (Wang et al., 2001). This process is becoming less important in the industrial nations because of the replacement of soap with detergents (Agarwal, 1990).

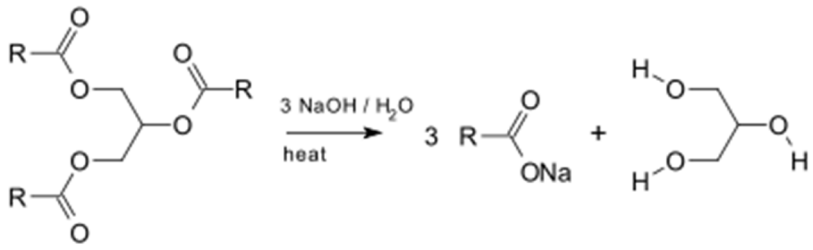

Fig. 5 Saponification of fats.

Glycerol is also produced by various routes from propylene. The epichlorohydrin process is the most important; it involves the chlorination of propylene to give allyl chloride, which is oxidized with hypochlorite to dichlorohydrin, which reacts with a strong base to give epichlorohydrin. Epichlorohydrin is then hydrolyzed to give glycerol. In 2001, around 25% of the world glycerol produced by the chemical industry was via the oxidation or chlorination of propylene (Wang *et al*., 2001). However, this route has become less common because of environmental concerns and the increase in the price of propylene. In addition, because of the emphasis on biodiesel, where glycerol is a waste product, the market for glycerol is depressed, and the epichlorohydrin process for glycerol synthesis is no longer economical on a large scale. It is projected that, by the year 2020, glycerol production will be six times more than the demand.

On the other hand, glycerol has a potential as a feedstock for new industrial fermentations. Glycerol has been subjected to bioconversion in diverse value products,

such as butanol (Biebl, 2001), citric acid (Imandi et al., 2007), propionic acid (Barbirato et al., 1997), succinic acid (Lee et al., 2001; Papanikolaou and Aggelis 2003), formate (Jarvis et al., 1997), hydrogen (Ito et al.,2005; Sabourin-Provost and Hallenbeck, 2009), 1,2-propanediol, ethanol (Ito et al., 2005), dihydroxyacetone (Bauer et al., 2005), or biosurfactants (Nitschke et al., 2005), as well as the high value chemical 1,3-propanediol (Colin et al., 2000; González-Pajuelo et al., 2005; Himmi et al., 1999; Nakamura and Whitedy, 2003; Papanikolaou et al., 2000), this last being the most capital one (Mu et al., 2008). Special attention has been paid to the microbial production of 1,3-propanediol from glycerol as this diol's proprieties gives it many applications in the production of new polymers.

As mentioned before, raw glycerol from the biodiesel process, also known as crude glycerol or glycerin, is a co-product from the production of biodiesel. It is a viscous, yellow/brown liquid, hard to purify to a pharmaceutical grade and therefore of low economic value (Yazdani et al., 2007). In general, glycerol is only about 62% to 85% (w/w) of the crude stream (Thompson and He, 2006; Mu et al.2008). The balance is water, methanol (usually used in Europe) or ethanol (conventionally used in America), fatty acids (soaps) and catalyst residues (salts). Raw glycerol also contains a variety of elements such as calcium, magnesium, phosphorous, or sulfur (Thompson and He 2005). The wide range of purity values can be attributed to the different feedstocks used by biodiesel producers. Thompson and He (2006) reported that mustard, rapeseed, canola, crambe, and soybean generated lower levels (62%-67.8%) of glycerol, while waste vegetable oil had the highest level (76.6%) of raw glycerol.

Biodiesel companies are the major sources of raw glycerol accumulation, generating 10g of glycerol per 100g of biodiesel produced (Barbirato et al., 1998) , but there are other important sources. The bioethanol industry is also responsible for raw glycerol accumulation. The most common processes in industry generate 4g of glycerol for 48g of ethanol produced (Yazdani et al, 2007).

The volumes of pure glycerol are huge and increasing. In 2003, glycerol prices were around $1200 per ton. In 2006, prices were around $600 per ton and falling (Miller-Klein Associates 2006). In November 2009, in Europe, spot prices were at €260-350/ton of glycerin.

The value of crude glycerol has also dropped. In 2006, the prices in the US were quoted at $0 - $70 per ton (Miller-Klein Associates 2006). In small-scale production, raw glycerol has a zero value to the producers. In these cases, three things typically happen to the crude stream: producers pay for transport to a purification unit, the crude stream is thrown into a landfill or, in a few cases, it is stored in containers waiting for a solution. Since crude glycerin is unsuitable for most glycerol markets, it is now regarded as a waste product that needs to be disposed of at a cost.

Waste glycerol from biodiesel manufacturing has not only reduced world glycerol market prices, but has also generated environmental concerns associated with contaminated glycerol disposal.

1.3. 1,3-Propanediol and its applications

1,3-Propanediol is an organic compound with the formula $CH_2(CH_2OH)_2$. This three-carbon diol is a colorless viscous liquid miscible in water. Figure 6 show the **s**pace-filling model for the 1,3-propanediol molecule.

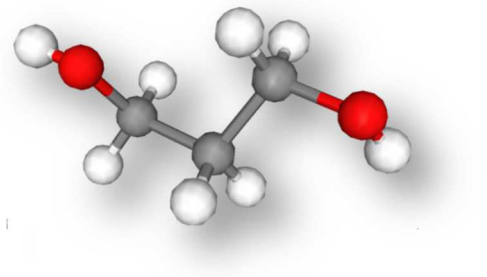

Fig. 6 Space-filling model for 1,3-propanediol molecule.

1,3-propanediol (1,3-PD), as a bifunctional organic molecule, has interesting properties for synthesis reactions, in particular as a monomer for polycondensations to produce polyesters, polyethers and polyurethanes.

In the past, its high production cost strongly restricted its uses. This situation started to change with Shell and DuPont, and their announcement of a 1,3-propanediol-based polyester, polytrimethyleneterephthalate, termed PTT (Shell) or 3GT (DuPont). This copolyester is a condensation product of 1,3-PD and terephthalic acid. It has excellent properties, such as good stain resistance or low static generation, and it is particularly suitable for fiber and textile applications [19, 20]. This special engineering plastic has replaced the traditional polyethylene terephthalate (PET) and polybutylene terephthalate (PBT). PTT can be produced in an environmentally friendly way and at a very competitive price as compared to that of PET and PBT (Zeng and Biebl, 2002). Because of this new application, 1,3-propanediol demand has improved and, today, it is a bulk polymer.

1,3-propaediol also has other interesting applications as a polymer constituent. 1,3-propanediol can provide better properties for solvents (increased flexibility), adhesives, laminates, resins (low intrinsic viscosity, less solvent for coating), detergents (preventing phase separation and loss of enzyme activity), and cosmetics (long-lasting but not sticky moisturizing effect). Finally, the biodegradability of natural plastics containing 1,3-propanediol is higher as compared to those of the fully synthetic polymers (Witt et al., 1994).

1,3-propanediol is produced by two methods: chemical synthesis and microbial conversion. Biosynthesis is particularly attractive in that it typically uses renewable feedstock and does not generate toxic byproducts.

1.4. Chemical Processes for 1,3-Propanediol

Nowadays, there are two chemical processes with high economic viability in industry use, the process of Degussa, now owned by DuPont, and the process of Shell.

The process of Degussa uses the conventional preparation method starting from acrolein, which is obtained by catalytic oxidation of propylene. Acrolein is hydrated at moderate temperature and pressure to 3-hydroxypropionaldehyde which, in a second

reaction, is hydrogenated to 1,3-PD over a rubidium catalyst under high pressure (90 bar), Fig. 7.

This acrolein process provides a yield which does not exceed 65% of the starting compound, due to the simultaneous formation of 1,2-propanediol. Furthermore, acrolein is a very harmful reagent (González-Pajuelo et al. 2005).

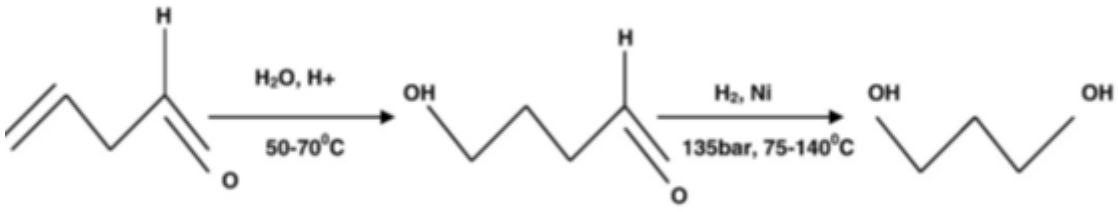

Fig. 7 Chemical synthesis of 1,3-propanediol by Degussa method starting from acrolein (adapted from Saxena et al. 2009).

The process of Shell starts from ethylene oxide, which is prepared by the oxidation of ethylene. Ethylene oxide is transformed with synthesis gas in a hydroformylation process to 3-hydroxypropanal as well (Fig. 8), but for this reaction an extreme high pressure (150 bar) is required. The aldehyde is extracted from the organic phase with water and subjected to hydrogenation using nickel as a catalyst, again under high pressure.

In this process, a yield of 80% is obtained. As the market price of ethylene oxide is also lower than that of the acrolein, the Shell process can be regarded as economically more favorable.

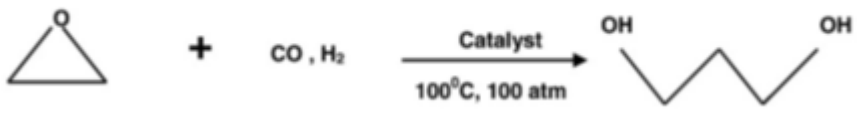

Fig. 8 Chemical synthesis of 1,3-propanediol: Shell method starting from ethylene oxide (adapted from Saxena et al. 2009).

This is reflected in the much higher production volume reported for the production of 1,3-propanediol from ethylene oxide, which amounted to 45,000 t/a in 1999, while acrolein amounted to 9000 t/a. The relatively high production costs with the acrolein process have probably induced the Dupont Company to invest in research efforts to further develop the biological process.

1.5. Microbial Formation of 1,3-Propanediol

The biological conversion of glycerol to 1,3-propanediol is increasingly becoming a practical alternative to the chemical production route due, most of all, to the fact that it is environmental friendly and to its industrial safety (low temperature and pressure is required), as well the relatively cheaply available feedstock (glycerol or glucose) and high theoretical molar yield. The renewable nature of this source of production is another aspect that improves the viability of the biotechnological production of 1,3-propanediol.

For almost 120 years a bacterial fermentation has been known in which glycerol is transformed into 1,3-propanediol, but only since 1990 has its biotechnological potential been recognized, and more intense research initiated (Zeng and Biebl, 2002). Since 1996, the production of 1,3-propanediol by microorganisms has been reported by different genera.

Natural microorganisms such as *Klebsiella* (Zhao et al., 2006; Yang et al., 2007), *Clostridia* (Barbirato et al., 1998; Himmi et al., 1999; Raynaud et al., 2003; Papanikolaou et al., 2004), *Citrobacter* (Barbirato et al., 1998), *Enterobacter* (Zhu et al., 2002) and *Lactobacilli* (Schutz and Radler, 1984) produce 1,3-propanediol by anaerobic and micro-aerobic fermentation. Among these organisms, *Clostridium butyricum* and *Klebsiella pneumoniae*, appear to be the best producers, because of their appreciable substrate tolerance, yield, and productivity.

Glycerol is fermented by a dismutation process involving two parallel pathways (Fig. 9). In one of the pathways, glycerol is transformed into dihydroxyacetone by glycerol dehydrogenase, which then undergoes normal glycolysis to form pyruvate, and

can finally be converted into various by-products such as acids and alcohols. Glycerol is converted in the second pathway by a coenzyme B12-dependent glycerol dehydratase to 3-hydroxypropionaldehyde (3-HPA), which is then reduced to 1,3-propanediol under the consumption of reducing power $NADH_2$ with 1,3-propanediol:NAD oxidoreductase as the catalyst. The second metabolic pathway, which maintains the redox balance of the cell, is necessary to convert glycerol to 1,3-propanediol, and glycerol dehydratase is the key limitation enzyme for this biological process.

In the conversion of glycerol into 1,3-propanediol, the reducing equivalents generated by the oxidative branch, during the production of organic acids, determines the amount of 1,3-propanediol that can be formed by the reductive branch.

The theoretical maximum yield that can be obtained from the anaerobic fermentation of glycerol occurs when acetate is the only by-product (Saxena *et al.*, 2009; Chotani *et al.*, 2000). The theoretical maximum 1,3-propanediol yield is 0.72 mol/mol glycerol, which was calculated for a culture without hydrogen and butyric acid production (Zeng, 1996).

For this combination, the fermentation equations can be written as:

$$CH_2OH–CHOH–CH_2OH + H_2O \longrightarrow CH_3COOH + CO_2 + H_2 + 4\,[H] \quad (1)$$

$$2\,CH_2OH–CHOH–CH_2OH + 4\,[H] \longrightarrow 2CH_2OH–CH_2–CH_2OH + 2H_2O \quad (2)$$

$$3CH_2OH–CHOH\text{-}CH_2OH \longrightarrow CH_3COOH + CO_2 + H_2 + 2CH_2OH–CH_2–CH_2OH + H_2O \quad (3)$$

The production of by-products not only reduces the amount of carbon available, but can also inhibit the growth of microorganisms.

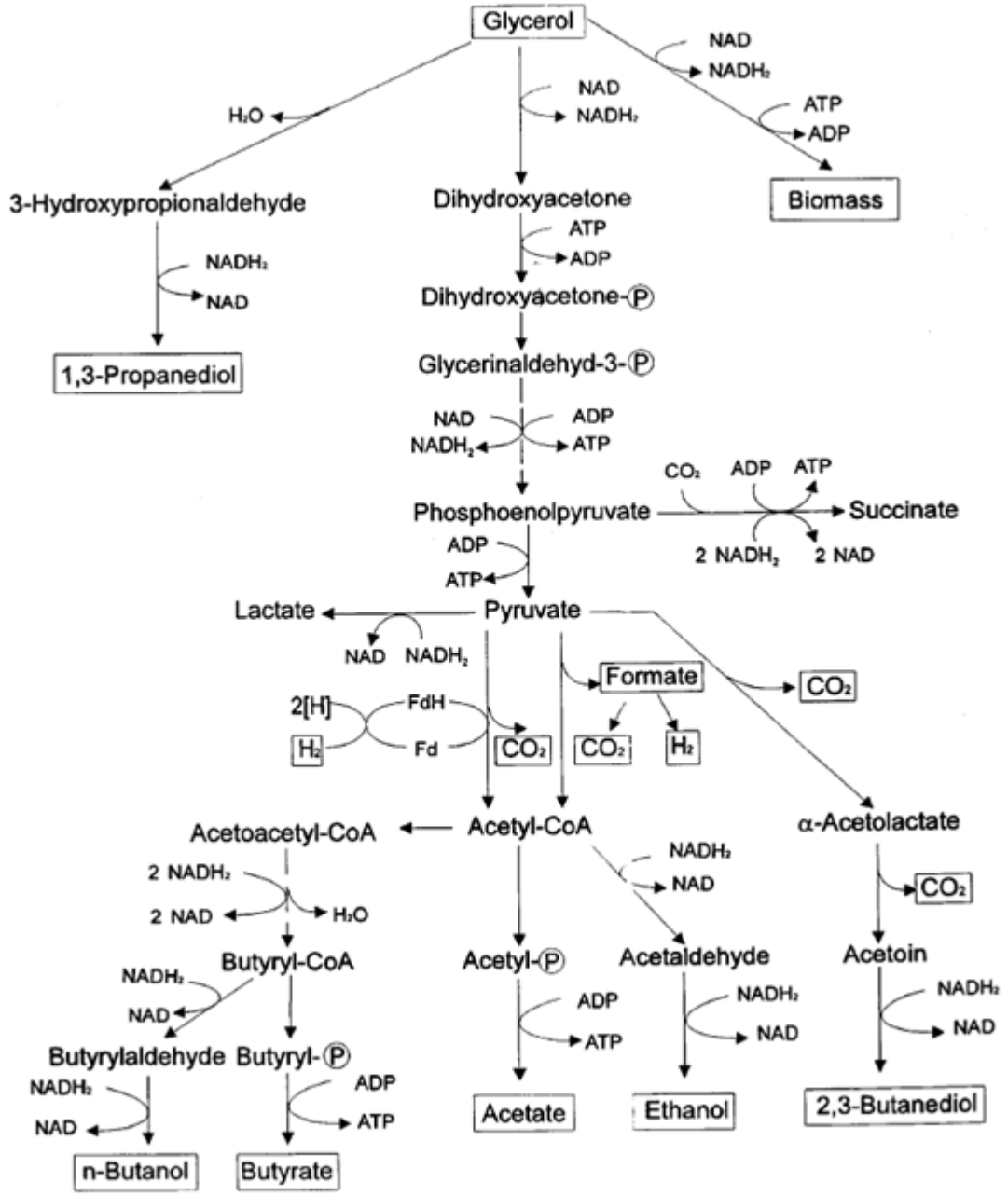

Fig. 9 Metabolic pathways of glycerol metabolism (in Biebl *et al*., 1999).

In glycerol fermentation, other by-products are also formed such as ethanol, lactic acid, succinic acid, and 2,3-butanediol, by the enterobacteria *Klebsiella pneumoniae*, *Citrobacter freundii* and *Enterobacter agglomerans*; butyric acid by *Clostridium butyricum*; and butanol by *Clostridium pasteurianum*. All these by-products are associated with a loss in 1,3-propanediol production relative to acetic acid, in particular ethanol and butanol, which do not contribute to the $NADH_2$ mechanism.

Interest in the bioconversion of glycerol into 1,3-propanediol has increased as glycerol has at times been a surplus product. In the last decade, enormous efforts are been made to optimize this process and improve the yield reaction. 1,3-propanediol production from genetically modified strains have been studied in order to improve the performance of natural producers in glycerol fermentations. However, the inhibitory effect of 1,3-propanediol in the process is difficult to optimize. Another problem of the genetically modified microorganism is the difficulty in carrying out fermentations on an industrial scale with these strains, due to their extreme sensibility and fragility. Biotechnology may be the key to 1,3-propanediol production if petroleum resources become exhausted (Cho et al., 2006).

Currently, the bioconversion of glycerol into 1,3-propanediol is an interesting alternative pathway to conventional chemical synthesis. The process eliminates two current problems in three different industries. In the biodiesel industry, the waste glycerol problem finds a use as a potential renewable feedstock for 1,3-propanediol production. As a result, the glycerol market could see the present scenario of collapse ending. On the other hand, a green technology is available for 1,3-propanediol producers.

There are different ways to optimize the microbial production of 1,3-propanediol from glycerol, and significant progress has already been achieved. The main concerns for optimizing glycerol bioconversion into 1,3-propanediolare are the formation of undesired by-products, the microbial tolerance for high concentrations of 1,3-propanediol and glycerol, and the low productivity of the bioreactor.

By-product control has been investigated for *K. pneumoniae* (Mu et al. 2006, Zeng et. al 1993) and *C. butyricum* (Colin et al. 2000). In *K. pneumonia* continuous fermentations, the production of ethanol is restricted to the limitation conditions of glycerol (Zeng et. al 1993). If the bacteria are limited by other nutrients, or by inhibition through the fermentation products, ethanol is almost completely suppressed (Zeng et al. 1994). However, under conditions of high glycerol excess and severe product inhibition, other by-products, in particular lactic acid and 2,3-butanediol, appear in the medium, diminishing the propanediol yield (Zeng et al. 1994; Menzel et al. 1997).

The production of butyric acid in *C. butyricum* fermentations also decrease under an excess of glycerol. Nevertheless, butyric acid production does seem to be dependent on the growth rate (Zeng et al. 1994).

In addition to the liquid products, the hydrogen gas released from pyruvate cleavage to acetyl-CoA is also significant for the 1,3-propanediol yield (Biebl and Zeng 2002). In *C. butyricum* fermentations, the reducing equivalents from this reaction are transferred to ferredoxin and may be further transferred to NAD by the NAD: ferredoxinoxidoreductase, instead of being released as molecular hydrogen, thus contributing to additional 1,3-propanediol formation. The enzyme is particularly active under excess substrate conditions (Solomon et al. 1994).

Product inhibition is another concern in optimized glycerol bioconversion into 1,3-propandiol. The strongest inhibitor seems to be 3-hydroxypropionaldehyde. This compound is normally an intracellular intermediate that does not accumulate. However, under conditions of high substrate excess, 3-hydroxypropionaldehyde could be excreted into the medium.

C. butyricum excretes only very small amounts of 3-hydroxypropanal and *K. pneumoniae* is able to reduce the accumulated 3-hydroxypropanal further to 1,3-propanediol. *Enterobacter agglomerans* is, however, killed by the aldehyde as soon as a concentration of 2.2 g/L has been reached (Barbirato et al. 1996).

1,3-propanediol is the least toxic product in glycerol fermentation, but still limits the final concentration. Wild-type strains can produce around 60-70 g/L of 1,3-propanediol. Based on Colin et al.'s (2000) research, *C. butyricum* can tolerate up to 83.7 g/L 1.3-propanediol when added externally. These values may represent the maximum achievable product concentration with non-modified strains (Bibl and Zeng 2002). To improve 1,3-propanediol's tolerance, many authors have worked on modified strains (Malaoui and Marczak 2001, Nakamura and Whited 2003, Reimann and Biebl 1996).

Abbad-Andaloussi et al. (1995) increased the 1,3-propanediol production of *C. butyricum* by chemical mutagenesis. This mutant was also strongly reduced in hydrogen formation and resulted in a final 1.3-propanediol concentration of 70 g/l.

To process optimization, different culture techniques, such as batch, fed-batch, chemostat and two stage chemostat cultures, have been practiced and evaluated (Gonzalez-Pajuelo et al 2005; Himmi et al 1999; Papanikolaou 2000; Reimann and Biebl 1996; Saint-Amans 1994). The productivity, but not the final 1,3-PD concentration, could be substantially increased by continuous cultures with immobilization (Sun-Ae Jun et al. 2010) or cell retention (Reimann et al. 1998)).

1.6. Bioconversion of Glycerol by *Clostridium butyricum*

Clostridium is a type of bacteria which is about 2,700 million years old. It existed before the appearance of oxygen on Earth and this is why *Clostridium* species cannot withstand oxygen.

It has been well known for almost 60 years that glycerol is fermented by anaerobic bacteria, among others by *Clostridium*, to 1,3-propanediol (1,3-PD), 2,3-butanediol, ethanol and acetic acid.

Microorganisms of this genus are Gram positive, relatively large, heterotrophic, endospore forming and motile rods. Most of them are mesophilic, though some are psychotropic or thermopholic. The natural environment of clostridia is anaerobic habitats with organic nutrients. Clostridia bacteria are spread on soils, feeding stuffs, aquatic sediments and the gastrointestinal tracts of animals and humans, where they can survive and multiply. They have the ability to form spores that ensures its survival in adverse conditions for long periods of time. A single strain of bacteria can multiply as long as they are in a culture medium containing nutrients and the environmental conditions necessary to grow.

The *Clostridium* genus consists of approximately 100 species that include common free-living bacteria as well as important pathogens.

The considerable biochemical activity of *Clostridium* is connected with the extended range of extracellular enzymes which they produce. These bacteria can cause the fermentation of organic compounds such as sugars and produce hydrogen and organic compounds like organic acids (especially butyric and acetic acids), butanol and

acetone. The metabolism of amino and fatty acids by clostridia results in the formation of foul-smelling degradation products (Buckel, 2005). Moreover, Clostridia can produce chiral products, which are difficult to obtain by chemical synthesis, and degrade a number of toxic chemicals. Solventogenic clostridia, such as *C. acetobutylicum*, *C. beijerinckii* or *C. saccharobutylicum*, can use a wide range of substrates, from monosaccharides, including many pentoses and hexoses, to polysaccharides (Hang et al. 2011, Lee et al. 2008). A variety of enzymes involved in the degradation of hemicellulose and starch have been identified in different strains. Cellulolytic Clostridia, typified by *Clostridium thermocellum*, produce a multi-enzyme cellulase complex able to degrade cellulose, hemicellulose and starch (Mitchell, 1997).

A remarkable development of clostridial toxins and spores has been their utility in the treatment of human diseases. Botulinum neurotoxin is used as a therapeutic agent for various neurological disorders, including dystonias, involuntary muscle disorders, pain, and other maladies. Spore systems of Clostridia are being developed for the delivery of therapeutics to tumors (Johnson, 1999).

The nutrient requirements for the growth of clostridia are simple. Complex nitrogen sources are generally required for good growth and solvent production.

The non-pathogenic Clostridia have a large potential industrial application. They are used for the production of butyric acid (Zhang et al., 2009; Nicolaou et al., 2010), and some solvents such as butanol, acetone, isopropanol (so-called solventogenic clostridia) (Dürre, 1998, Ezeji et al., 2005; Ezeji et al., 2007) and hydrogen (Skonieczny and Yargeau, 2009; Kothari et al., 2010).

The fermentation of glycerol by *C. butyricum* results in the production of 1,3-PD, but also in secondary products. In the metabolic pathways of anaerobic fermentation by *C. butyricum*, by-products such lactate, butyrate, acetate, ethanol, CO_2 and H_2 were synthesized (Fig. 10). The production of these by-products reduces the amount of carbon available and can inhibit the growth of *C. butyricum.*

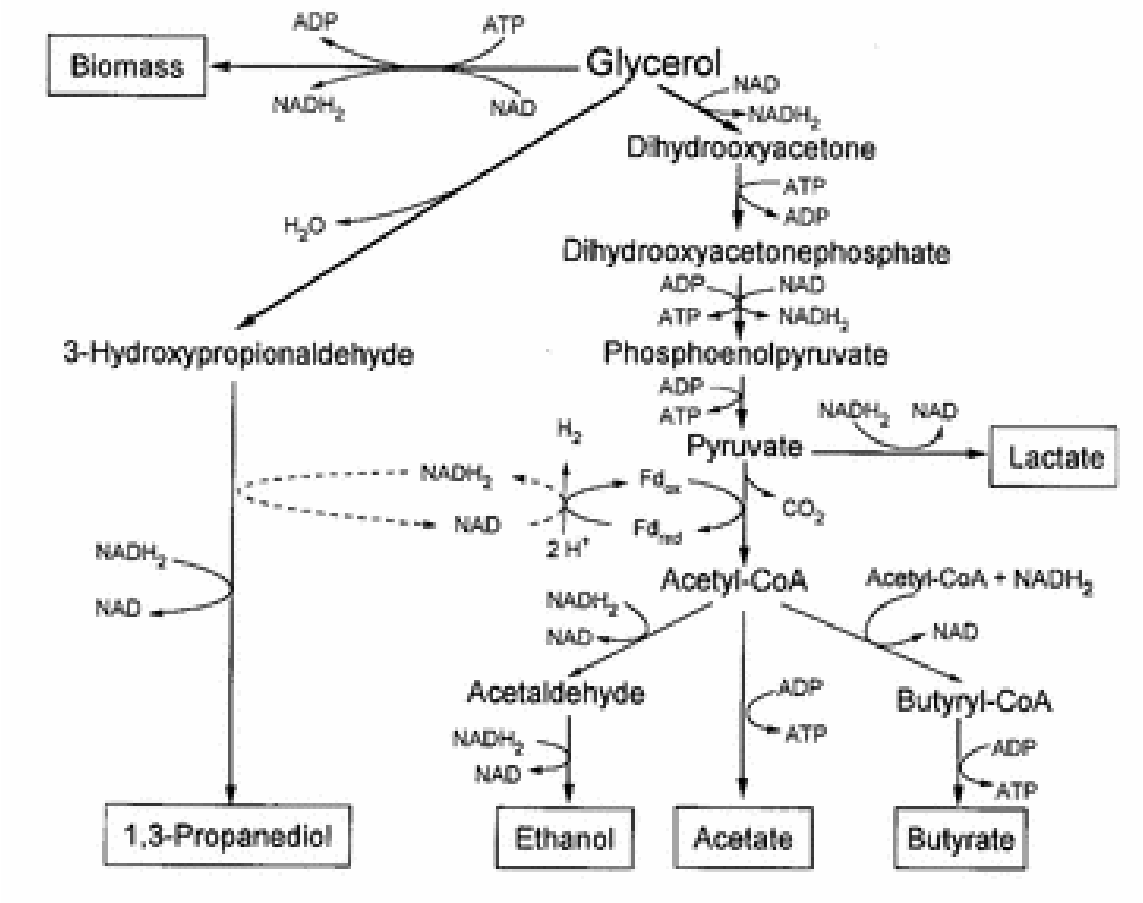

Fig. 10 Metabolic pathways of anaerobic fermentation by *C. butyricum* (in Zeng et al. 1996).

The fermentation of glycerol by *C. butyricum* has mainly been investigated with one of the following three strains: DSM5431, 3266 and strainVPIE5 (Biebl and Sproer, 2002). Table 1 summarizes some of these results.

Table 1. Different fermentation modes for 1,3-propanediol production by different *C. butyricum* strains.

Fermentation Mode		Microorganism	1.3-PD (g/L)	$Y_{PD/S}$ (mol/mol)	Q (g/L/h)	Reference
Batch		*C. butyricum* DSM 5431	56.0	0.62	2.2	Biebl *et al.* (1992)
		C. butyricum VPI 3266	35.0	0.65	0.6	Saint-Amans *et al.* (1994)
		C. butyricum CNCM1211	67.0	0.63	-	Himmi *et al.* (1999)
		C. butyricum CNCM1211	63.7	0.62	-	Colin *et al.* (2000)
		C. butyricum VPI 1718	11.3	0.60	-	Chatzifragkou et al. (2010)
Fed-batch		*C. butyricum* DSM 5431	58.0	0.68	2.7	Günzel et al. (1991)
		C. butyricum VPI 3266	65.0	0.69	1.0	Saint-Amans *et al.* (1994)
		C. butyricum DSM 5431	70.3	0.68	1.5	Abbad-Andaloussi et al. (1995)
		C. butyricum E5	65.6	0.65	1.2	Petitdemange *et al.* (1995)
		C. butyricum DSM 5431	70.4	0.68	1.4	Reimann and Biebl (1996)
		C. butyricum mutnt 2/2	70.5	0.66	0.9	Reimann and Biebl (1996)
		C. butyricum DSM 5431	45.0	0.51	2.14	Asad-ur-Rehman *et al.* (2008)
		C. butyricum DSM 2478	7.0	-	0.15	Moon *et al.* (2010)
Continuous	**Single stage**	*C. butyricum* mutant 2/2	34.2	0.70	5.13	Reimann *et al.* (1998)
		C. butyricum VPI 3266	30.0	0.65	10.3	González-Pajuelo *et al.* (2005)
		C. butyricum VPI 3266	30.0	0.60	– -	González-Pajuelo *et al.* (2005)
	Two stage	*C. butyricum* F 2b	46.0	0.53	3.4	Papanikolaou et al. (2000)
		C. butyricum F 2b	43.5	0.49	1.33	Papanikolaou et al. (2008)

The maximum 1,3-PD concentration was reached in fed-batch cultures by *C. butyricum* DSM 5431 and *C. butyricum* mutant 2/2 with produced values around 70g/L.

In continuous cultures, the production of 1,3-PD by *C. butyricum* mutant 2/2 decreased to 30.0 g/L, the productivity, however, was five times higher.

Concerning the production of by-products, Chatzifragkou et al. (2010) reported the formation of lactic acid during batch and continuous fermentations carried out with *C. butyricum* VPI 1718. In fermentations with 20g/L of pure glycerol, Chatzifragkou et al. (2010) obtained 3.1 g/L of butyrate, 0.7 g/L of acetate and 0.5g/L of lactate.

Apart from Chatzifragkou et al. (2010), all studies reported that the main and only by-products were butyric and acetic acid, the butyric acid being approximately 4 times higher than the concentration of acetic acid.

For batch fermentations, some of the authors (Papanikolaou et al. (2000), Abbad-Andaloussi et al. (1995)) observed a direct relationship between cell growth and acetic and butyric acids, while for continuous experiments, Papanikolaou et al. (2000) described changes in the yields and the specific production rates. According to the authors, low and medium specific growth rates favored butyric acid production, whereas high dilution rates improved acetic acid production. Abbad-Andaloussi et al. (1996) also reported that, at high growth rates, the carbon flux through the butyrate biosynthesis was disrupted due to the thiolase (converting acetyl-CoA to acetoacetyl-CoA), resulting in the movement of the cellular metabolism towards the biosynthesis of the acetic acid. Moreover, at neutral pH values and high dilution rates, the acetic acid pathway is preferred due to its low toxicity. Zeng et al., (1994), also documented a direct relationship between the initial substrate concentration and the production of acetic acid.

2. References

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CHAPTER 2. AIM AND OUTLINE OF THE THESIS

1. Aim and outline of the thesis

The aim of this work was to investigate the production of 1,3-propanediol (1,3-PD) from biodiesel-based waste glycerol. In that sense, fermentation strategies using the microorganism *Clostribium butyricum* DSM 10703, will be developed. For this purpose the current work is developed in four specific objectives:

1. To carry out experiments with pure glycerol (model solutions) analysing the influence of operating conditions (temperature, pH, substrate concentration) both in final 1,3 propanediol concentration and fermentation yield. Different operation modes will be planned: batch, fed-batch and continuous.

2. To perform experiments with raw glycerol from an industrial biodiesel production process in order to establish the influence of substrate concentration and/or other potential inhibitory compounds on yield and productivity. Batch, fed-batch and continuous operation modes will also be used in order to get the best fermentation strategy.

3. To establish an adequate pre-treatment sequence for raw glycerol in order to reduce/remove the main toxic compounds that influence the glycerol fermentation stage. Results will be compared with that obtained when pure and untreated raw glycerol solutions were tested.

4. To study the immobilization of microorganisms by means of bioparticles formation by entrappment with a polymeric matrix. Operation in stirred tank and fluidized bed bioreactor will be compared.

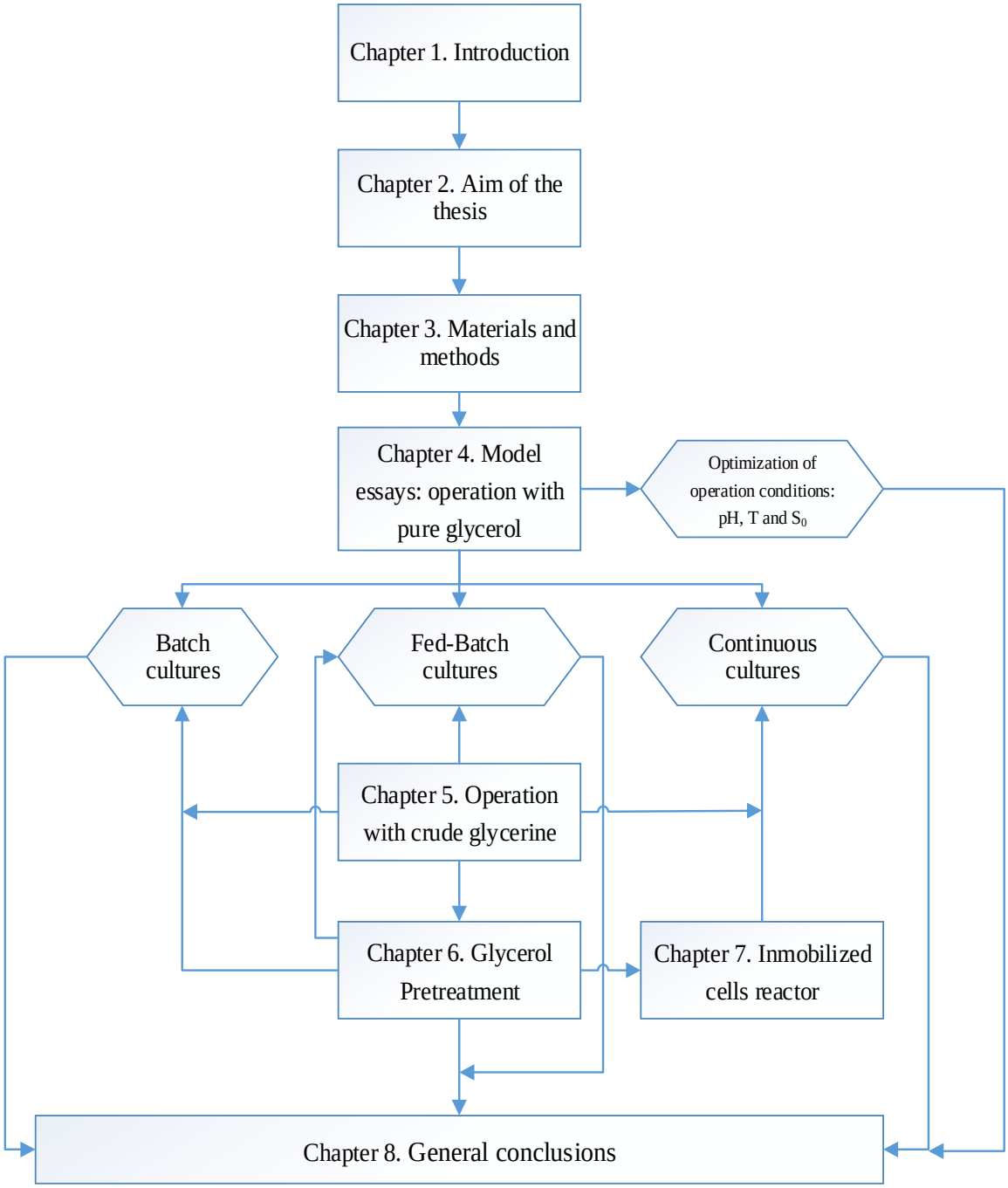

Figure 1. Outline diagram the thesis.

CHAPTER 3. MATERIALS AND METHODS

1. Microorganism maintenance and growth conditions

1.1. Microorganism

The organism used in this study was *Clostridium butyricum* DSM 10702, obtained from the German collection of microorganisms (DSMZ).

Name:	***Clostridium butyricum*** Prazmowski 1880

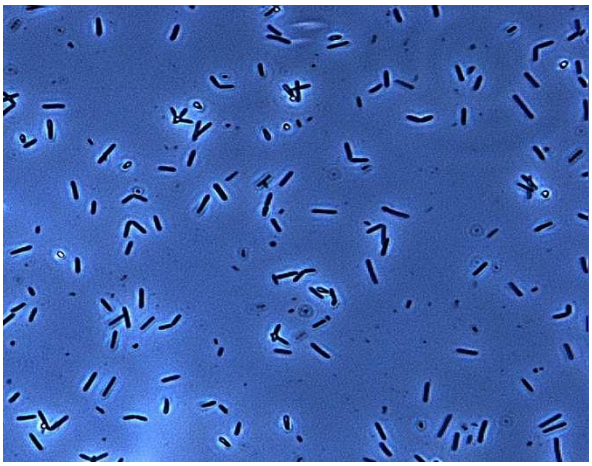

Fig.1 *Clostridium butyricum* (100x).

DSM No.:	10702
Information:	Strain Rowett. Intestine of pig. **Type strain.** Taxonomy/description (1300, 1892). Butyricin 7423 (2118). Phylogenetic relatedness (1336). (Medium 104b, Medium 110 or Medium 634, 37°C, anaerobic)
Isolated from:	intestine of pig
Risk group:	2 (1 before 2013)

1.2. Maintenance

The strain was maintained, in Reinforced Clostridial Medium (RCM, Fluka) in Hungate tubes (18 x 150 mm), in spore form and conserved at -20ºC under anaerobic conditions. Before inoculating the preculture, a heat shock was conducted at 80ºC for 10 minutes, stimulating germination spores.

1.3. Media and cultivation conditions

Precultures and bioreactor cultures were performed in the medium described by Papanikolaou *et al* (2000) that contained: K_2HPO_4 1 g/L, KH_2PO_4 0.5 g/L, yeast extract 1 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $(NH_4)_2SO_4$ 2 g/L, $CaCl_2$ 15 mg/L, $FeSO_4.7H_2O$ 5 mg/L, micromineral solution SL_7 2 mL/L. The composition of the solution SL_7 was as follows: $ZnCl_2$ 70 mg/L, $MnCl_2.4H_2O$ 100 mg/L, H_3BO_3 60 mg/L, $CoCl_2.2H_2O$ 200 mg/L, $CuCl_2.2H_2O$ 20 mg/L, $NiCl_2.6H_2O$ 25 mg/L, $Na_2MoO_4.2H_2O$ 35 mg/L, HCl 37% 0.9 ml/L. Crude glycerol, from the transesterification process for biodiesel production, was kindly supplied by ACOR and contained the following components: glycerol, 88% (w/v); sodium salts, 5% (w/w), water 4.3% (w/w), and non-glycerol organic matter 2.11% (w/w).

Precultures were grown in 100 mL serum bottles with rubber septa for syringe operation. The bottles were filled with 50 mL preboiled (with nitrogen during 5 min) growth medium and sealed under nitrogen atmosphere before autoclaving them (fig. 2). The preculture was then incubated at operation temperature without agitation for 20h.

Fig. 2 Preculture preparation.

2. Bioreactor experiments

Batch, fed-batch and continuous experiments were carried out in a 2 L bioreactor Biostat Bplus, Fig. 3.

2.1. Description of fermenter

The fermenter is equipped with a stirring motor, filter and nitrogen regulator; cooler (gas condenser) and sensors of temperature, pH and foam level. For pH adjustment, it has input valves for acidic and basic solutions.

Fig. 3 Bioreactor Biostat Bplus 2L

The reactor was sealed and autoclaved with the growth medium inside and, immediately after, while the medium was still boiling, anaerobic atmosphere was created by the introduction of nitrogen with a flow rate of 2.0 L/min. Clamps were open as outflow tracts to the atmosphere and the containing oxygen fermentor is replaced by

nitrogen. The nitrogen flow was maintained until the fermenter reached operating temperature and closed after inoculation. The operating temperature varied, depending on the planned experiment, between 33 and 37 °C.

The reaction volume was 1 L, containing 0.9 L of growth medium and inoculated with 0.1 L of preculture. Agitation was set at 200 rpm and the pH was controlled by the automatic addition of 3 M KOH. The temperature was regulated by a thermostatted water circulation system. The fermentation time was determined by biomass evolution, and the experiments ended when the biomass reached the stationary phase.

3. Analytical methods

3.1.Determination of glycerol, 1,3 - propanediol and organic acids

Glycerol and fermentation products were analyzed by HPLC using an Aminex Biorad HPX87H column (300 mm x 7.8 mm) at 60ºC, in combination with an R.I. Detector (differential refractometer 410 from Waters). The eluent used was sulphuric acid 0.01 N flowing at 0.6mL/min. The samples of the fermentation broth were previously centrifuged, and the supernatant filtered through 0.20 μm pore size nylon filters.

In these conditions, the residence times in minutes for the end products, glycerol, acetic acid, butyric acid and 1,3propanediol are, respectively, 13.2, 14.9, 17.4 and 21.5, as shown in Fig. 4.

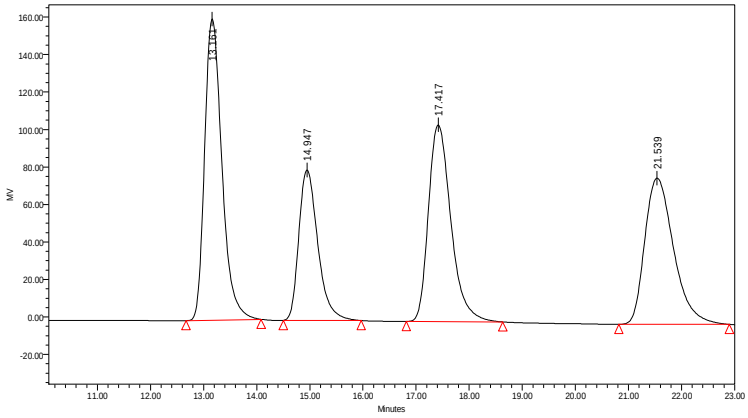

Fig.4 Separation products from glycerol fermentation by *C.butylicum* 10702. Residence times for starting order: glycerol, acetic acid, 1,3-propanediol and butyric acid for a 300 cmx7.8 mm Biorad Animex HPX87H column, using an RI detector at T =60 °C and flow 0.6 mL/min.

3.2.Biomass measurement

Biomass concentration was determined by two methods: dry-weight and optical density.

3.2.1 Optical density

The biomass growth was determined by periodic measurements of the optical density (OD) at an absorbance of 600 nm in a HITACHI U200 UV/visible (Hitachi Ltd, Tokyo, Japan) spectrophotometer. The OD was measured immediately after collecting the sample, and the samples presenting an OD value higher than 0.9 were diluted with de-ionized water.

3.2.2 Dry-weight

Dry-weight determination was carried out with 1.5 mL of sample, centrifuged and dried to a constant weight at 100ºC. A linear relationship between optical density and cell dry-weight concentration was established, showing a linear behavior for

concentrations below 1600 mg/L. The relationship obtained was: one OD unity corresponds to 853.89 mg/L of biomass.

$$Weight\ dry\ \left(\frac{g}{L}\right) = 0.853 * OD$$

3.2.3 Microscopic observation of cultures

Microorganism morphology observation was followed in a microscope (LEICA DM 400B) equipped with halogen and mercury lamp and four lenses (5x, 10x, 20x and 100x). A camera attached to the microscope and LEICA APPLICATION SUITE software for image analysis were used to obtain the photographs.

3.3. Crude glycerol characterization

Crude and treated glycerol samples were analyzed for glycerol, moisture, salts, ash and non-glycerol organic matter content. Glycerol content was measured by means of a titrimetric method (AOCS Ea 6-94), moisture content by means of titration (Mettler Toledo Karl Fischer Titrator DL35), salt content by volumetric determination, using potassium dichromate as indicator and silver nitrate as titrant, and ash content through calcination (2 hours at 550 ºC in a furnace). Finally, non-glycerol organic matter content was estimated as the percentage remaining from 100%.

3.4. Carbon recovery

Carbon recovery (Rc) calculation was carried out at the steady state obtained during the continuous cultures. In order to proceed with this calculation, the dry bacterial mass was considered to be represented by the chemical formula $C_5H_7NO_2$. It was considered that for 1 mol of acetic acid produced, 1 mol of CO_2 was synthesized, while the formation of 1 mol of butyric acid resulted in the production of 2 mol of CO_2 (Papoutsakis 1984).

4. References

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CHAPTER 4. MODEL ASSAYS: OPERATION WITH PURE GLYCEROL

1. Introduction

There are numerous ways to optimize the microbial production of 1,3-propanediol from glycerol, and significant work has already been developed in that sense. The main concerns for optimized glycerol bioconversion into 1,3-propanediol are the undesired formation of by-products, the microbial tolerance for high concentrations of 1,3-propanediol and glycerol, and the low productivity of the bioreactor.

Batch culture systems contain a limited initial amount of nutrients. In general, this operation mode provides high product concentrations , though this is limited by the initial substrate concentration and the product toxicity.

On the other hand, the use of fed-batch cultures in industry takes advantage of the fact that the concentration of the limiting substrate may be maintained at a low level, thus avoiding the repressive effects of high substrate concentration.

Continuous cultures have the advantage of surpassing inhibition by substrate and product and reaching a higher productivity. However, low product concentrations are achieved as compared with batch and fed-batch systems.

In this chapter the influence of operation variables (temperature, pH and initial glycerol concentration) on the concentration and selectivity of 1,3-propanediol biosynthesis by C. butyricum DSM 10702 was studied.

Furthermore different culture techniques (batch, fed-batch and chemostat cultures) were tested. All experiments were carried out with pure glycerol (Sigma 99% (wt/wt)) as substrate in order to function as model assays to compare with further crude glycerol experiments and investigate the inhibitor effect of toxins and inhibitors in crude glycerol from the biodiesel waste industry.

2. Methodology

The organism used in this study was *Clostridium butyricum* DSM 10702, whose specifications were shown in chapter 3 as part of Materials and Methods.

2.1.Batch fermentations

Batch experiments were carried out in a 2L bioreactor Biostat Bplus, Fig.1. The fermenter was equipped with a stirring motor (R), filter and nitrogen regulator (V_2). cooler (gas condenser) (C), and temperature (T) and pH (P) sensors. For pH adjustment, it has input pumps for acidic and basic solutions.

The fermentation time was determined by biomass evolution and the experiments ended when the biomass reached the stationary phase.

2.2.Fed-batch fermentations

Fed-batch fermentations were performed in a 2-L reactor (BiostatBplus) equipped with pH, temperature and level sensors. pH was controlled at 6.5 by the automatic addition of 3 M KOH and temperature was regulated at 37ºC by a thermostatted water circulation system. A peristaltic pump was attached to feed the supply (fig.1).

2.3.Continuous fermentations

Continuous cultures were carried out in a 600 mL glass bioreactor, with a working volume of 500mL. The cultures were stirred at 200 rpm, pH was controlled at 6.5 by the automatic addition of 3 M KOH and temperature was regulated by a

thermostatted water circulation system. The reaction volume was 0.5 L, containing 0.45 L of growth medium and inoculated with 0.05 L of preculture.

To create anaerobic conditions, the sterilized synthetic medium in the vessel was flushed with sterile O_2-free nitrogen until room temperature was attained. The culture was first grown batchwise and continuous feeding was started once the exponential growth phase was reached. During the experiments, the feed medium was maintained under N_2 at 20 mbar, to avoid O_2 getting in. The bioreactor gas outlet was protected with a pyrogallol arrangement (Vasconcelos et al. 1994). The steady state condition was considered to have been attained when at least four working volumes of the culture medium had been passed through the chemostat and the optical density measured was constant throughout four measurements.

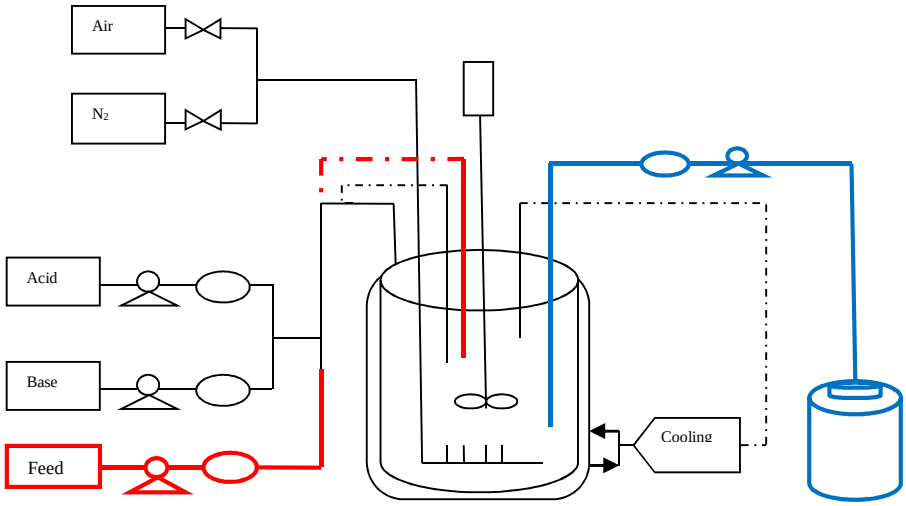

Fig. 1 Bioreactor diagram. – Feed supply arrangement, – level sensor and chemostat outlet.

3. Batch Fermentations

A first set of experimental runs, based on a factorial design of experiments, were conducted with pure glycerol to obtain the operating conditions that maximize both 1,3-PD concentration and yield factor. The inhibitory effect of the initial substrate concentration on fermentation to 1,3-PD was also studied for pure glycerol. Finally,

experimental data were fitted to a kinetic model to obtain the kinetic and biological parameters.

In order to minimize experimentation, the experiments were performed using a design that took into account the most representative operating variables (temperature, pH and substrate concentration).

Table 1. Experimental design**:** range of variables assayed.

Temperature (ºC)						
	33			37		
pH	6.5		7.0	6.5		7.0
S_0	30	50	70	30	50	70

To establish the influence of the operating conditions on fermentation, a fractional factorial design was carried out considering temperature, pH and initial glycerol concentration as the main factors, and 1,3 PD, acetic acid and butyric acid concentrations as the responses. A linear multiple regression model has been proposed to adjust the responses. Moreover, the effect of the operating parameters on yield and selectivity has also been considered. The statistical significance of the effects was analyzed by ANOVA tests ($p < 0.05$) using the software Statgraphics Centurium XVI.

The experimental results corresponding to the fermentation of pure glycerol by *C. butyricum* DSM 10702 are presented in Table 2 for the different operating conditions studied. Concentrations of 1,3 PD and the main by-products (acetic and butyric acids) are reported, as well as yield, selectivity and productivity after 50 h fermentation.

Table 2. Effect of temperature, pH and initial pure glycerol concentration on batch fermentation by *Clostridium Butyricum* DSM 10702.

Temperature	pH	S_0 (g/L)	1,3 PD (g/L)	HAc (g/L)	HBut (g/L)	R_C (%)	$Y_{PD/S}$ (g/g)	$Y_{HAc/S}$ (g/g)	$Y_{HBut/S}$ (g/g)	$S_{P/HAc}$	$S_{P/HBut}$	QPD (g/(L·h)
33	6.5	30	14.0	1.4	2.1	71.2	0.47	0.047	0.070	10.0	6.7	0.28
		50	21.4	3.9	3.8	70.6	0.43	0.078	0.076	5.5	5.6	0.43
		70	37.3	3.6	3.8	77.5	0.53	0.051	0.054	10.4	9.8	0.75
	7	30	14.8	1.8	2.2	76.3	0.49	0.060	0.073	8.2	6.7	0.30
		50	25.4	3.2	3.7	78.5	0.51	0.064	0.074	7.9	6.9	0.51
		70	38.2	3.8	4.5	80.7	0.55	0.054	0.064	10.1	8.5	0.76
37	6.5	30	14.8	3.1	2.1	80.3	0.49	0.103	0.070	4.8	7.0	0.30
		50	23.3	1.9	5.4	75.6	0.47	0.038	0.108	12.3	4.3	0.47
		70	40.0	3.0	6.0	85.7	0.57	0.043	0.086	13.3	6.7	0.80
	7	30	15.6	2.0	2.4	81.1	0.52	0.067	0.080	7.8	6.3	0.32
		50	28.2	2.7	4.0	85.1	0.56	0.054	0.080	10.4	7.1	0.56
		70	40.5	3.6	6.9	89.3	0.58	0.051	0.099	11.3	5.9	0.81

Note: QPD is the productivity after 50 h fermentation.

3.1.1,3-propanediol concentration and yield

Influence of temperature

Regarding the operating temperature, it was observed that the concentration of 1,3 PD increased with the temperature, ranging from 14.0 g/L (initial substrate concentration of 30 g/L, pH 6.5 and 33 ºC) to 40.5 g/L (70 g/L of glycerol, pH 7, 37 ºC). The same positive effect of temperature was observed in the yield values, which varied from 0.43 to 0.58 g of 1,3-PD formed per g of glycerol consumed, close to the theoretical maximum yield (0.72 mol/mol, e.g. 0.60 g/g). Productivity also rose with temperature, ranging from 0.28 g/(L·h) to 0.81 g/(L·h) after 50h of fermentation (A-P. Zeng 1996).

Influence of pH

As for the pH, an increase from 6.5 to 7 involved a slight increase in 1,3 PD concentration. Using an initial glycerol concentration of 70 g/L, an increase in pH from 6.5 to 7 at 33ºC led to a rise in both the 1,3 PD concentration (from 37.3 g/L to 38.2 g/L) and the yield factor (from 0.53 to 0.55). However, at 37ºC, the differences observed were not considerable for the higher substrate concentration: 40.0 g/L and 40.5 g/L of 1,3 PD and yield factors of 0.57 and 0.58 g/g at pH 6.5 and 7, respectively. Although an optimum pH value of 7.0 is reported in the literature, no considerable differences were found in the results reported in Table 1. Therefore, further experiments were carried out at the lower pH in order to diminish the addition of chemicals (Colin et al 2000).

Influence of initial substrate concentration

From the values shown in Table 1, it is clear that an increase in the initial substrate concentration involved an increase in the concentration of 1,3 PD,

independently of the operating temperature and pH. The higher 1,3 PD concentrations corresponded to 70 g/L initial glycerol.

These results are in agreement with the mass balance for carbon. The percentage of carbon recovery (% R_C), substrate consumed that is retrieved in carbon products, was 85.7 % (37 ºC, pH 6.5, 70 g/L). This data was calculated taking into account the fact that 1 mol CO_2 is formed per mol of acetic acid and 2 mol CO_2 per mol of butyric acid. Carbon in biomass was not considered because of the low biomass yield (Y_{XS} = 0.02 g/g, 1.5 g/L). Other authors (Barbirato et al. 1998; Himmi et al. 1999) reported slightly lower percentages of carbon recovery (about 83%), although biomass production was higher.

Finally, the productivity also depended on the initial substrate concentration, and as the initial glycerol concentration increased, productivity also increased. The highest productivity values were obtained at 70 g/L glycerol and 37 ºC: 0.80 g/(L·h) at pH 6.5 and 0.81 g/(L·h) at pH 7.

3.2.By-products and process selectivity

In relation with the organic acids obtained as by-products of the fermentation process (acetic and butyric acids), the results are also shown in Table 1, where the parameters $S_{PD/HAc}$ (g 1,3 PD per g acetic acid) and $S_{PD/HBut}$ (g 1,3 PD per g butyric acid) represent the process selectivity.

The concentrations of acetic acid obtained in the experimental runs presented a random tendency, a clear influence of the operating conditions not being observed. For instance, at 33 ºC and pH 7.0, the acetic acid concentration increased from 1.8 g/L to 3.8 g/L when the initial substrate concentration increased from 30 g/L to 70 g/L. The same effect was observed at 37 ºC and pH 7.0: the acetic acid concentration increased from 2.0 g/L to 3.6 g/L. Although, at pH 6.5, an erratic trend was observed for both temperatures, working at 37 ºC and 70 g/L glycerol, the acetic acid concentration was 3.0 g/L, lower than the value obtained at pH 7.0.

On the other hand, the concentration of butyric acid increased when the initial glycerol concentration rose for all pH values tested. At 33 ºC, butyric acid concentration ranged from 2.1 g/L (30 g/L glycerol and pH 6.5) to 4.5 g/L (70 g/L glycerol and pH 7.0), whereas, at 37 ºC, the butyric acid concentration varied from 2.1 g/L (30 g/L glycerol andpH 6.5) to 6.9 g/L (70 g/L glycerol and pH 7.0). It can also be observed that the concentration of butyric acid increased with temperature.

Regarding the selectivity, the highest values corresponded to 37 ºC, 70 g/L glycerol and pH 6.5: $S_{P/HAc}$ was 13.3 and $S_{P/BuH}$ 6.7.

3.3.Statistical analysis

As previously explained, a fractional factorial design was used to find significant factors affecting the 1,3 PD production process. In this sense, a first order model has been proposed:

$$Y = \beta_0 + \sum \beta_i \cdot x_i$$

where Y is the response (i.e., 1,3 PD concentration, acid concentration,…), β_0 and β_i are the coefficients for linear regression, and x_i is the independent variable. In order to carry out a systematic study, three independent variables: temperature, pH and initial glycerol concentration, were selected. Statistical analysis was carried out using the ANOVAF-test. Table 3 summarizes the coefficients of the model and the significance of each coefficient for 1,3 PD and butyric acid concentrations.

For 1,3 PD concentration, the following equation with the significant terms ($p < 0.05$) was obtained:

$$1,3\ PD = -47.78 + 0.475T + 3.997pH + 0.605S_0 \qquad (1)$$

As can be seen in Table 3, the F- and p-values were 124.62 and 0.0000, respectively, with a value of R^2 = 0.97 and R^2 (Adj) = 0.97, which indicates that the model is significant at the 95% confidence level.

Table 3. Statistical analysis from batch fermentation experiments.

Source	1,3 PD†			Butyric acid‡		
	Coefficient	F-value	p-value	Coefficient	F-value	p-value
Intercept	-47.78			-10.93		
A (T)	0.475	3.39	0.1027	0.280	6.41	0.0391
B (pH)	3.997	3.75	0.0890	0.173	0.04	0.8502
C (S_0)	0.605	366.72	0.0000	0.077	16.77	0.0021
Model		124.62	0.0000		12.99	0.0019

†R^2 = 0.97, R^2 (Adj.) = 0.97, significant at the 95% confidence interval

‡ R^2 = 0.83, R^2 (Adj.) = 0.77, significant at the 95% confidence interval

For butyric acid, a similar equation was found with the following significant terms:

$$Butyric\ acid = -10.931 + 0.280\ T + 0.077\ S_0 \qquad (2)$$

The model for butyric acid concentration was also found to be significant (95% confidence level), the F- and p- values being 12.99 and 0.0019, respectively. The regression coefficient R^2 was 0.83 (R^2 adjusted = 0.77).

For acetic acid concentration, no significant model was obtained.

In short, the experimental results showed that the operation with the highest values of each variable (37 °C, pH 7 and 70 g/L glycerol) provided the highest concentration of 1,3 PD (40.5 g/L) and the highest yield factor ($Y_{PD/S}$ = 0.58 g 1,3 PD/g glycerol), as well as acceptable values of selectivity and organic acid concentration: $S_{P/HAc}$ = 11.3 for acetic acid and $S_{P/HBut}$ = 5.9 for butyric acid, which means 3.6 g/L of acetic acid and 6.9 g/L of butyric acid. However, it should also be remarked that, in the experimental runs conducted at 37 ºC, 70 g/L glycerol and pH 6.5, the 1,3 PD concentration (40.0 g/L) was quite similar, but slightly lower by-product concentrations were produced (3.0 g/L acetic acid and 6.0 g/L butyric acid). Moreover, lower reagent addition was necessary to maintain the pH.

Therefore, 37 °C, pH 6.5 and 70 g/L were the fermentation conditions selected as optimum for 1,3-PD production from pure glycerol using *Clostridium butyricum* DSM 10702. The evolution of the fermentation process regarding glycerol uptake, cell growth, 1,3-PD and by-product concentrations is depicted in Fig. 2.

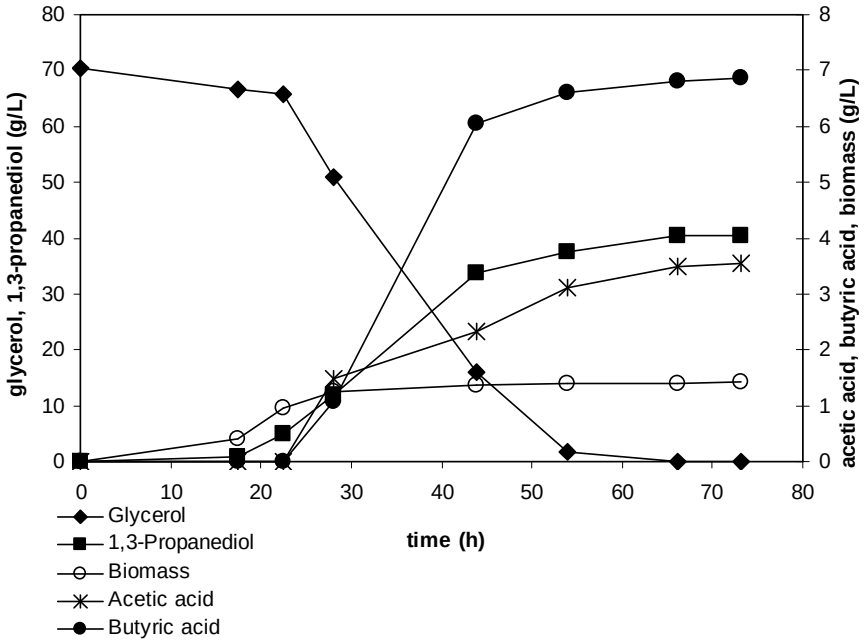

Fig. 2 Batch Fermentation profiles for pure glycerol at the best operating conditions (37 ºC, pH 6.5 and 70 g/L of glycerol).

3.4.Tolerance of C. butyricum to initial substrate concentration

As previously explained, the concentration of 1,3 PD increased with the initial substrate concentration. In order to determine the maximum initial substrate concentration that the microorganism can tolerate while still maintaining high 1,3 PD concentration in culture medium, a set of experimental runs were performed varying the concentration of pure glycerol from 50 g/L to 160 g/L at pH 6.5 and 37 ºC. Results corresponding to the evolution of 1,3-PD concentration and yield factor are shown in Fig. 3.

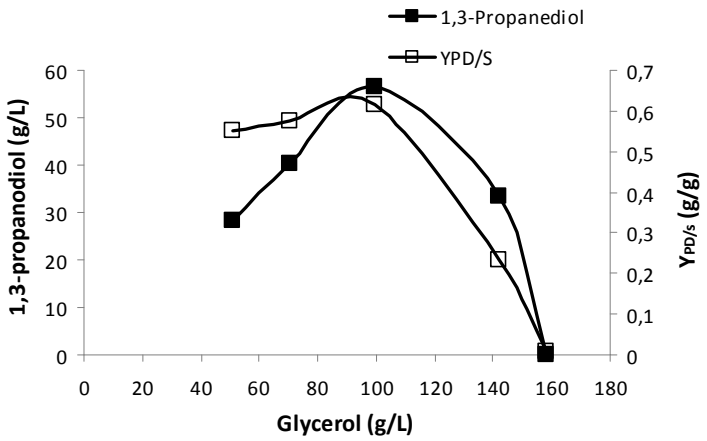

Fig. 3 Effect of glycerol concentration on 1,3-propanediol concentration and yield factor ($Y_{PD/S}$) for pure glycerol.

As can be seen in Fig. 3, the maximum value of 57g/L and a yield factor of 0.61 g/g of 1.3-propanediol was reached for an initial concentration of glycerol of 100 g/L. However, the diol production sharply decreased for concentrations higher than 100 g/L and it was almost zero when 160 g/L of substrate was used.

Although the maximum yield factor corresponded to 100 g/L of pure glycerol, the organic acids produced were considerably higher (4.5 g/L acetic acid, 9.2 g/L butyric acid) than those obtained using 70 g/L of glycerol (3.6 g/L acetic acid, 6.9 g/L butyric acid). Furthermore, in the fermentation with an initial substrate concentration of

70g/L, all substrate was consumed after 55 h. In the case of 100 g/L of initial glycerol concentration, an amount of 8 g/L remained in the fermentation broth.

Regarding organic acids, the concentration increased as the substrate concentration increased, up to a maximum that corresponded to the maximum concentration of glycerol tolerated by the microorganism. Thereafter, the acid concentrations decreased.

Therefore, it may be considered that, for practical applications, glycerol up to 100 g/L is an adequate substrate for 1,3-propanediol production using *C. butyricum*. Although 100 g/L of substrate provides higher 1,3-propanediol concentration and yield, it also provides higher concentrations of organic acids and an amount of glycerol remains unconsumed, a fact that, in terms of product separation, could represent a drawback.

Colin et al. (2000) studied the inhibition of *C. butyricum* CNCM 1211 by glycerol in batch fermentations at pH=7 and T=37 ºC. According to the report, the production of 1,3-propanediol increased linearly with the initial concentrations from 20 to 140 g/L and decreased for an initial concentration of 150 g/L.

3.5.Kinetic Model

The production of 1,3 PD from glycerol by *C. butyricum* is a primary metabolic process and direct relations can be established between the specific production rate of 1,3 PD, the specific substrate uptake, and the specific growth rate of the biomass.

Although there are several expressions which relate the specific growth rate to the substrate concentration, the Contois model can be suitable for this type of fermentation process. This model considers that the biomass growth rate depends on both substrate and biomass concentration, and growth is inhibited by the cell mass. For the batch experimental runs, the following equations can be applied:

Uptake rate of substrate $$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\,\mu X \quad (3)$$

Kinetic Contois model $$\mu = \frac{\mu_{max} S}{B X + S} \quad (4)$$

Growth yield coefficient $$X - X_0 = Y_{X/S}\,(S_0 - S) \quad (5)$$

Product yield coefficient $$P - P_0 = Y_{PD/S}\,(S_0 - S) \quad (6)$$

Equations (3) to (6) were solved numerically to estimate the kinetic parameters and the yield factors. The simulation software EcosimPro© (that incorporates the NPL non-linear algorithm) was used, minimizing the sum of the squares of the differences between the predicted value given by the model and the corresponding experimental value. The estimated kinetic and biological parameters obtained are shown below:

Pure glycerol: $\mu_{max} = 0.08\ h^{-1}$, $B = 8\ g/g$;

$Y_{X/S} = 0.02\ g/g$; $Y_{P/S} = 0.57\ g/g$

The results from Contois model adjustment are in accordance with previously published data for the cultivation of *Clostridium butyricum* in both pure and raw glycerol. The estimated parameter μ_{max} took a value of 0.08 h^{-1}. This value is considerably lower than those found in the literature (Chatzifragkou et al., 2011). However, this low value is in accordance with the low biomass growth observed in the experimental runs. The yield coefficient Y_{PX} took a value of 29. The value of the B parameter was 8 g/g. The B parameter value depends on the cell density and the initial amount of substrate (Papanokolaou and Aggelis 2003).

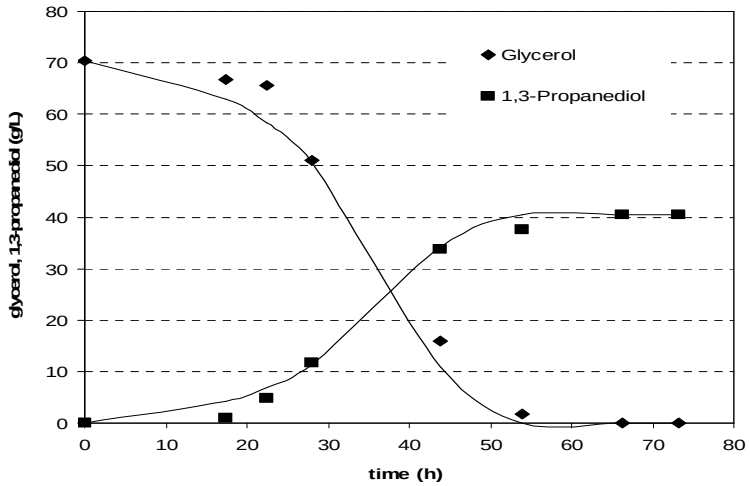

Fig. 4 Experimental (solid symbols) and estimated data (lines) for 1,3-propanediol production from 70 g/L of pure glycerol. Operating conditions: 37 ºC and pH 6.5.

Fig. 4 compared the experimental data and the calculated curves for pure glycerol and 1,3 PD concentrations corresponding to batch experiments at 37 ºC, pH 6.5 and an initial substrate concentration of 70 g/L. As can be seen, there is a good agreement between the experimental and predicted values.

4. Fed-batch operation

The previous batch experiments indicated that, with an initial high substrate concentration, the conversion became slow and glycerol remained in the culture at the end of the fermentation, which would make the downstream separation process more difficult, as the boiling points and chemical properties of 1,3-PD and glycerol would be close to each other. Therefore, it was important to keep the glycerol concentration within a proper range to eliminate the inhibition of the substrate. A fed-batch culture was consequently proposed, in which glycerol and yeast extract were fed to keep the glycerol in a comparatively low concentration during the fermentation. Different feed strategies were tested to evaluate the best feed rate.

In all the experiments, the fed-batch cultivation was initiated as a batch with an initial glycerol concentration of 70g/L in the bioreactor. At 24h, when the culture was growing exponentially and the unconverted substrate glycerol concentration had fallen below 50g/L, fresh sterilized glycerol feeding (99%(w/w) pure glycerol and 1%(w/w) yeast extract) was initiated. The feeding stopped at 90h of fermentation time. Two different feeding strategies were tested, as reported in Table 4.

Table 4. Experimental protocol of the different strategies of fed-batch operation.

Strategies	S_0 (g/L)	$V_{initial}$ (L)	Feed rate (g/h)	Cyclic feeding Δt (h)
1st	70	1	6	10
		1	3	10
		1	1.4	10
2nd	70	1	6	continuously
		1	1.4	continuously
		1	1.4	continuously

Results of 1st strategy

In the first strategy, feeding was supplied in cycles of 10 h. The feeding was continuously added from 24 h (growing batchwise step) to 34 h, after which the batch cultivation was resumed again for the next 10 h in order to allow the use of unconverted glycerol. At 44 h, additional glycerol was added when the glycerol in the vessel was nearly exhausted.

This strategy provided low concentrations of 1,3-PD independently of the feed rate since, in the second fed step (44-54 h), the production of 1,3-PD could not be resumed as the cells were kept in low glycerol concentration for too long and could not adapt to the relatively high glycerol concentration.

Ahmed et al. (2012) evaluated different fed-batch feeding strategies by varying the initial concentration of glycerol in the fermenter from 50 to 100 g/L, the feed rate of glycerol from 2.5 to 10 g/L, and the cycle feeding time from 12h to 17h. As in the present study, a high feed flow involved a glycerol accumulation in the culture medium.

Results 2nd strategy

In the 2nd strategy tested, the feed medium was continuously added from 24h to 90h of fermentation time. Three feed rates were tested, 6 g/h, 3 g/h and 1.4 g/h of glycerol. For the fermentations with the highest concentrations of substrate (6 and 3 g/L of glycerol), a low production of 1,3-PD was observed. This was due to the fact that the addition rate of glycerol was faster than its consumption rate, which meant that the glycerol concentration went above the critical concentration of 100 g/L. Furthermore, the addition of glycerol could not keep up with its consumption. The strategy of continuously feeding 1.4 g/h of glycerol provided the best results and was assumed as the optimal feed.

Fermentation results for the 1.4 g/h feeding strategy are shown in Fig. 6. The maximum 1,3-propanediol production was 37.3 g/L, with the corresponding 1,3-propanediol production yield where 0.53 g of 1,3-propanediol were formed per g of glycerol consumed. The 1,3-propanediol molar yields obtained in the previous batch fermentations were higher than those obtained in the fed-batch fermentations. In addition, the butyric acid concentration was also higher in fed-batch system (8.3 g/L for fed-batch vs. 6 g/L for batch cultures), whereas the acetic acid concentration was similar).

These results are consistent with those previously reported by Chen et al. (2003) and Mu et al. (2006), where higher product yields were also obtained in batch fermentations.

Mu et al. (2006) reached 0.62 mol/mol (0.51 g/g) in pure glycerol batch cultures of *Klebsiella pneumoniae* while in fed-batch systems, the 1.3-PD molar yield was only 0.49 mol/mol. In accordance with what has been reported, the total amount of by-

product concentration was 24.6 g/L. In the present study, the total amount of by-products in the fed-batch system was only 10.7 g/L and a higher 1,3-PD molar yield was obtained (0.63 mol/mol).

Chen et al. (2003) also achieved a higher molar yield in *Klebsiella pneumoniae* batch cultures (0.56 mol/mol) than in fed-batch systems of the same species, where 0.54 mol/mol was obtained. Furthermore, the author reported a productivity of 0.80 g/(L.h) in anaerobic conditions, a value very close to that reached in the present work (0.88 g/(L.h) for 42 h of fermentation time).

Despite the lower concentration of 1.3-PD in the fed-batch system, the total amount of 1,3-propanediol formed was 49.3 g, higher than the total amount reached in batch fermentations (40.5 g). Nevertheless, these results will be useful as control experiments in fed-batch fermentations using crude glycerol, where the fed-batch strategy could minimize the effect of the inhibitors present in the substrate.

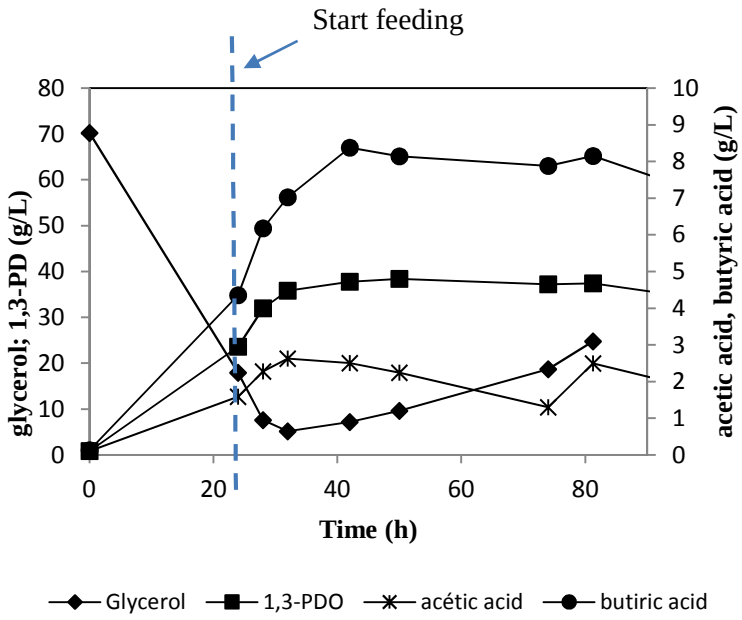

Fig. 6. Fed-Batch fermentation of pure glycerol *Clostridium butyricum* DSM 10702. Glycerol feeding rate: 1.4 g/h.

5. Continuous fermentations with pure glycerol

Notwithstanding the high production of 1,3-PD achieved in batch and fed-batch experiments, it suffers from certain drawbacks, such as inhibition by both substrate and product and the simultaneous formation of by-products, which need to be overcome to make this pathway economically viable. To eliminate product and substrate inhibition during fermentation, the normal approach is the removal of the inhibitory products from the fermentation broth by either plug flow or continuous operation. Continuous cultures do not usually offer an advantage of very high product concentrations but, on the other hand, they do offer the distinct advantage of high productivity.

In this section, a continuous strategy was evaluated to produce 1,3-propanediol from pure glycerol by *C. butyricum*. In order to optimize the process, different dilution rates were tested for growth in the microorganism at dilution rates varying from 0.05 to 0.2 h^{-1}. The effect of glycerol feed concentration was also evaluated with different initial concentrations of glycerol in the feed (30 and 70 g/L) .

Table 5. Experimental design**:** range of feed concentration and dilution rate assayed.

S_{Feed} (g/L)	D (h^{-1})
30	0.05
	0.10
	0.20
70	0.05
	0.10
	0.20

Fermentations with S_0=30g/L

Figure 7 shows the kinetics of biomass, 1,3-propanediol, acetic and butyric acid production, as well as glycerol consumption, from the growing batch stage (first 24 hours) until 600 h of continuous fermentation with different dilution rates.

For experiments fed with 30 g/L of substrate, the 1,3-propanediol concentration was the major fermentation product, reaching 13.7 g/L, and acetic and butyric acids were the main by-products. Acetic acid concentration was constant through 550 h of fermentation, although the dilution rate increased from 0.05 to 0.2 h^{-1}. On the other hand, butyric acid concentration was unstable in the first 200 h, varying from 1.5 to 2.5 g/L. Once the steady state had been reached, the butyric acid concentration remained around 3 g/L until the dilution rate changed from 0.1 to 0.2 h^{-1}. From this point on, the butyric acid concentration decreased until a new steady state was achieved at 540 h, reaching 1.3 g/L. Notwithstanding the variation of the butyric acid concentration, no significant change was observed in the butyrate/acetate ratio, indicating that the increase in the dilution rate, from 0.05 to 0.2 h^{-1}, did not perturb the carbon flux through the butyrate biosynthesis. Furthermore, the butyrate concentration was always substantially higher than the acetate concentration.

The biomass concentration was constant until D=0.2 h^{-1} was set. After that, the biomass concentration decreased severely to 0.7 g/L.

Residual glycerol was below 8 g/L until the dilution rate increased to 0.2 h^{-1}.

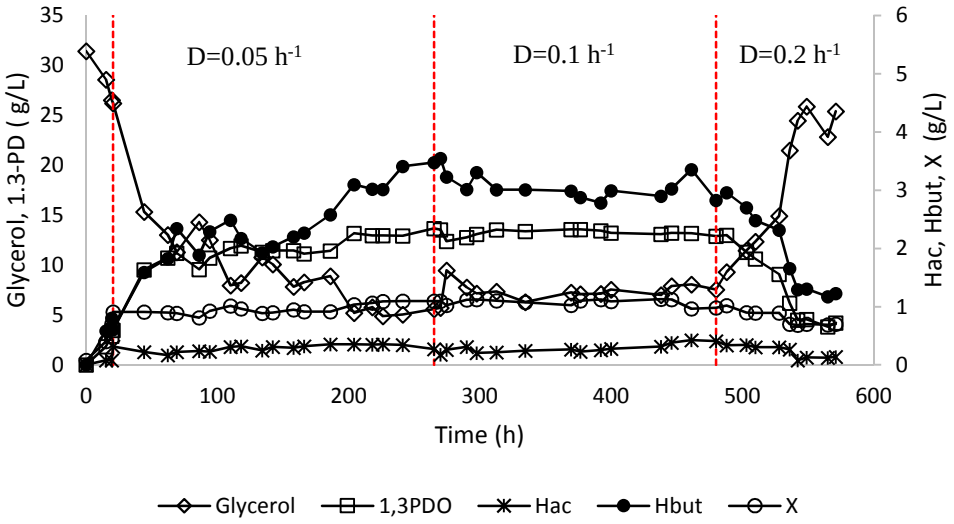

Fig. 7 Biomass (X, g/L), 1,3-propanediol (1,3 PD, g/L), acetic acid (Ac, g/L) and butyric acid (But, g/L) production and glycerol (S, g/L) consumption by *Clostridium butyricum* during growth in continuous bioreactor experiment at different dilution rates (D=0.05; 0.1 and 0.2 h^{-1}) and S_0=30g/L.

1,3-propanediol concentration was approximately constant up to a dilution rate of 0.10 h^{-1}, leading to an increase in the volumetric productivity for D=0.1 h^{-1} (Fig 8), with a maximum value of 1.34 g/L.h. For D values under 0.1 h^{-1}, conversion of glycerol into 1,3-propanediol was around 0.52 g (0.63 mol/mol) 1,3-propanediol/g glycerol consumed, and slightly higher for D=0.1 h^{-1}, indicating that the carbon flux through the 1,3-propanediol pathway is strongly regulated.

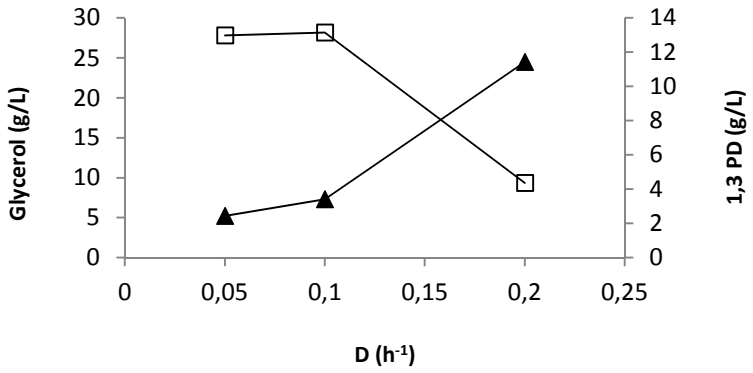

Fig. 8 Effect of dilution rate on substrate consumption (triangle) and 1,3-propanediol (open square) production during continuous fermentations of *C. butyricum* using 30g/L of substrate.

Fermentations with S_0=70g/L

Figure 9 shows the effect of the increase in the glycerol feed concentration in continuous fermentation, at different dilution rates. Time 0 hours corresponds to the change of the feed supply from 30 g/L to 70 g/L. As in the continuous fermentations fed with 30g/L of glycerol, the dilution rate was first set to 0.05 h^{-1} and, once the steady state had been reached, was then increased. At D=0.05 h^{-1}, the butyric acid concentration was around 3.9 g/L, slightly higher than the concentration reached when 30g/L of glycerol was fed (approx. 3.5 g/L). The butyric acid concentration sharply decreased when the dilution rate was increased to 0.1 h^{-1}. The acetic acid concentration followed the same behavior, dropping from 1g/L at D=0.05 h^{-1} to 0.38 at D=0.1 h^{-1}. Once again, no change in the butyrate/acetate ratio was observed.

The biomass concentration was also affected by the glycerol feed concentration, since, when substrate concentration was changed from 30 to 70g/L, an increase in the biomass concentration was observed for the lower dilution rate. For both glycerol feed concentrations, a decrease in the biomass concentration was associated with an increase in the residual glycerol in the fermentation broth.

At a high dilution rate, residual glycerol had reached 50 g/L and severe decreases in the biomass and 1,3-propanediol were observed, pointing to a washout of the reactor at dilution rates higher than 0.2 h^{-1}, Fig. 9. González-Pajuelo et al (2004) also observed the washout of the reactor in the same conditions (70 g/L of glycerol fed at a dilution rate of 0.2 h^{-1}) using *C. butyricum* VPI 3266.

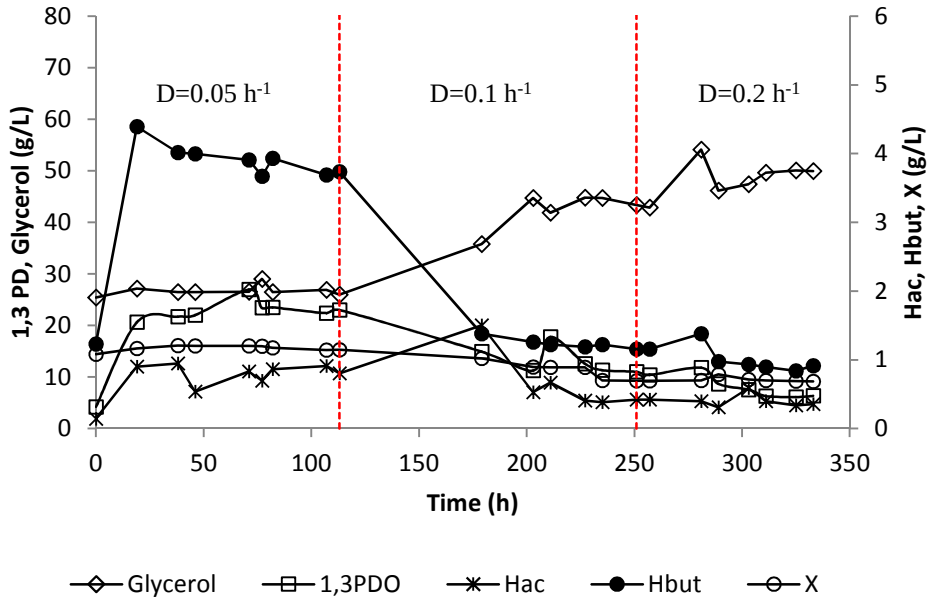

Fig. 9 Biomass (X, g/L), 1,3-propanediol (1,3 PD, g/L), acetic acid (Ac, g/L) and butyric acid (But, g/L) production and glycerol (S, g/L) consumption in continuous bioreactor experiment at a different dilution rates (D=0.05, 0.1, 0.2 h^{-1}) and 70g/L of glycerol in the feed medium.

Figure 10 shows the effect of the dilution rate on glycerol consumption and 1,3-propanediol synthesis during continuous fermentations fed with 70 g/L of substrate. For lower dilution rates, an increase in glycerol feed concentration led to an increase in 1,3-propanediol concentration, which reached 23.05 g/L. 1,3-propanediol yield was also affected by increasing the glycerol feed concentration and 1,3-PD yield was higher for lower values of dilution rate.

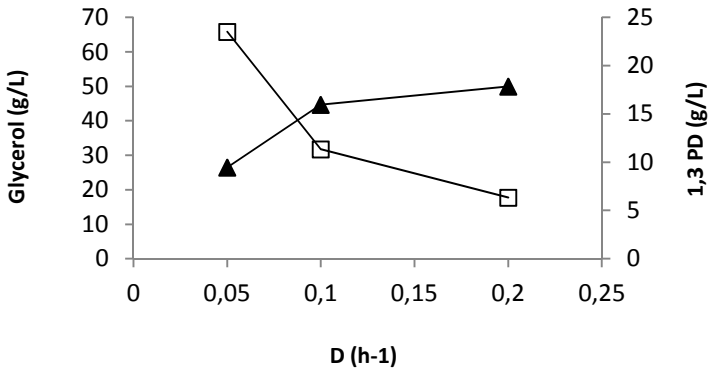

Fig.10 Effect of dilution rate on glycerol (triangle) consumption and 1,3-propanediol (open square) production during continuous fermentations using 70g/L of pure glycerol.

Table 5 summarizes the steady-state results (concentrations of substrate and product and carbon recovery) for continuous fermentation carried out under different dilution rates and initial substrate concentration. The highest concentration of 1.3-PD was obtained for 70 g/L of feed glycerol at D=0,05 h^{-1}. In these conditions, a 1.3-propanediol molar yield of 0.64 mol/mol was obtained, a value very close to the theoretical maximum yield of 0.70 mol/mol reported by Zeng et al. (1996). For a feed concentration of 30 g/L, the 1,3-propanediol yield was not affected by increasing the dilution rate from 0.05 h^{-1} to 0.1 h^{-1} and 1,3-PD molar yield was also around 0.64 mol/mol, meaning that the 1,3-PD yield was not affected by the increment in the glycerol concentration. Additionally, the highest productivity was obtained for D=0.10 h^{-1} and 30 g/L of substrate. For 70 g/L of feed glycerol concentration at D=0.05 h^{-1}, the productivity was similar though slightly lower, but a high 1,3-PD concentration was obtained.

The carbon recovery was calculated to verify the reliability of the obtained results. For 30 g/L of fed glycerol and dilution rate 0.05-0.1 h^{-1}, the carbon balance is approximately 96 %, which leads to the conclusion that the uncertainty on measures is acceptable. In the case of D=0.2 h^{-1}, the carbon recovery was higher than 100 %. Other authors (Abbad-Andaloussi et al. 1996) also reported carbon recovery higher than 100 %. Besides theoretical assumptions in R_c calculations (biomass formula, CO_2 and H_2 production), the origin of the errors introduced in this case may be related to the low production of acetic acid that, for D=0.2 h^{-1}, was outside the calibration range.

At fermentations fed with 70 g/L of glycerol, the percentage of carbon recovery was around 88 %, in accordance with values reported and validated in the bibliography (Papanikolaou et al. 2008).

Table 5 Steady-state growth parameters, carbon recovery during an anaerobic continuous fermentation of pure glycerol at different dilution rates.

Fed	D (h^{-1})	S_0 (g/L)	S_f (g/L)	X (g/L)	1,3 PD (g/L)	HBut (g/L)	HAc (g/L)	Rc (w/w)	Q_P (g/(L·h)
30g/L	**0.05**	31.4	5.2	0.9	13.7	3.5	0.27	95.6	0.7
	0.10	31.4	6.3	1.1	13.4	3.0	0.24	95.6	1.3
	0.20	31.4	25.4	0.7	4.2	1.2	0.14	108.4	0.8
70g/L	**0.05**	71.3	26.5	1.2	23.5	3.9	0.86	91.2	1.2
	0.10	71.3	44.7	0.7	11.3	1.2	0.38	86.9	1.1
	0.20	71.3	49.9	0.7	6.4	0.9	0.35	85.2	1.3

González-Pajuelo et al (2004) study the effects of the dilution rate from 0.05 to 0.3 h^{-1} and substrate feed from 60 to 70 g/L on continuous cultures by *C. butyricum* VPI 3266. González-Pajuelo et al (2004) obtained similar molar yields (0.65 mol/mol), irrespective of glycerol feed concentration or dilution rate. This study also reported a switch in the acetate/butyrate rate with a dilution rate higher than 0.10 h^{-1}. In the present study, as mentioned before, no change in metabolic flux was observed in acetic and butyric acids, in accordance with the report of Abbad-Andaloussi et al. (1996), where the switch in the acetate/butyrate ratio was only at dilution rate values higher than 0.3 h^{-1}.

6. Conclusions

The conversion of glycerol into another commodity is essential to increase and maintain the economic viability of biodiesel production using vegetable oils, and, in this sense, the fermentation by *Clostridium butyricum* to 1,3-propanediol is an attractive process.

Control experiments were carried out in batch, fed-batch and continuous systems, using pure glycerol (99% purity, Sigma) as substrate, to assess the possible effect of raw glycerol impurities. In all these systems, *C. butyricum* was able to produce considerable amounts of 1,3-propanediol from pure glycerol. In all the fermentations, 1,3-propanediol was the main product and acetic and butyric acids were by-products of the reactions.

High 1,3-propanediol concentration and yield factor were obtained in batch fermentations, higher than values reported in the literature under similar experimental conditions, with a low biomass growth (2.76 g/mol) and relatively low values of by-product formation. The greatest concentration of 1,3-propanediol (40.0 g/L), and the highest yield factor ($Y_{PD / S}$= 0.57 g/g), were obtained at 37 °C, pH = 6.5 and 70 g/L glycerol. Moreover, the maximum tolerance of *C. butyricum* to the inhibitory effect of the substrate was 100g/L. The kinetic Contois type-model successfully simulated the batch fermentation of glycerol by *Clostridium butyricum* DSM 10702.

In fed-batch fermentations, 37.3 g/L of 1,3-propanediol were formed with a corresponding production yield of 0.53 g of 1,3-propanediol per g of glycerol consumed. A total amount of 49 g of 1,3-propanediol was attained. Acetic and butyric acid concentrations, respectively, reached 2.5 g/L and 8.3 g/L.

In continuous cultures, the maximum 1,3-propanediol obtained was 23.51 g/L at low dilution rate (D=0,05 h^{-1}) and 70 g/L of glycerol in the fed stream. Lower values of by-products were produced: 3.93 g/L of butyric acid and 0.86 g/L of acetic acid. The continuous strategy does not provide, as expected, the highest amounts of 1,3-propanediol. However, the lower production of by-products represents an advantage of the continuous strategy in comparison with the batch and fed-batch systems. Furthermore, high productivity was obtained in continuous cultures.

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CHAPTER 5. ASSAYS WITH CRUDE GLYCEROL

1. Introduction

Crude glycerol, also known as raw glycerol or glycerin, is a co-product of biodiesel production. It is a viscous, yellow/brown liquid hard to purify to a pharmaceutical grade and therefore of low economic value (Yazdani et al., 2010).

The characteristics of crude glycerol vary depending on the feedstock used in the biodiesel production process and on the process itself. Crude glycerol as feedstock for possible value-added compound synthesis depends on its physical, chemical, and nutritional properties. Thus, it is essential to characterize crude glycerol.

Most industrial biodiesel processes use a 6-to-1 molar ratio of alcohol to oil (Thompson and He, 2005) in order to drive the reaction to completion. Up to 80% of the excess of alcohol will end up in the glycerol layer after the reaction. Subsequently, the alcohol will be recovered by the producers for reuse. Further refining of the crude glycerol will depend on the economy of production scale and/or the availability of a glycerol purification facility.

In this chapter, the microbial production of 1,3-propanediol by *C. butyricum* DSM 10702 was studied in batch, fed-batch and continuous systems using crude glycerol from biodiesel manufacturing. Crude glycerol often contains sodium salts and heavy metal ions which may interfere with cell division and reduce the capability of *C. Butyricum* (Gonzalez-Pajuelo et al. 2004). Growth inhibition experiments by substrate using crude glycerol were carried out and the data compared with pure glycerol numbers.

2. Characterization of "crude glycerol"

In general, glycerol is about 62% to 85% (w/w) of the crude stream (Thompson and He, 2006; Mu et al., 2006). The balance is water, methanol (usually used in Europe) or ethanol (conventionally used in America), fatty acids (soaps) and catalyst residues (salts). Crude glycerol also contains a variety of elements such as calcium, magnesium, phosphorous, or sulphur (Thompson and He 2005).

The crude glycerol used in this work was kindly supplied by the company ACOR, (Spain). The characterization below was carried out in our laboratory and summarized in Table 1.

Table 1. Characterization of biodiesel crude glycerol in %wt.

Sample	Glycerol	Salts	Ash[a]	MONG[b]
Crude glycerol	88.45	5.23	5.28	2.18

[a] Salts included in ash
[b] MONG = Organic non-glycerol matter

3. Methodology

The organism, medium fermentation and reactor specifications were the same as those used in batch fermentations using pure glycerol as substrate, shown in chapter 4.

4. Batch fermentations with crude glycerol

Once the best operating conditions were established for pure glycerol solutions, batch fermentations were performed at 37ºC and pH 6.5 using crude glycerol (70g/L) as substrate and the sole carbon source. The results shown in Fig. 1 indicate that the glycerol was completely consumed and that the biomass reached a value of about 1.2

g/L after 60 h fermentation. The concentration of 1,3-propanediol reached a maximum of 35.9 g/L, slightly lower than the concentration reached using pure glycerol (40.0 g/L), probably due to the presence of impurities in the raw glycerol (salts and/or non-glycerol organic matter) that could affect the 1,3-propanediol production. The yield factor was 0.51 g 1,3-PD/g glycerol, also lower than that reached in the fermentation of pure glycerol (0.57 g 1,3 PD/g glycerol). However, the 1,3-PD yield was quite similar to that found in the literature when fermenting 90 g/L of crude glycerol at 33ºC and pH 7.0 by *C. butyricum* F2b (Papanikolaou et al., 2008), and higher than the yield found by Gonzalez-Pajuelo et al. (2004) in batch fermentations of *C. butyricum* VPI 3266 with initial concentration between 33.5-44.1 g/L at 35 ºC without pH regulation.

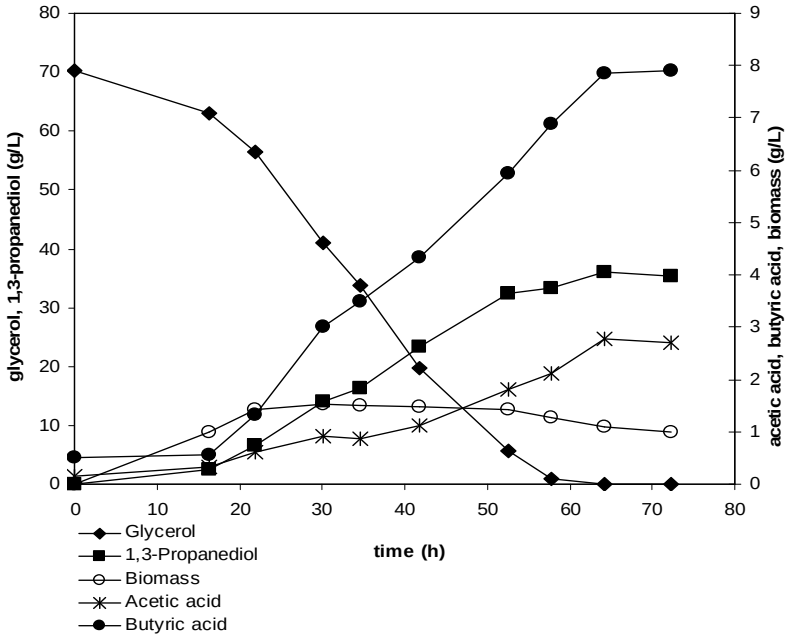

Fig. 1 Batch Fermentation profiles for crude glycerol at the optimal operating conditions (37 ºC, pH 6.5 and 70 g/L of glycerol)

The total amount of by-products produced in crude glycerol fermentation by *C. butyricum* DSM 10702 was 10.6 g/L. The main by-product was butyric acid (7.9 g/L) followed by acetic acid (2.7 g/L). In fermentations, under the same conditions, using pure glycerol as substrate (chapter 4), the total amount of by-products produced was

slightly lower (9 g/L). Butyric acid concentration was 6.0g/L for pure glycerol fermentation, a value lower than that reached with crude glycerol. Acetic acid was quite similar in both cases. The yield factors were 0.03 g HAc/g crude glycerol and 0.11 g HBut/g crude glycerol.

Tolerance of C. butyricum to initial substrate concentration

As described in chapter 3, the concentration of 1,3-PD increased with the initial substrate concentration up to 100g/L of pure glycerol. In order to determine the maximum initial crude glycerol concentration that the microorganism can tolerate while still maintaining high 1,3-PD concentration in the culture medium, a set of experimental runs were performed varying the concentration of raw glycerol from 50 g/L to 150 g/L at pH 6.5 and 37ºC.

Fig. 2 shows the results corresponding to the evolution of 1,3 PD concentration and yield factor.

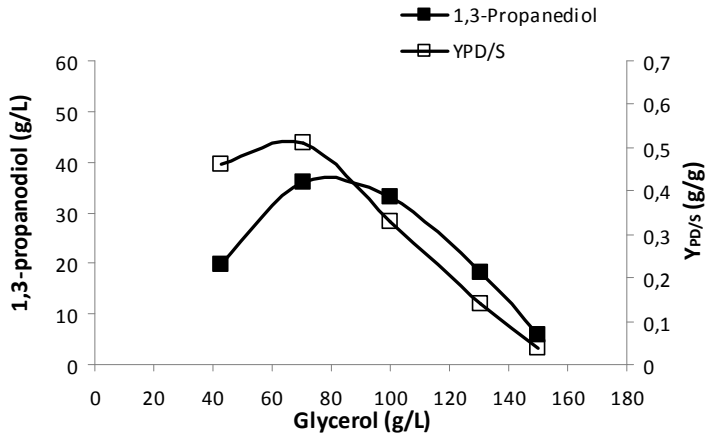

Fig.2 Effect of glycerol concentration on 1,3-propanediol concentration and yield factor ($Y_{PD/S}$). for crude glycerol.

Regarding the fermentation of crude glycerol, the maximum production of 1,3-propanediol corresponded to an initial glycerol concentration of 70 g/L and it was

almost zero when 150 g/L of crude glycerol was used. The maximum yield factor was 0.51 g 1,3 PD/g crude glycerol (70 g/L initial glycerol concentration), lower than that obtained with pure glycerol solutions (0.60 g 1,3 PD/g pure glycerol for 100 g/L of fermented glycerol), probably due to the presence of impurities in the crude glycerol.

Regarding organic acids, the concentration increased as the substrate concentration increased, reaching a maximum that corresponded to the maximum concentration of glycerol tolerated by the microorganism. Thereafter, the acid concentrations decreased. The maximum concentrations of acetic and butyric acids with crude glycerol (70 g/L) were: 2.7 g/L and 7.9 g/L, respectively; whereas, working with pure glycerol (100 g/L), concentrations were slightly higher: 4.5 g/L and 9.7 g/L, respectively. Therefore, it may be considered that the industrial crude glycerol (up to 70 g/L) is an adequate substrate for 1,3-propanediol production using the *C. butyricum* DSM 10702 strain, and could be used without further purification.

Papanikolaou et al. (2008) studied the production of 1,3-PD in batch fermentation by *C. butyricum* F_2b, using crude glycerol from a fatty acid methyl ester production unit as the sole carbon source. The initial substrate concentrations studied were 39 and 90 g/L. According to the report, the mass yield value for 1,3-PD production was similar for both initial concentrations (0.53 g/g) and comparable to the yield found in the present study for 70 g/L (0.51 g/g). The high tolerance of initial substrate concentration may be explained by the different compositions of the crude glycerol, which depends on the biodiesel fabrication process.

Concerning the mass yield of by-products, it was lower in the fermentations carried out by Papanikolaou and co-authors (2008) for butyric acid, and similar for the acetic acid mass yield. Nevertheless, the biomass production was considerably higher on the mentioned report. In general, the mass yield of 1.3-PD is around 0.45-0.54 g/g, acetic acid between 0.03-0.10 g/g, and butyric acid around 0.10-0.20 g/g (Gonzalez-Pajuelo et al. 2004, Papanikolaou et al., 2000, 2003, 2008, Saxena et al. 2009, Zeng and Biebl 2002, Zeng 1996). The high initial crude glycerol concentration reported without inhibition was 90g/L for *C. butyricum* F_2b fermentation (Papanikolaou et al., 2008).

Kinetic Mode

As in chapter 3, the Contois model was chosen. The equations (3), (4), (5) and (6) from chapter 3 section "*Kinetic Model*" were applied. In the same way, equations (3) to (6) were solved numerically in order to estimate the kinetic parameters and the yield factors. The simulation software EcosimPro© (that incorporates the NPL non-linear algorithm) was used, minimizing the sum of the squares of the differences between the predicted value given by the model and the corresponding experimental value.

The estimated kinetic and biological parameters obtained are:

Crude glycerol: $\mu_{max} = 0.07\ h^{-1}$, B = 9 g/g;
$Y_{X/S} = 0.01$ g/g; $Y_{P/S} = 0.51$ g/g

*Pure glycerol: $\mu_{max} = 0.08\ h^{-1}$, B = 8 g/g ;
$Y_{X/S} = 0.02$ g/g; $Y_{P/S} = 0.57$ g/g *(from chapter 4)

The results from the Contois model adjustment are in accordance with previously published data for the cultivation of *Clostridium butyricum* for both pure and crude glycerol. The estimated parameter μ_{max} took values of 0.08 and 0.07 for pure and crude glycerol, respectively. The maximum specific estimated growth rate was similar for both substrates, although slightly higher for pure glycerol, indicating a low inhibition of the microorganism by crude glycerol. The value of the B parameter ranges from 8 and 9 for pure and raw glycerol, respectively, and depends on cell density and the initial amount of substrate. The B parameter value also indicated some inhibition of *C. butyricum* by crude glycerol.

Figure 3 shows the experimental data and the calculated curves and 1,3-PD concentrations corresponding to batch experiments at 37ºC, pH 6.5 and an initial substrate concentration of 70 g/L crude glycerol.

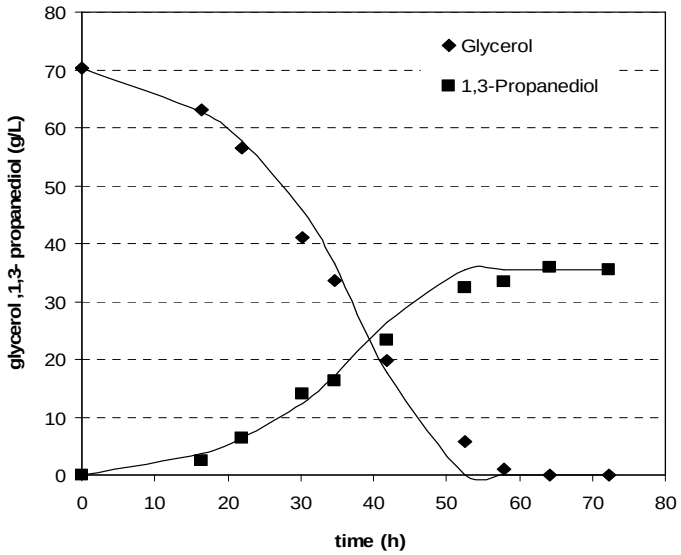

Fig. 3 Experimental (solid symbols) and estimated data (lines) for 1.3-propanediol production from 70 g/L of crude glycerol. Operating conditions: 37 ºC and pH 6.5.

There is good agreement between the experimental and predicted values. The kinetic Contois type-model successfully simulates the batch fermentation of glycerol by *Clostridium butyricum* DSM 10702.

In conclusion, the fermentation of crude glycerol without further purification produces significant amounts of 1.3-propanediol (35.9 g 1.3PD, $Y_{PD/S}$= 0. 0.51gg^{-1}) at 37°C, pH = 6.5 and 70 g/L glycerol. The maximum tolerance of *C. butyricum* DSM 10702 to the inhibitory effect of the substrate was 70 g/L.

The results were found to be very satisfactory, when compared with bibliographic reports (Gonzalez-Pajuelo et al. (2004), Moon et al. (2010), Papanikolaou et al. (2008)).

Some *Clostridium* strains were reported to be strongly inhibited by the use of untreated crude glycerol (Gonzalez-Pajuelo et al., (2004), Petitdemange et al., (1995).

Furthermore, *C. butyricum* DSM 5431 was reported not to grow at all in the presence of crude glycerol (Asad-ur-Rehman et al., 2008, Petitdemange et al., 1995). Petitdemange et al., (1995) also showed that, in ten isolated strains of *C. Butyricum*, only four were able to grow on crude glycerol.

Moon et al. (2010) studied the effect of crude glycerol types on different microorganisms. The *Clostridium* strains tested were *C. butyricum* DSM 2477, *C. butyricum* DSM 2478 and *C. butyricum* DSM 15410. For batch fermentations, using untreated crude glycerol (80 wt. % glycerol) as substrate, *C. butyricum* DSM 2477 only produced 2.9 g/L of 1.3-PD (0.26 g 1,3-PD/g crude glycerol). *C. butyricum* DSM 2478 had the best performance, reaching 6 g/L of 1.3-PD (0.35 g 1,3-PD/g crude glycerol) while *C. butyricum* DSM 15410 did not grow and 1,3-PD was not produced at all. Comparing the performance of *C. butyricum* DSM 10703 with the results of the abovementioned reviews, it can be stated that *C. butyricum* DSM 10703 is one of the best 1,3-PD producers from crude glycerol without pretreatment.

5. Fed-batch fermentations with crude glycerol

With the aim of reducing the inhibitory effect of high substrate concentrations and increasing the amount of 1,3-propanediol in the broth, a fed-batch supply of crude glycerol was evaluated. The strategy followed was chosen in chapter 4 in section 3.2. “Fed-batch fermentations with pure glycerol”. The initial glycerol concentration was 62 g/L. Feed was started at the beginning of the exponential phase (24h fermentation time) and stopped at 100h of fermentation time. The feeding was carried out by adding continuously 1.4 g/h of feed medium, a strategy that provided the best results with pure glycerol in chapter 4.

The fermentation results are shown in Fig.1. The maximum 1,3-propanediol concentration was 36.1 g/L with the corresponding 1,3-propanediol production yield of 0.49 g of 1,3-propanediol formed per g of glycerol consumed.

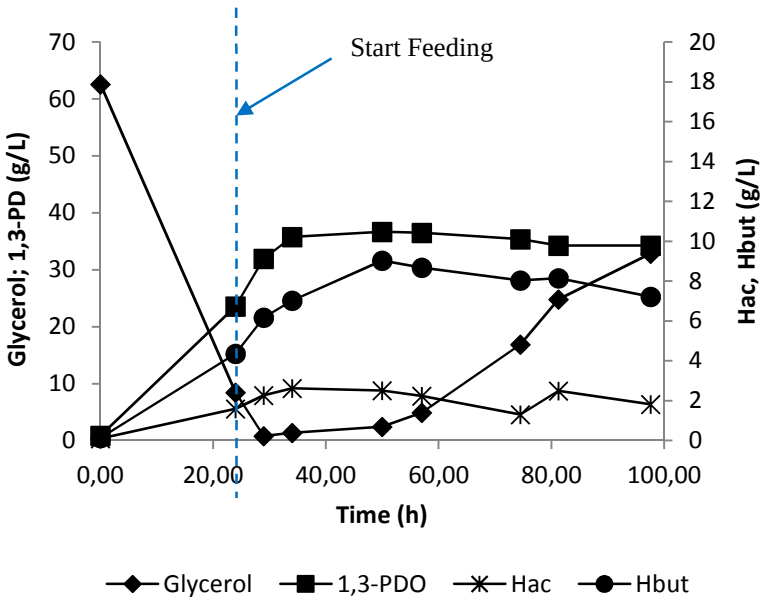

Fig. 4. Fed-Batch fermentation of crude glycerol from oil transesterification by *Clostridium butyricum* DSM 10702.

The total amount of 1,3-PD produced was 48,2 g, higher than that reached in batch fermentation (35.9 g) under the same conditions. The productivity of 1,3-PD in the first 40 hours was also higher in the fed-batch experiments.

In comparison with fed-batch fermentation using pure glycerol as substrate (chapter 4, section 3.2. "Fed-batch fermentations with pure glycerol"), the results were similar in terms of 1,3-PD concentration (37.3 and 36.1 g/L for pure and raw glycerol, respectively). However, in terms of by-products, the butyric acid concentration was higher for the fermentation using raw glycerol as substrate (9.0 g/L for raw glycerol vs. 8.3 g/L for pure glycerol). The substrate uptake rate ($r_s = -\Delta S/\Delta t$) estimated during the batch exponential growth phase was similar for both fermentations, although slightly higher for crude glycerol fermentation. The volumetric productivity was approximately 0.90 g/(L.h), comparable to that of 0.88 g/(L.h) on the fed-batch pure glycerol fermentation (Q_{PD} calculated for the first 40 h of fermentation time).

The results were similar to those obtained by Ahmed et al. (2012) in fed-batch fermentation feeding by 12 h pulses of 4.5 g/h of glycerol over 50 h of fermentation time. Ahmed et al. (2012) reported reaching 35.36 g/L of 1,3-propanediol with a

corresponding production yield of 0.48 g of 1,3-PD/g of glycerol. The total amount of by-products formed was 14.28 g/L, a value slightly higher than the amount produced in the present work, 12 g/L (9.5 g/L of butyric acid and 2.5 g/L of acetic acid).

Mu et al. (2006) reached 53 g/L of 1,3-propanediol in fed-batch fermentation by *Klebsiella pneumoniae* using crude glycerol (85 % w/w). Despite the high 1,3-PD concentration, a high concentration of by-products was also produced (15,6 g/L), and the 1,3-PD molar yield obtained was low (0.47 mo/mol).

6. Continuous fermentation with crude glycerol

In this section, crude glycerol is used as substrate for *Clostridium butyricum* DSM 10702 continuous fermentation into 1,3-propanediol. Different dilution rates were tested in order to optimize the fermentation.

First set of experiments

Continuous cultures were conducted in the same conditions as the continuous culture model assays described in chapter 4: 500 mL glass bioreactor, stirred at 200 rpm, pH controlled at 6.5 by the automatic addition of 3 M KOH, and temperature regulated by a thermostatted water circulation system. The reaction volume was 500 mL, containing 450mL of growth medium inoculated with 50mL of preculture.

To create anaerobic conditions, the sterilized synthetic medium in the vessel was flushed with sterile O_2-free nitrogen until room temperature was attained. The culture was first grown in batch and continuous feeding was started after 24h of fermentation time, when the exponential growth phase was reached. During the experiments, the feed medium was maintained under N_2 at 20 mbar to avoid O_2 entering. The bioreactor gas outlet was protected with a pyrogallol arrangement (Vasconcelos et al. 1994). The steady state was obtained after continuous flow of at least four working volumes of the medium through the vessel. As in model assay fermentations with pure glycerol

(chapter 3), three different dilution rates were tested: 0.05, 0.1 and 0.2h^{-1}, at three different glycerol concentrations in the feed (30, 50 and 70g/L).

Table 2. Experimental design**:** range of feed concentration and dilution rate assayed for raw glycerol experiments.

Feed (g/L)	**D (h^{-1})**
30	0.05
	0.10
	0.20
70	0.05
	0.10
	0.20

Second set of experiments

Continuous cultures were carried out in a 2L bioreactor (biostat Bplus, Sartorious) equipped with temperature, pH and level sensors and four peristaltic pumps. The pH was controlled at 6.5 by the automatic addition of 3 M KOH, and the temperature was regulated at 37ºC by a thermostatted water circulation system (fig.5). Fermentations were carried out with a working volume of 1500mL, containing 1350mL of growth medium inoculated with 150mL of preculture. To create anaerobic conditions, the bioreactor containing the sterilized synthetic medium was flushed with sterile O_2-free nitrogen until room temperature was attained. The culture was first grown batchwise and continuous feeding was started when the exponential growth phase was reached. Once again, the feed medium was maintained under N_2 flow, at 20 mbar, to preserve anaerobic conditions. In this set of experiments, the bioreactor gas outlet was not protected to avoid the entry of air.

Table 3. Experimental design of the second set of experiments. Feed concentration and dilution rate assayed for raw glycerol experiments.

Feed (g/L)	D (h^{-1})
50	0.05
	0.75
	0.10
	0.15

Results

First set of experiments (from D=0.05 to 0.2 h^{-1}, $\Delta D=0.05h^{-1}$)

Fermentations with S_0=30g/L

As can be observed in Fig. 5, the carbon substrate was almost completely consumed at the low dilution rates (<0.1h^{-1}). A slight increase in the biomass concentration was shown when the dilution rate was raised from 0.05 h^{-1} to 0.1 h^{-1}. Product and by-products showed similar concentrations for the two different dilution rates, 0.05 and 0.1 h^{-1}.

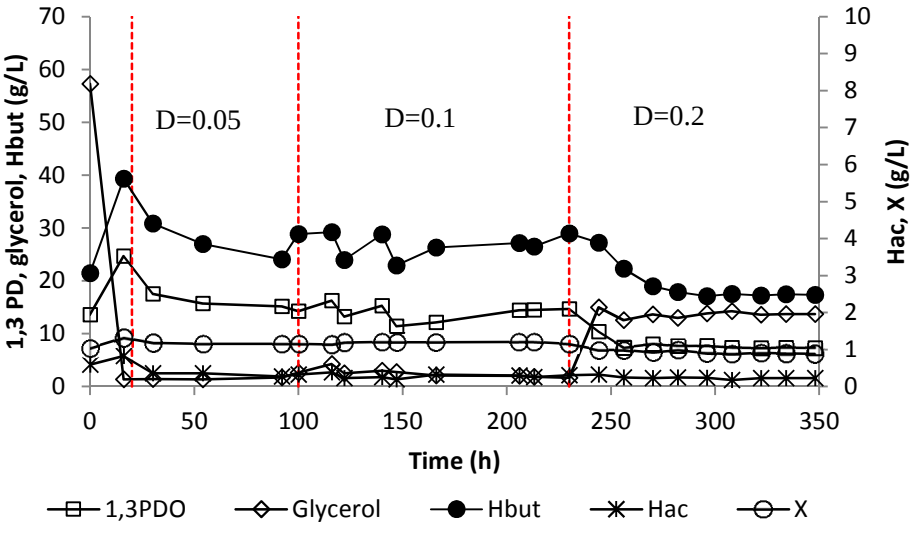

Fig. 5 Evolution of biomass (X, g/L), 1,3-propanediol (1,3 PD, g/L), acetic acid (Hac, g/L) and butyric acid (Hbut, g/L) production and glycerol (S, g/L) consumption by *Clostridium butyricum* during growth in a continuous bioreactor experiment at the different dilution rates and S_{feed}=30g/L.

The 1,3-propanediol concentration reached 15 g/L for dilution rates up to 0.1 h^{-1}. The by-products, acetic and butyric acids, presented concentration values of around 0.3 and 4 g/L, respectively. As in continuous fermentation using pure glycerol as substrate, the butyrate concentration was always substantially higher than that of acetate and no significant butyrate/acetate ratio was observed. Furthermore, the 1,3-propanediol concentration was slightly higher than that obtained with pure glycerol, and a high operation stability in terms of biomass, products and by-products was observed throughout the different steady state dilution rates.

The biomass concentration was constant through 230 h, corresponding to the change of dilution rate value from 0.1 to 0.2 h^{-1}. Residual glycerol was below 2 g/L for dilution rate values of 0.05-01 h^{-1} and around 14 g/L for D value 0.2 h^{-1}. Observing the low product and biomass concentrations and the high residual glycerol concentrations, this points to the washout of the reactor for dilution rates higher than 0.2 h^{-1}.

Figure 6 shows 1,3-propanediol and glycerol concentrations for the different dilution rates. The residual glycerol concentration was similar for the dilutions rates 0.05 and 0.1 h^{-1}. 1,3-propanediol production was very similar to the range of dilution rates mentioned before, with the extra advantage of high productivity. At D=0.2 h^{-1} an increase of residual glycerol was observed and, consequentially, a decrease in the 1,3-propanediol concentration.

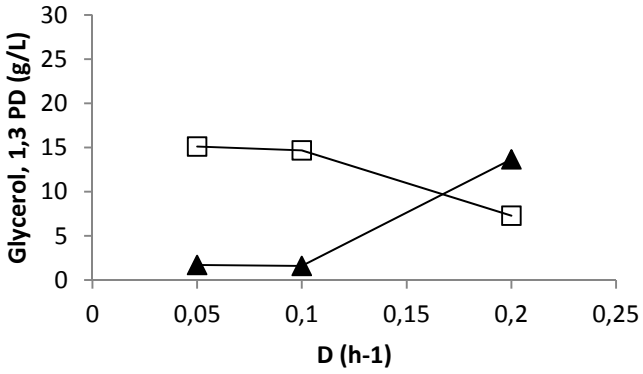

Fig.6 Effect of dilution rate on crude glycerol (triangle) consumption and 1,3-propanediol (open square) production during continuous fermentations of *C. butyricum* DSMZ 10702 with S0=30g/L in the feed medium.

Comparing with continuous pure glycerol experimentation (Chapter 4), the effect of crude glycerol was minimal and did not inhibit 1.3-PD production. In fact, the 1.3-PD was slightly higher when crude glycerol was used as substrate, independently of the dilution rate.

Fermentations with S_0=70g/L

As in continuous fermentations fed with 30g/L of crude glycerol, the biomass concentration was constant for the dilution rates 0.05 and 0.1 h^{-1} (Fig. 7). On the other hand, the residual carbon substrate was not completely consumed, remaining around 17g/L of crude glycerol in the effluent for D=0.05 h^{-1} and around 23 g/L for D=0.1 h^{-1}. The maximum 1.3-PD concentration achieved was 27.57 g/L for a dilution rate value of 0.05 h^{-1}.

As for by-product formation, butyric acid was the major by-product with a maximum concentration of 6 g/L for D=0.05 h^{-1}, which decreased the dilution rate increased. On the other hand, acetic acid formation increased up to D=0.1 h^{-1}, although the butyric acid concentration was always higher than the acetic acid concentration. The decrease in butyrate/acetate may be related to a change in the metabolic carbon flux reported by many authors in continuous fermentations with high glycerol feed concentrations at high dilution rates (Abbad-Andaloussi et al., 1996, Gonzalez-Pajuelo et al., 2005, Papanikolaou et al., 2000).

Although the batch fermentations showed a direct relationship between cell growth and acetic and butyric acid production, the continuous culture experiments showed that, at low and medium specific growth rates, butyrate production was preferred; whereas high dilution rates seemed to favor acetate production, a behavior already noticed by Papanikolaou (2000).

At D=0.2 h^{-1}, product, by-products and biomass concentrations sharply decreased, while residual glycerol concentration increased, up to a point close to reactor washout.

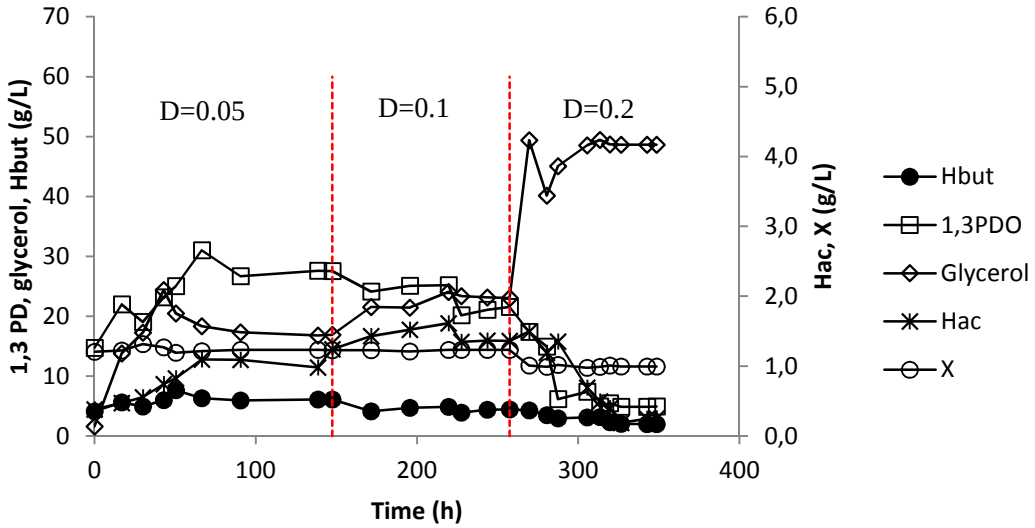

Fig. 7 Kinetics of biomass (X, g/L), 1,3-propanediol (1,3 PD, g/L), acetic acid (Ac, g/L) and butyric acid (But, g/L) production and glycerol (S, g/L) consumption of *Clostridium butyricum* during growth in continuous bioreactor. Culture conditions: different dilution rates (D=0.05; 0.1 and 0.2 h^{-1}), pH=6.5, T=37ºC and S_0=70g/L.

Fig. 8 shows the effect of the dilution rate on crude glycerol consumption and 1,3-propanediol production during continuous fermentations fed with 69g/L of crude glycerol. Residual glycerol increased as the dilution rate increased, while the 1,3-propanedial concentration diminish. Additionally, residual glycerol was considerably higher than the glycerol that remained in the effluent in experiments fed with 30 g/L of substrate.

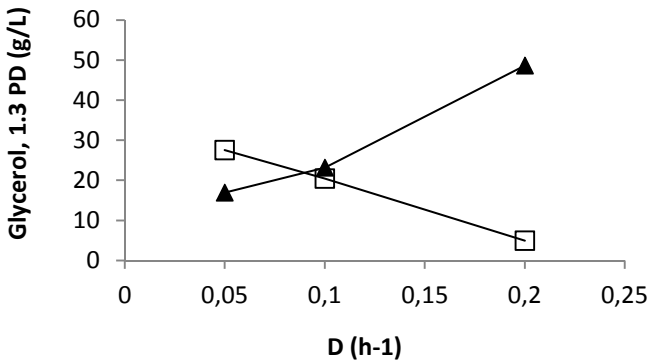

Fig. 8 Effect of dilution rate on crude glycerol consumption (triangle) and 1,3-propanediol production (open square) during continuous fermentations of *C. butyricum* DSMZ 10702 with S0=70g/L in the feed medium.

The 1,3-propanediol volumetric productivity of the six single-stage continuous cultures conducted is presented in Table 2. The maximum productivities achieved were 2.11 g/L/h for 70 g/L of glycerol in feed and D=0.05 h^{-1}. This productivity almost duplicates that obtained under the same conditions when pure glycerol was used (1.1 g/(L.h), chapter 4). Furthermore, the productivity achieved in experiments using crude glycerol as substrate was always higher than that obtained with pure glycerol. Several authors have reported that the presence of traces of methanol on crude glycerol from biodiesel manufacturing may produce a stimulating effect on microorganism cell growth, which could explain the high productivity achieved in crude glycerol fermentations.

The carbon recovery percentage was in a standard range, thus the uncertainty on measures is acceptable.

Table 2. Steady-state growth parameters, carbon recovery during an anaerobic continuous fermentation of raw glycerol at different dilution rates.

Fed concentration (g/L)	D (h^{-1})	S_f (g/L)	X (g/L)	1,3PD (g/L)	HBut (g/L)	HA (g/L)c	Rc (%)	Q_{PD} (g/L.h)
30	**0.05**	1.71	1.15	15.1	3.44	0.27	93.3	0.76
	0.10	1.62	1.22	14.7	4.13	0.30	96.6	1.47
	0.20	13.68	0.89	7.3	2.48	0.22	94.6	1.46
69	**0.05**	16.90	1.23	27.5	6.09	1.24	94.5	1.38
	0.10	23.13	1.23	21.1	4.40	1.37	87.8	2.11
	0.20	48.64	0.99	4.9	2.00	0.25	87.3	0.99

Second set of experiments

A set of experiments using an intermediate concentration of substrate, 50 g/L, was carried out in order to optimize the continuous process and improve the production of 1,3-propanediol. An analysis about the influence of the dilution rate on the production of 1,3-PD, fed with 50 g/L of raw glycerol, was performed. Regarding the

preceding results, a new range of dilution rate values was established, between 0.05 and 1.5 h^{-1}. Results at the steady state are collected in Table 3.

Table 3. Steady state parameters and yields for biomass (X), 1,3-PD, Butyrate and Acetate.

D (h^{-1})	S_0 (g/L)	S_f (g/L)	1,3-PD (g/L)	$Y_{X/S}$ (g/g)	$Y_{1,3PD/S}$ (g/g)	$Y_{HBut/S}$ (g/g)	$Y_{HAc/S}$ (g/g)	Q_{PD} (g/L.h)
0.050	49.7	7.10	24.3	0.084	0.57	0.125	0.024	1.21
0.075	49.7	8.78	24.1	0.125	0.59	0.143	0.027	1.80
0.100	49.7	6.13	24.9	0.141	0.57	0.106	0.016	2.48
0.150	49.7	12.63	21.5	0.142	0.58	0.129	0.023	3.22

Regarding the results from Table 3, the conditions which provide the maximum yield (0.59 g/g) for the continuous production of 1,3-PD, using crude glycerol as substrate, are the ones with a dilution rate of 0.075 h^{-1}. This yield is higher than results obtained in similar conditions found in the bibliography (Gonzalez-Pajuelo et al. 2004, Papanikolaou et al. 2000, 2008). Papanikolaou et al. (2000) got a global yield of 0.55 g/g in single-stage continuous cultures by *Clostridium butyricum* F2b. González-Pajuelo et al (2004) reached 0.51 g 1.3-PD/g crude glycerol in fermentations using *Clostridium butyricum* VPI 3266 at a dilution rate value of 0.1 h^{-1}.

Nevertheless, the most interesting option could be at D=0,15 h^{-1}, since this almost reaches the same 1,3-PD yield as the previous one (0.58 g/g in this case), but with the benefit of less butyrate and acetate produced. Moreover, higher dilution rate values also provide a higher productivity.

7. Conclusions

In this chapter, the production of 1,3-propanediol by *C. butyricum* DSMZ 10702, using waste glycerol from biodiesel industry, was investigated.

Batch, fed-batch and continuous fermentation experiments were carried out. In batch fermentation with 70 g/L of crude glycerol, the substrate was completely consumed and the biomass reached a value of about 1.0 g/L after 60 h fermentation. The concentration of 1,3-propanediol reached a maximum of 35.9 g/L, slightly lower than the concentration reached using pure glycerol (40.0 g/L) . The yield factor was 0.51 g 1,3-PD/g glycerol, also below the value reached in the fermentation of pure glycerol (0.57 g 1,3 PD/g glycerol). The main by-product was butyric acid (7.9 g/L) followed by acetic acid (2.7 g/L). In this case the butyric acid production was higher than when pure glycerol was used, while the acetic acid concentration was quite similar for both substrates.

The maximum initial crude glycerol concentration tolerated by *C. butyricum*, yet maintaining a high production of 1,3-PD, was investigated with a set of experimental runs where the concentration of crude glycerol varied from 50 g/L to 150 g/L at pH 6.5 and 37 ºC. The maximum tolerance of *C. butyricum* to the inhibitory effect of the substrate was 70 g/L, lower than the 100 g/L for pure glycerol experiments. The kinetic Contois type-model successfully simulates the batch fermentation of glycerol by *Clostridium butyricum* DSM 10702.

Fed-batch experiments were performed in order to reduce the inhibitory effect of high substrate concentrations. The maximum 1,3-propanediol concentration was 36.1 g/L, close to the 37.3 g/L reached in fed-batch pure glycerol experiments. Acetic and butyric acid concentrations reached, respectively, 2.5 g/L and 9.0 g/L.

In continuous cultures, the maximum 1,3-propanediol obtained was 27.5 g/L at a dilution rate of 0.05 h^{-1} and 70 g/L of glycerol in the fed stream. However, for D=0.15 h^{-1} and S_0=50g/L, 21.5 g/L of 1,3-propanediol were reached with a lower production of butyrate and acetate and tripling the productivity at the same time.

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CHAPTER 6. ASSAYS WITH PRETREATED GLYCEROL

1. Introduction

In the 1,3-propanodiol biotechnological process, microorganisms are exposed to various environmental stresses that influence their metabolism, such as osmotic pressure caused by high concentration of glycerol and the presence of toxins and inhibitors. In chapters 3 and 4 it has been shown that a high concentration of glycerol prevents normal bacteria growth and decreases the efficiency of the 1,3-propanodiol synthesis. The optimal initial glycerol concentration found for fermentations using crude glycerol as substrate (70g/L) was lower than the optimal initial concentration when pure glycerol was used (100g/L). The crude glycerol impurities introduced into the process appear to be the principal cause.

Different technical grades of glycerol are available in the market. The three main qualities of glycerol commercially available depend on their purity. These are: crude glycerol, technical glycerol, and refined glycerol. Crude glycerol usually has between 40 and 88 wt % of glycerol, and contains high amounts of methanol, soaps, and salts. Technical glycerol is a high purity product where most of its pollutants have been totally removed. This glycerol is free of methanol, soaps, salts, and other components. Refined glycerol is a pharmaceutical quality product which can be used in foods, personal care, cosmetics, pharmaceutical products, and other special applications.

Industrially crude glycerol is refined through a filtration process, followed by mixing with chemical additives which allow the precipitation of salts and, finally, different qualities of commercial glycerol are obtained by a vacuum fractional distillation process. Crude glycerol can also be refined by filtration through a series of ion exchange resins, a method with low energy consumption.

Distillation is the most commonly used method for purifying glycerol. This technology produces high purity glycerol at high yields. However, glycerol distillation is an energy-intensive process due to its high heat capacity, which requires a high supply of energy for vaporization (Posada, Cardona and Rincon, 2010).

Ion exchange has also been applied to purifying glycerol (Berrios and Skelton, 2008). The separation by ion exchange resins is an easy and safe process that requires low energy consumption. Nevertheless, a high salt content makes this technique

economically unfeasible on an industrial scale. The cost of the chemical regeneration of the resins becomes high when the salt content is higher than 5wt%, an amount commonly found in the glycerol obtained from the biodiesel industry (Berríos and Skeltron, 2008, Posada and Cardona, 2010).

A commercially available technology for crude glycerol purification obtained during the production of biodiesel was jointly developed by Rohm and Haas and Novasep Process (Posada and Cardona, 2010). The process, called Ambersep BD50, uses a chromatographic separator to remove large amounts of salts and free fatty acids. A refined stream is then processed in an evaporator / crystallizer unit, which removes the salts in a crystalline form. This avoids the production of effluents in the glycerol purification plant. Thus, a glycerol stream at a purity of 99.5 wt % is obtained. If high quality glycerol is required (e.g., 5 to 10 parts per million of salt content), it is possible to use an ion exchange demineralization unit. This process has lower energy requirements as compared to the traditional distillation process.

In this chapter, a pretreatment was studied, based on ion exchange resin for biodiesel-based crude glycerol, to eliminate a number of impurities generated during the transesterification process and reduce the concentration of growth inhibitors such as salts and pigment. Two different pretreatments were tested based on ion exchange resins.

As explained above, the chemical regeneration cost of the resins becomes high when the salt content is higher than 5 wt%. The crude glycerol used in this study contains 5.23% of salts. However, since *C. butyricum* DSM 10702 has a high tolerance to the inhibitors present in crude glycerol, a complete purification process is not intended, but a low cost pretreatment that removes some impurities with inhibitory effects in the fermentation step. Therefore, in this case, the use of resins can still be feasible to purify crude glycerol.

First, serum bottles of batch fermentations by *C. butyricum* DSM 10703 were run, at different initial substrate concentrations, to select the most effective pretreatment in terms of 1,3-propanediol concentration, yield, productivity, and high substrate tolerance. Once the best pretreatment had been selected, batch and fed-batch experiments were carried out in a 2L bioreactor using pretreated crude glycerol.

2. Experimental set-up and procedures

2.1. Pretreatments

The feedstock (crude glycerol) was supplied from a typical biodiesel plant (ACOR industry, Valladolid, Spain) with an 88 wt% of purity (see crude glycerol characterization in Table 1, Chapter 4). The purification of crude glycerol involved a refining process using an ion exchange resin which removes the impurities contained in the mixture. In this sense, four different ion exchange resins were tested: LEWATIT S8528, LEWATIT GF 303, LEWATIT GF 404 and LEWATIT GF 505.

LEWATIT S8528 is a weakly acidic, macroporous cation exchange resin based on crosslinked polyacrylate. This resin is commonly used for decarbonisation of drinking water and liquids which are either used as foodstuffs or in the production of foodstuffs. Lewatit S8528 has been chosen because it has a low regenerant requirement and is therefore an economical option.

LEWATIT GF 303, 404 and 505 are ion exchange resins, especially suitable for the purification of crude glycerol. This set of resins is the commercial process proposed by the Lewatit® GreenFuels product line for glycerine purification.

- Lewatit GF 303, a strongly acidic, uniform bead sized, cation exchange resin, especially suitable for the chromatographic separation of salts from glycerine by ion exclusion.
- Lewatit GF 404, a strongly acidic, macroporous cation exchange resin with beads of uniform size and high mechanical and osmotic stability. It is especially suitable for removing traces of cations from glycerine which has been subjected to the preliminary deashing with Lewatit GF 303.
- Lewatit GF 505, a medium, basic macroporous anion exchange resin with beads of uniform size and high mechanical and osmotic stability. Lewatit GF 505 is especially suitable for removing traces of anions and colour from glycerine which has been subjected to preliminary deashing with Lewatit GF 303.

Pretreatment with LEWATIT S852

The trials using LEWATIT S8528 were carried out in a glass column (50 cm high and 2.7 cm in diameter) filled with the resin, in upward, at room temperature (≈25 ºC). A 250mL solution of 80 wt% crude glycerol was pumped through the column by a peristaltic pump (Watson Marlow 520). The ratio between crude glycerol and resin was 100g resin/100g glycerol.

Pretreatment with LEWATIT GF resins

The ion exchange GF resins were investigated by passing the feed through a column of resin supported in a glass tube 50 cm high and 2.7 cm in diameter in upward, fig. 1. The flow was controlled by peristaltic pumps (Watson Marlow 520) and the outlets were restricted to ensure a head of liquid above the resin at all times. The initial loadings and flows for the three GF resins were as recommended in the LEWATIT trade literature. The initial resin sample size was 80 g and the flow rate, based on the LEWATIT recommendations of 0.25 L/h. for a purification of 99.8 % and an operation temperature of 80 ºC. However, since there is no need for a high purification level, the temperature was controlled at 40 ºC, in order to keep pretreatment costs down.

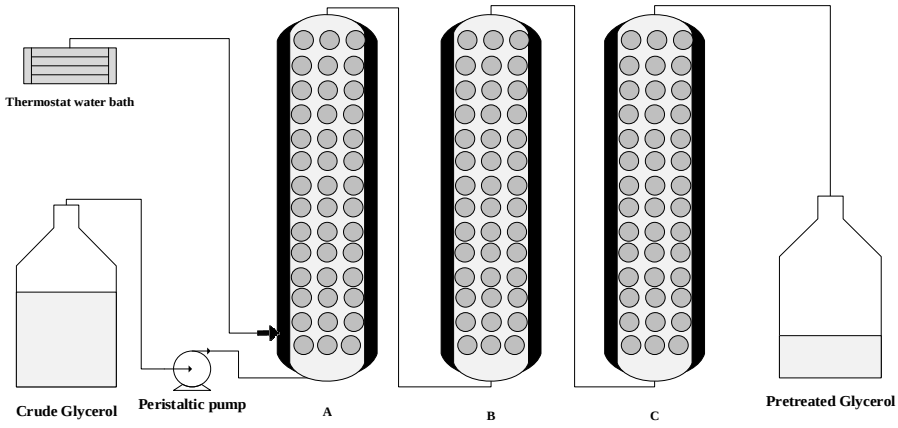

Fig.1 Process diagram for crude glycerol pretreatment with LEWATIT GF resins. Column A-GF303; column B-GF404 and column C-GF505.

2.2. Fermentation Experiments

Serum Bottle experiments

A first set of experiments was carried out in 100mL serum bottles with a working volume of 50mL under anaerobic conditions. Once inoculated, the serum bottles were incubated at 37ºC without agitation during 24h. The cells were allowed to grow for 48h in an orbital shaker incubator (COMECTA) at 200 rpm. In this case, the pH was not controlled.

Fermentation in bioreactor

In the second set of experiments, batch and fed-batch fermentations were performed in a 2L reactor (Biostat Bplus). The pH was controlled at 6.5 by the automatic addition of 3M KOH and the temperature was regulated at 37ºC by a thermostatted water circulation system.

In batch fermentations, the reaction volume was 1L, containing 0.9 L of growth medium inoculated with 0.1L of preculture. The fermentation time was determined by biomass evolution and the experiments ended when the biomass reached the stationary phase.

Fed-batch cultures were started batchwise, with approximately 70 g/L of substrate and, after 24 h of fermentation, feed was continuously added to the fermenter. All fed-batch fermentations were carried out for 90h.

3. Results

3.1. Pretreatments

Crude Glycerol, obtained from biodiesel manufacturing, was subjected to two different pretreatment processes and the main results are shown in Table1: sample A results are from the treatment of crude glycerol with the weakly acidic ion exchange resin LEWATIT S8528, based on crosslinked polyacrylate. In the second process, crude glycerol was treated with LEWATIT GF resins. First, a sample of crude glycerol was treated with LEWATIT GF 303 for the separation of salts, sample B-1. Sample B-1 was then treated with LEWATIT GF 404 for polisher and cation removal, resulting in sample B-2. Sample B-2 was then treated with LEWATIT GF 404 for decolorization and was called sample B-3.

Table 1. Characterization of various grades of pretreated biodiesel crude glycerol.

Resin	Sample	Glycerol (%wt)	Salts (%wt)	Ash[a] (%wt)	MONG[b] (%wt)
	Crude glycerol	88.45	5.23	5.28	2.18
S8528	A	57.90	3.52	3.67	1.68
GFs	B B-1	58.40	4.76	4.81	1.40
	B B-2	49	4.70	4.79	1.27
	B B-3	47.60	4.30	4.39	1.21

[a] Salts included in ash

[b] MONG = Organic non-glycerol matter

The results show that the pretreatment using the weakly acidic ion exchange resin LEWATIT S8528 removes more salts from the biodiesel waste glycerol than the pretreatment using the LEWATIT GF resins. In theory, LEWATIT GF 303 resin is

suitable for eliminating the salts present in crude glycerol almost completely. The high amount of salts obtained after washing the glycerine through GF 303 resin could be explained by the low operating temperature set at 40ºC instead of the value recommended in the literature (80ºC) - as mentioned above, we were looking to work in friendly operating conditions.

In terms of MONG, the pretreatement using the GF resins was the most effective, eliminating 45% of MONG from the crude glycerol.

3.2. Fermentation experiments

Serum Bottle experiments

The efficiency of crude glycerol pretreatments for growth and 1,3-propanediol production by *C. butyricum* DSM 10702 was evaluated by a set of experiments carried out in 100mL serum bottles.

Two initial substrate concentrations, 70 and 100 g/L, were tested to estimate the effectiveness of the pretreatments at standard and high concentrations. The main results were collected in Table 2. The results show that both pretreatments provide higher concentrations of 1,3-propanediol at low and high concentrations of substrate. In the fermentation using glycerol sample A at 100g/L of initial substrate concentration, the microorganism showed inhibition. Since sample A contains a lower concentration of salts, it would seem that the inhibition of *C. butyricum* was mostly caused by other impurities. These impurities are probably MONG contents, since simple B, which has a lower concentration of MONG and higher concentration of salts, has not showed inhibition for S_0=100g/L.

Homan et al. (1990) reported that *C. butyricum* was inhibited at 12 g of sodium ions. Chatzifragkou et al. (2010) evaluated the impact of some impurities of biodiesel-based crude glycerol, reporting no inhibition effect on the microbial growth or 1,3-PD production when 30% (w/w glycerol) of NaCl was added. On the other hand, according to same authors, 2% of double bond acids caused a total inhibition of the microorganism.

Though high concentrations of 1,3-propanediol have been achieved in option B, pretreatment A (LEWATIT S8528) has the advantage of being a more economical and simple process, as only one resin is used and no heating is required.

Since both pretreatments showed different advantages and drawbacks, the pretreatment choice should take into account the concentration of the initial glycerol that will be used. For fermentations carried out with 70g/L of substrate (optimal initial substrate concentration, based on Chapter 4), the most appealing option is the pretreatment with LEWATIT S8528, as similar product concentrations to option B are reached, but at lower costs.

Table 2. Crude glycerol and pretreated crude glycerol fermentation by *C. butyricum* DSM 10702 in 100mL serum bottles at different initial concentrations.

Sample	S_0 (g/L)	S_f (g/L)	1,3-PD (g/L)	HAc (g/L)	HBut (g/L)	t(h)
Crude Glycerol	70.6	56..8	6.1	0.10	0.183	48
	101.2	98.2	0.52	0.00	0.791	48
A	70.1	53.0	7.1	0.057	0.134	48
	102.6	91.7	4.1	0.256	0.000	48
B	70	42.0	8.8	0.323	1.369	48
	100.7	84.5	10.5	0.604	1.510	48

Fermentation in bioreactor

Batch Fermentations

In order to investigate *C. butyricum* DSM 10702 grown in crude glycerol, and previously treated with LEWATIT S8528 resin (PCG), anaerobic batch fermentation was conducted in a 2L bioreactor (fig. 2) and the data was compared with background data of pure and crude glycerol fermentations (Table 3).

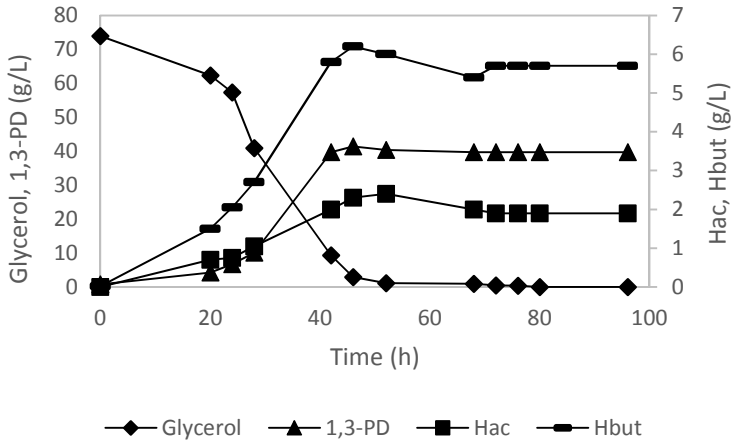

Fig. 2 Batch fermentation profiles of pretreated crude glycerol by *Clostridium butyricum* DSM 10702.

Table 3. Influence of raw glycerol impurities on biomass and 1,3-propanediol formation in batch fermentations by *C. butyricum* DSM 10702.

	Concentration (g/L)	Biomass (OD_{600})	1,3propanediol		
			Concentration (g/L)	Productivity (g/L/h)	Yield (g/g)
Pure Glycerol (99% purity)	70.0	1.8	40.5	0.80	0.58
Crude Glycerol (88% purity)	70.3	1.9	35.9	0.83	0.51
*PCG (LEWATIT S8528)	73.9	2.3	41.4	1.03	0.56

*PCG-Pretreated Crude Glycerol

Pretreated crude glycerol was completely consumed in less than 50 hours and a high concentration of 1,3-propanediol was obtained.

The use of pretreated crude glycerol increased the 1,3-propanediol concentration and yield (41.4 g/L and 0.56 g/g respectively), reaching values close to that of pure glycerol (40.5 g/L and 0.58 g/g, respectively). The fermentation of the by-products,

acetic and butyric acids, present similar concentrations in both cases, 3.0 and 6.0 g/L for pure glycerol and 2.3 and 6.8 g/L for pretreated crude glycerol, respectively. An increase in the productivity (g/L/h) and the biomass concentration of pretreated glycerol as compared to that of pure glycerol can be attributed to the presence of certain growth-promoting nutrients, such as potassium or magnesium ions, frequently present in biodiesel waste glycerol. When pretreated crude glycerol was used, a considerable decrease in fermentation time was observed. A complete consumption of glycerol was also achieved in both cases.

There are only a few reports of pretreatments of crude glycerol for use as substrate in 1,3-PD biosynthesis. One of those, Asad-ur-Rehman et al. (2008a), reached a mass yield of 0.52 using pretreated crude glycerol (through an n-hexanol washing process) in batch fermentation at 32ºC, pH 7.0 and 50g/L of initial substrate concentration. The pretreatment proposed in the present work allowed *C. butyricum* fermentation to reach a mass yield production of 0.56.

Fed-Batch fermentations

In order to extend the knowledge concerning fermentations using pretreated glycerol, anaerobic fed-batch cultures were conducted using PCG. Fed-batch cultures were started batchwise at an initial concentration of 70g/L. The time of the exponential phase (24h) was set as the beginning of feeding. The results are shown in Fig. 3.

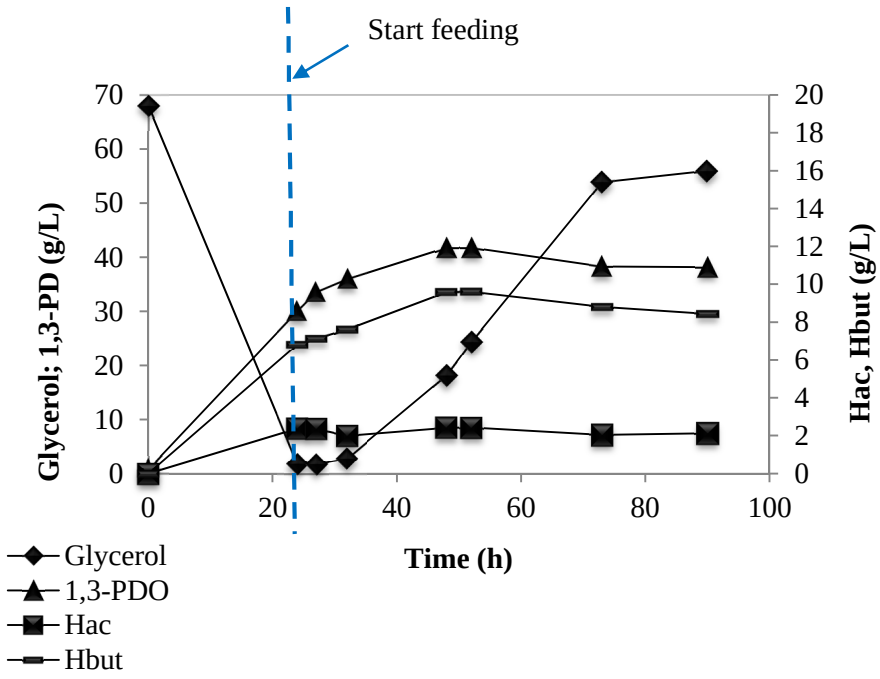

Fig. 3 Fed-Batch fermentation of pretreated crude glycerol by *Clostridium butyricum* DSM 10702.

The glycerol consumption was incomplete and 54.2 g of 1,3-propanediol was accumulated in the fermentation broth. The incomplete glycerol consumption might be attributed to the exhaustion of the nutrients before the complete utilization of the glycerol, given that the concentration of 1,3-propanediol and acetic and butyric acids remained below the inhibitory value. Biebl (1991), at a pH of 6.5 for *C. butyricum* species, indicated that the inhibitory levels of 1,3-propanediol, acetic and butyric acids are 64, 27 and 19 g/L, respectively. Gunzel *et al.* (1991) achieved a complete consumption of glycerol in a fed-batch culture using a medium containing 5 g of $(NH_4)SO_4$/100 g of glycerol. However, in this case, only 2 g of $(NH_4)SO_4$ was used.

Table 4 presents the results of the fed-batch fermentations using the three different grades of glycerol. In terms of 1,3-propanediol yield, the fermentation using PCG not only outdoes the crude glycerol fermentation results, but also exceeds the pure glycerol fermentation yield. As previously explained, this fact may be due to the presence of certain growth-promoting nutrients present in crude glycerol that were not removed in the pretreatment step. Significant differences were also observed in the formation of different by-products. The amount of butyric acid formed, when PCG was used as substrate, was higher than the amount found in the pure glycerol fed-batch

fermentation. On the contrary, the formation of acetic acid was higher when pure glycerol was used as the substrate. As for the volumetric productivity (Q_{PD}), better results were observed, once again, for the PCG fermentation.

Asad-ur-Rehman et al. (2008a) also carried out fed-batch fermentations with pretreated glycerol (n-hexane washing system), obtaining good results when compared with those obtained with untreated glycerol (0.49 g 1,3-PD/g pretreated glycerol), but not reaching the results obtained when pure glycerol was used (0.51 g 1,3-PD/g pure glycerol).

Table 4. Comparison between yield and productivity of anaerobic fed-batch cultures of *C. butyricum* at 37ºC and pH 6.5 using different grades of glycerol as substrate.

Parameters	**Type of glycerol**		
	Pure	Crude	PCG
$Y_{PD/S}$ (g/g)	0.53	0.49	0.55
Q_{PD} (g/(L.h))	0.77	0.80	0.88
$Y_{X/S}$ (g/g)	0.021	0.019	0.027
$Y_{Hac/S}$ (g/g)	0.031	0.034	0.027
$Y_{Hbut/S}$ (g/g)	0.112	0.110	0.119

Values calculated to t=50h.

4. Conclusion

The production of 1,3-propanediol by *C. butyricum* DSM 10702 using pretreated glycerol as a sole carbon source was feasible in the two pretreatments tested. The inability of *C. butyricum* DSM 10702 to withstand the inhibitory effects of high concentrations of crude glycerol was overcome by a simple process of adsorption with ion exchange resins. Although both pretreatments tested presented a positive response in the fermentation process, the pretreatment using the GF resins provided higher concentrations of 1,3-propanediol and a higher tolerance of the microorganism at high concentrations of substrate. Nevertheless, the pretreatment using LEWATIT S8528 represents a practical option with lower costs and a high production of 1,3-propanediol.

Judging from the serum bottle results, the presence of salts between 4 and 5 wt% in crude glycerol does not cause *C. butyricum* DSM 10702 inhibition. The assumption mentioned above is in accordance with the results of Chatzifragkou et al. (2010), where *C. butyricum* was not inhibited with the addition of 30% (w/w glycerol) of sodium salts for an initial substrate concentration of 20 g/L.

The principal inhibition agent seems to be in the non-glycerol organic matter, most likely the free fatty acids, such as oleic and linoleic acids (Chatzifragkou et al., (2010).

For the two different fermentation modes tested (batch and fed-batch systems), the pretreatment proposed improved the results reached in fermentations carried out with untreated crude glycerol.

Asad-ur-Rehman *et al.* (2008a) reported a pretreatment for sunflower oil biodiesel crude glycerol. Crude glycerol was washed three times with either n-hexanol or n-hexane at 300 rpm for 15 min at room temperature. Although the production of 1,3-propanediol by *C. butyricum* DSM 5431 using the pretreated crude glycerol was feasible, the use of n-hexanol during the solvent washing process on an industrial scale will depend mainly upon the costs of n-hexanol and its distillation, which are higher. The pretreatment with LEWATIT S8528 studied in this work represents a low cost process with positive results. To our knowledge, there have been no reports of the use of LEWATIT S8528 for crude glycerol.

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CHAPTER 7. IMMOBILIZED CELLS BIOREACTOR: ASSAYS WITH CRUDE GLYCEROL

1. Introduction

Efforts have been made to increase the concentration, yield and/or productivity of 1,3-propanediol bioconversion from crude glycerol. In previous chapters, operation with pure, crude and pretreated glycerol, including batch, fed-batch and continuous cultures, was investigated. In the literature, microaerobic culture (Chen et al. 2003), co-substrate fermentation (Biebl and Marten, 1995), and two-stage continuous fermentation (Papanikolaou et al. 2000) were carried out with the same objective.

In general, the maximum concentration of 1,3-propanediol (55-60 g/L) was obtained in batch and fed-batch cultures (Saxena et al., 2009). Continuous culture has the advantage of relatively high productivities, e.g. 1.33-3.4 g/(L.h) with 1,3-propanediol concentrations of 43.5-46 g/L at low dilution rates (Papanikolaou, 2000, 2008). However, the concentration of 1,3-propanediol is still insufficient to reduce the load of downstream separation.

In order to eliminate this problem and reduce the substrate and product toxic effects, the implementation of an appropriate immobilization technique (adsorption or entrapment of the cells on or in inert material) can be performed. High productivities can be obtained in immobilized cell reactors, due to the effective retention of the bacteria (Pflugmacher and Gottschalk, 1994).

The biosynthesis of 1,3-propanediol using crude glycerol as substrate was typically produced in suspended cultures. There are very limited studies with immobilized cell systems for 1,3-propanediol production (Gungormusler et al., 2011; Pflugmacher and Gottschalk, 1994; Wittlich et al., 2004 and Chen et al., 2006).

The immobilization technique has displayed excellent properties in the realm of bioconversion, though its use for 1,3-propanediol production has been rare. Griffiths and Bosley (1993) used POLYHIPETM polymers to immobilize *C. Freundii* for 1,3-propanediol production. Pflugmacher and Gottschalk (1994) immobilized *C. Freundii* on modified polyurethane particles and obtained a high productivity of 1,3-propanediol (8.1 g/L h).

Yeast cell immobilization by entrapment has most commonly been used in alginate gels. Among the advantages of this method are the ease of manipulation of the gelling properties and the gel strength (Sree et al., 2000). Furthermore, in alginate bead immobilizations, these can be re-solubilized for free cell counting and evaluation. The difference between the alginate and other gels such as agar, is that the former is thermally irreversible, which allows work to be done at high temperatures.

The aim of this chapter is to study the use of sodium alginate gel as an immobilization material on *C. butyricum* cell growth and 1,3-propanediol production from waste glycerol. The concentration and yield of 1,3-propanediol under batch and continuous cultures are reported and compared with the results of the free cell culture.

2. Experimental procedures

2.1. Immobilization Method

The microorganism, *Clostridium butyricum* DSM 10700, was immobilized by entrapment in calcium alginate gel, using aluminium as a hardener for a greater mechanical strength of the bioparticles. The immobilization was carried out in the following stages:

I. Preparation of sodium alginate solution.
II. Preparation of calcium chloride and aluminium chloride solution.
III. Preparation of sodium alginate and microorganism suspension.
IV. Bioparticle formation.

I. Preparation of sodium alginate solution.

The alginate solution has been used in a concentration of 2% (w/v). The sodium alginate used was alginic acid sodium salt from brown algae from Sigma Aldrich. The dissolution of 2% sodium alginate was prepared with deionised water and sterilized in autoclave at 121 ◦C for 15 min. Once autoclaved, the solution was perfectly uniform.

II. Preparation of $CaCl_2$ and $AlCl_3$ solution.

The solution was prepared with calcium chloride and aluminium chloride at 4 and 0.5%, respectively, with deionised water. The ratio volume of calcium chloride to each millilitre of sodium alginate was 5 to 1. The solution was sterilized in autoclave at 121 ◦C for 15 min.

III. Preparation of sodium alginate and microorganism suspension.

The required amount of microorganism (inoculum) was added to the sterile sodium alginate solution to obtain the desired concentration. The mix was homogenized by mechanical stirring. *C. butyricum* cells were pre-cultured into a 100mL medium in a 150mL serum bottle and anaerobically incubated at 37 ◦C for 20 h. Then, the 100mL suspension of preculture was centrifuged to eliminate the excess of growth medium and obtain a high biomass concentration. The microorganism was then well mixed with the sterilized alginate solution by stirring. Then 4% (w/v) chloride calcium and 0.5% chloride aluminium solution were added drop by drop to the mixed suspension.

IV. Bioparticle formation

Cell entrapment was carried out under aseptic conditions. Bioparticles were generated by ion exchange when the alginate chloride and biomass solution dripped on the solution containing calcium chloride and aluminium chloride. Aluminium chloride was used to harden the bioparticles in order to withstand the shearing forces to which they are subjected inside the reactor.

Experimental set-up for the formation of bioparticles comprises the following elements, Fig. 1:

1. Hypodermic needle, where drops are formed.
2. Peristaltic pump, which drives alginate suspension to the hypodermic needle.
3. Pressurised nitrogen system, which forms the particle size.

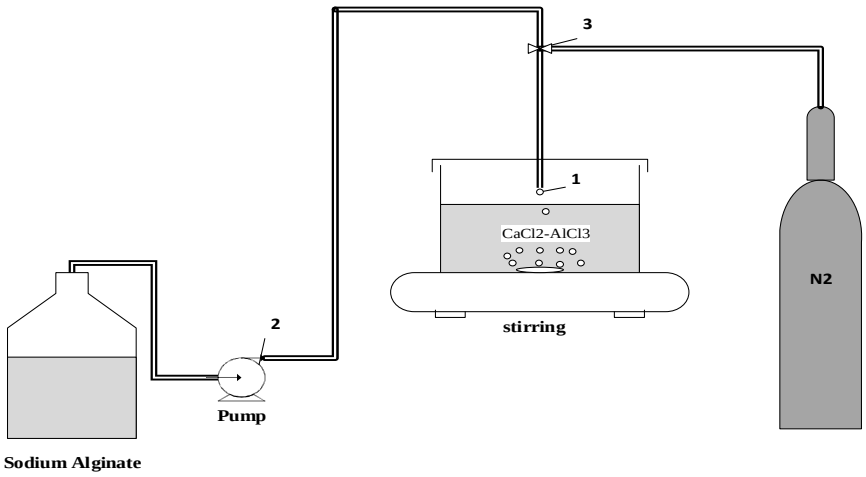

Fig.1. Schematic diagram of bioparticle formation.

There were three control points for the formation of bioparticles with an appropriate size, the flow of the suspension of alginate chloride and biomass, the flow of nitrogen, and the diameter of the needle.

Bioparticles formed after mild stirring for 40 min at room temperature. Excess free $CaCl_2$-$AlCl_3$ on the microcapsule surface was carefully washed away with sterilized water. The average diameter of each microcapsule was 2.6 mm.

2.2. Serum Bottle experiments

The cultivation of *C. butyricum* was described in the Section “Media and cultivation conditions”, in Chapter 2’s “Materials and Methods”. The 150 mL serum

bottles were filled with 50 mL pre-boiled growth medium and sealed under nitrogen before autoclaving them. After sterilization, 5 mL suspension of *C. butyricum* or 25 mL of bioparticles, was added to the 50 mL of fermentation medium (50 g/L glycerol). The cells continued to grow at 37 ºC and 200 rpm in a shaker incubator (COMECTA).

Two batches of fermentations with encapsulated cells in the serum bottles were performed. At the end of the first batch, the microcapsules were directly moved into 50mL of fresh medium and another batch was then started.

2.3. Fermentation in bioreactor

Firstly, bioparticles were pre-incubated at 37 ◦C, pH 6.5 for 24 h in order to obtain high cell density in the microcapsule. Then, a sterile water-jacketed glass column bioreactor (4 cm inner diameter×40 cm high) packed with 180 mL of bioparticles was established (Fig. 2).

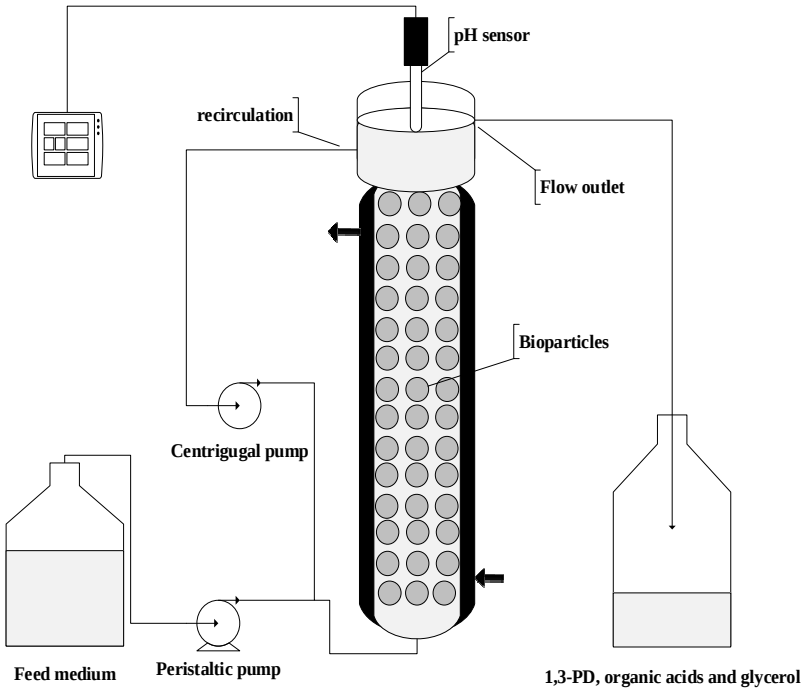

Fig. 2 Schematic diagram of immobilized bioreactor system.

At the top of the reactor, there was a cylinder enlargement (90 mm in height and 90 mm internal diameter), where the bioparticles were losing speed, decanting in this area and returning to the reactor. This area also contained the effluent outlet and the recirculation. The top of the fermenter was also provided with an inlet for the pH sensor and a filter for degasification.

The bioreactor was filled with the feeding solution. Bioparticles initially tended to float, since they have less density than the feed solution, and are thus on the surface. As the dissolution spread into the bioparticles, these increased in weight, having a higher density than the feed, and began to settle. When all the bioparticles were in the bottom of the reactor, the recirculating pump was connected at a low flow rate.

Reducing agents were not added and the medium was not gassed with N_2 during experimentation since conditions in the reactor were sufficiently anaerobic.

Fermentations were performed at 37 ◦C and initial pH 6.5. The pH was measured but not regulated. In order to establish well-mixed conditions, the medium effluent was continuously recycled.

2.4. Analytical procedure

pH measurement

pH measurement was performed with a Crison selective electrode connected to a Crison digital pH meter, pH/mV model 505. The pH meter was calibrated before each measurement with standard solutions of 4.00 and 7.02. The pH sensor was sterilized with an ethanol solution.

Determination of biomass, product and by-products concentrations into the bioparticle

In order to determine the biomass, glycerol and products concentrations in each bioparticle, spherical beads were randomly sampled and the surface liquid was

absorbed. Then, each bead was placed in a tube containing 1.5mL of 0.1 M citrate buffer at pH 4.8 and incubated for 15 min at room temperature to dissolve the alginate.

Scanning electronic microscopic study

For electronic microscopic scanning (SEM) micrographs, samples were taken from fresh and 7-day beads from the serum bottles batch experiments. The samples were freeze-dried for 10 h in the Freeze Drier. The samples were observed under SEM at various magnifications (Quanta 200 FEG, FEI).

3. Results

3.1. Electronic microscopic scanning of immobilized cells

The immobilization strategy was successful and the microorganism was trapped in the spherical beads shown in fig. 3.

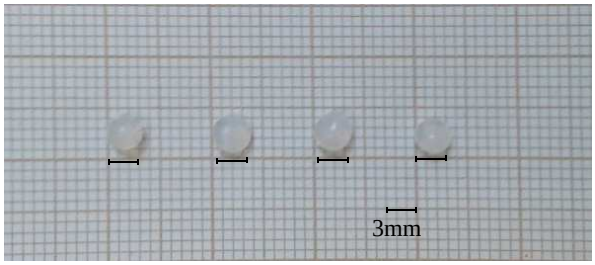

Fig. 3 Ca-alginate beads.

Figs. 4 show electronic micrographs of outer and inner surfaces of the beads right after immobilization and on after 7 days of fermentation.

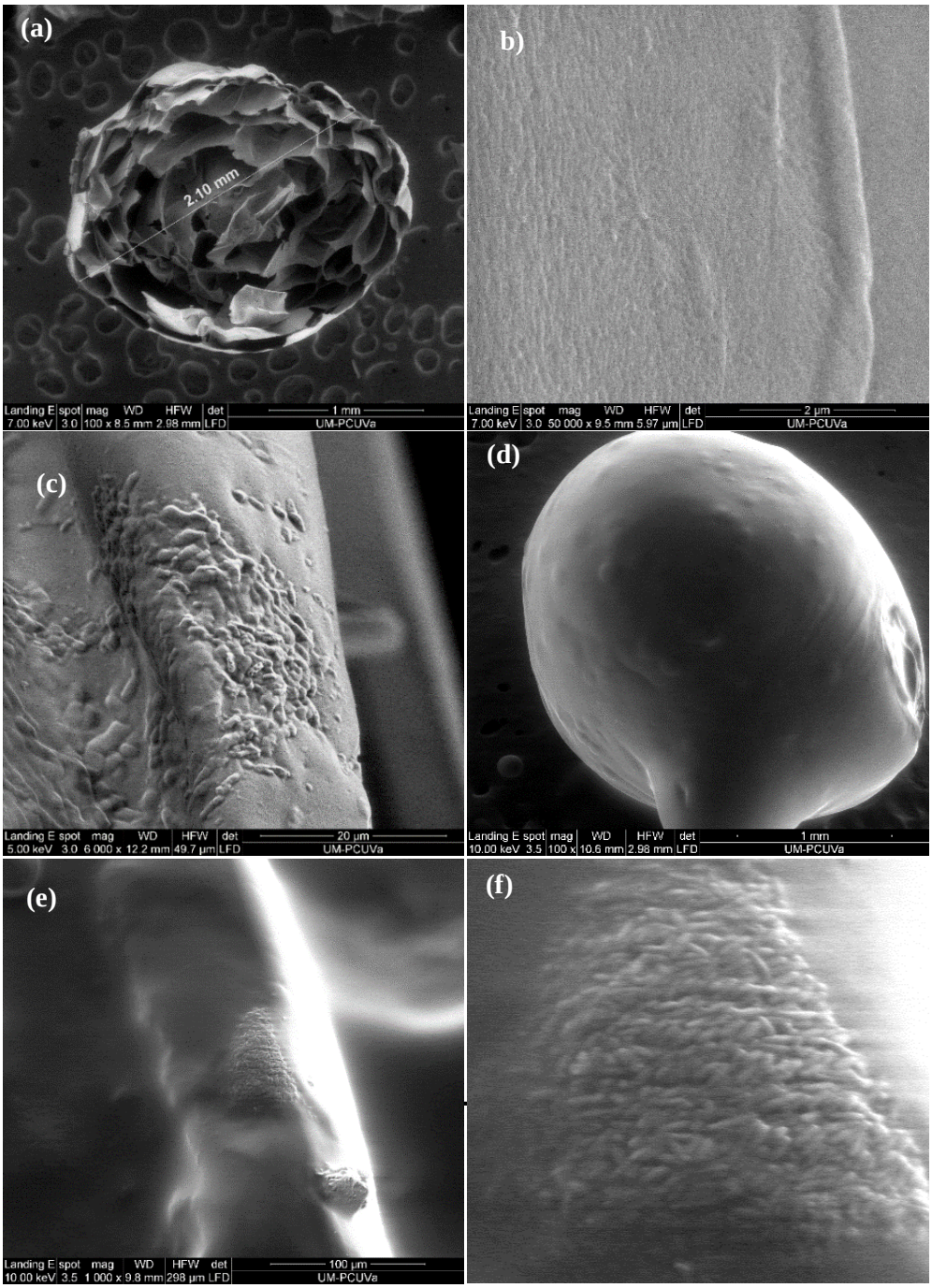

Fig. 4 Electronic photomicroscope of fresh and 7 days bio-beads at different magnifications: (a); structure of a half bead (b) outer surface of the 0 days beads; (c) inner surface of the 0 days beads (d) outer surface of the used beads after 7 days; (e) inner surface of the used beads after 7 days; (f) inner surface of the used beads after 7 days with a 200 % Zoom.

Fig. 4 a), b) and c) shows the structure and the cell entrapment in the alginate matrix, which included cells within a rigid network. Fig. 4 a) corresponds to a half 0 days bioparticle and b) to the outside matrix of the bead. In the outer surface of the fresh beads, fig. 4 c) was not visible *C: butyricum*. The cells were initially in low concentration dispersed inside the beads, fig. 4 c).

On the other hand, the outer surface of 7-day beads, shown in Fig. 4 d), presented spherical footprints that indicated the cells were grow in spherical colonies inside the bioparticles.

The inner surfaces of the beads after 7-days are shown in fig 4 e) and f). After 7 days, the cells concentration severally increased and several colonies were observed inside the bioparticles.

The Ca-alginate gel prevented the cells from diffusing into the liquid medium while still allowing penetration of the substrate. It is also obvious that the concentration of cells were severally grown on the inner surfaces of the Ca-alginate beads after the 7 day of operation.

The cell immobilization using alginate polysaccharide has an advantage due to cell immobilization and polymerization and good stability of the gel matrix under mild conditions.

As expected, the method used to prepared the beads for SEM analyses (lyophilisation) was not successful in the 7 days bioparticles since these samples contained glycerol. As result, the samples were hard to analyze resulting in blurry images.

Although the SEM analysis show a rigid matrix and the cell entrapment only inside the alginate matrix, some free cells were observed into the bulk of the liquid medium. This fact could be explained by an inefficient wash of the bioparticles and contamination of the reactor by *C. butyricum* remaining in the surface of the beads.

3.2.Serum bottles cultures

The initial glycerol concentrations were all 50 g/L in shake flask cultures with either free cells or encapsulated cells of *C. butyricum* DSM 10702. The cultivation time of the free cell culture lasted 72 h. When fermentation ends, a biomass of 1.68 g/L, as well as 11.56 g/L of 1,3-propanediol, was obtained. The main by-products were acetic and butyric acid.

In terms of immobilized cell cultures, two batches were performed. The results of the first batch were similar to those of the free cell culture, except that the final biomass concentration in the bioparticles was enriched to 3.84 g/L, which was approximately 2.3-fold higher than that of the free cell culture (1.68 g/L).

When the first batch was completed, the biobeads were saved and reused in the next batch. Fig. 3 shows results of the second batch, which had accumulated 13.77 g/L of 1,3-propanediol in the broth with a yield of 0.47 grams of 1,3-propanediol formed per gram of glycerol consumed.

The bioparticle analysis showed that the crude glycerol concentration remained low (1-1.8 g/L capsule) in the bio-bead during cultivation, while 1,3-propanediol was accumulated up to 8.3 g/L per bead at the initial stage. The main by-product was butyric acid, while acetic acid could not be detected by the chromatograph.

The final biomass concentration of the second batch was similar to that of the first batch. Furthermore, a small amount of biomass was detected on the fermentation broth. Therefore, the total amount of biomass per culture was calculated, taking into account both immobilized and free cells, as follows:

$$C_{Bioparticle\ sample} \times V_{Bioparticles} + C_{free\ cell} \times V_{broth}$$

$$3.80\frac{g}{L}\,bioparticles \times 0.025L + 0.5\frac{g}{L}\,broth \times 0.05L = 0.12\ g$$

Comparing this with the free cell cultures (0.084 g), the bioparticle fermentation reached a high biomass concentration. The presence of biomass in the fermentation broth, although in a low concentration, indicated that there was biomass transfer through the bio-bead.

The reuse of immobilized microorganisms has rarely been reported (Zhao et al., 2006, Jun et al., 2010). However, Zhao et al. (2006) reported the use of encapsulated cultures of *Klebsiella pneumoniae* in five repeated batch fermentations with high cell density and activity.

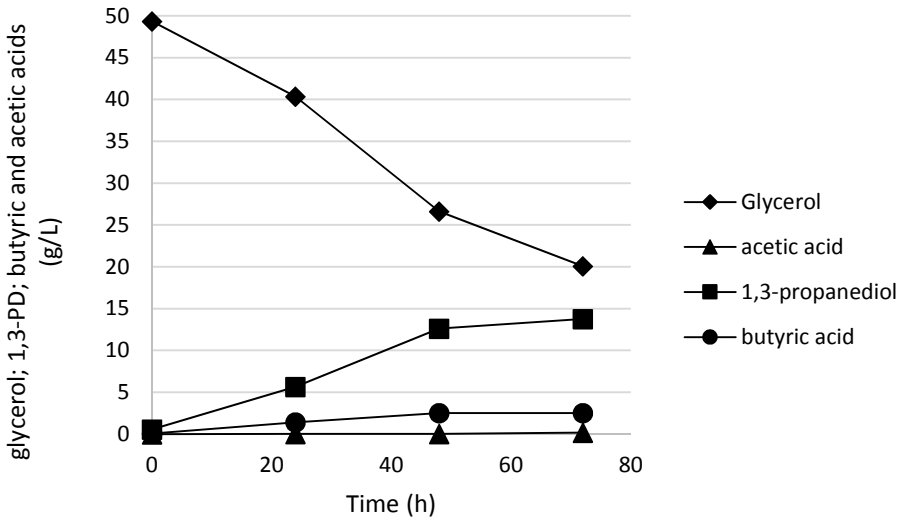

Fig. 5 Batch culture in the shake serum bottles at an initial glycerol concentration of 50 g/L.

The low concentration of glycerol favoured the growth of *C. butyricum* because it is inhibited by high concentrations (more than 70 g/L of crude glycerol). Meanwhile, low concentrations of glycerol inside the bioparticle might diminish 1,3-propanediol production as fewer reducing equivalents (NADH) would enter the reductive pathway of glycerol to 1,3-propanediol. Furthermore, the substrate in low concentrations inside the bioparticle could also explain the differences in by-product distribution between

immobilized cell culture and free cell culture, in which case the regulation of the enzyme system would favour butyric acid formation.

3.3.Continuous cultures

Continuous cultures were carried out in the fluidized-bed bioreactor under different dilution rates (0.05, 1.0, 1.5 h^{-1}). The initial concentration of glycerol was 50 g/L. In a first step, *C. butyricum* was grown batchwise until 24 h of fermentation time in order to improve biomass concentration in the bioparticles. Then, the feeding pump started and the continuous process began.

In the supernatant of the reactor, viable and dead cells could be detected throughout all the experiments, indicating that the immobilization was not complete and that a certain turnover of the cells occurred.

The results, at the different dilution rates, are shown in Fig. 4. A steady state was obtained after continuous flow of at least three working volumes of the medium through the vessel.

For a dilution rate of 0.05 h^{-1}, concentrations of glycerol (9.27 g/L) and 1,3-propanediol (20.35 g/L) in the effluent remained unchanged after 15 h, with a yield of 0.51g 1,3-propanediol formed/g glycerol consumed and a productivity of 1.02 g/Lh.

When the dilution rate was increased from 0.05 to 0.1 h^{-1}, a minor increase in glycerol consumption and a consequent diminishing products concentration was observed. On the other hand, 1,3-propanediol productivity almost duplicated.

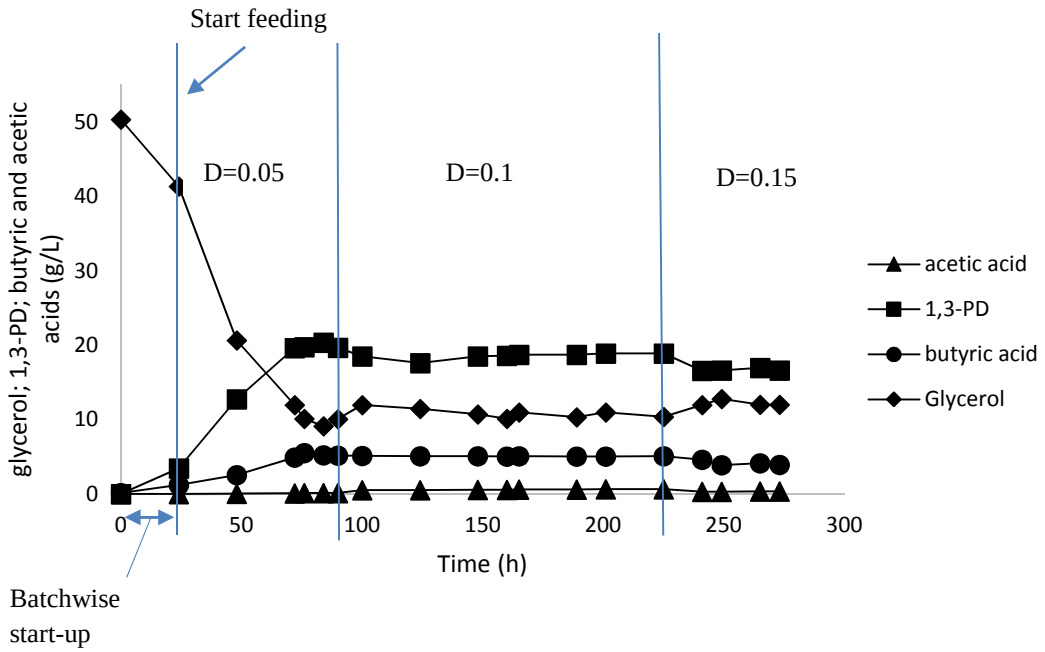

Fig. 6. Continuous fermentation of raw glycerol from oil transesterification by Clostridium *butyricum* DSM 10702.

Results under dilution rates of 0.05, 1.0 and 1.5 h^{-1} are summarised in Table1. As shown in this table, the higher the dilution rate (e.g. 10.3 g/L at 1.0 h^{-1} and 12.7 g/L at 1.5 h^{-1}), the greater the amount of residual glycerol in the effluent medium, whereas the concentration of 1,3-propanediol gradually decreased.

Table 1. Steady state parameters and concentrations in g/L for biomass (X), 1,3-PD, Butyrate and Acetate.

Fed	D (h^{-1})	S_f (g/L)	1,3PD (g/L)	HBut (g/L)	HAc (g/L)	Rc (%)	Q_{PD} (g/(L.h))
	0.05	9.10	20.54	5.51	1.12	98.98	1.02
50.7g/L	**0.10**	10.3	18.87	5.04	0.91	95.35	1.89
	0.15	12.7	16.93	3.91	0.33	90.27	2.54

In terms of the main product, the results were similar to those obtained in chapter 5 with free cell continuous fermentations of crude glycerol. Particularly, for D=0.15 h^{-1}, the productivity obtained with free cell cultures (2.73 g/(L.h)) was very close to the productivity operating with the bioparticles (2.54 g/(L.h)) for the same dilution rate. As for the by-products, in the assays carried out with the immobilized reactor, the concentration of acetic acid was considerably below the concentrations obtained in continuous free cell cultures for all dilution rates. The concentration of butyric acid was, however, similar in both cases.

The results demonstrated that the alginate bio-bead represents a good option for the immobilization system for the continuous bioconversion of 1,3-propanediol from glycerol. The productivity and yield of the continuous culture under 0.05 h^{-1} was close to the typical values of free cell cultures. Once the first steady state had been reached, a high stability of the culture was observed (higher than continuous free cell cultures) and a low variation in the results was noticed until the end of the experiment (275 h). Moreover, operations for the continuous culture and downstream process became more convenient and simple, since cells were separated from the fermentation broth by encapsulation.

In the literature, Pflugmacher and Gottschalk (1994) immobilized *Clostridium freundii* DSM 30040 on modified polyurethane particles to convert glycerol into 1,3-propanediol. Using feeding medium of 400 mM glycerol, the productivity of immobilized *C. freundii* was 4.1 g/L at the dilution rate of 0.3 h^{-1}, and 8.2 g/L h at 0.5 h^{-1}. Although the productivity was high, the maximum concentration of 1,3-propanediol achieved was 16.4 g/L.

Klebsiella pneumoniae ZJU 5205 was encapsulated in NaCS/PDMDAAC (sodium cellulose sulphate/poly-dimethyl-diallyl-ammonium chloride) microcapsules to produce 1,3-propanediol from pure glycerol (Zhao et al. 2006). According to the report, continuous fermentation was carried out with an initial substrate concentration of 40g/L, and 13.6g/L of 1,3-propanediol was achieved.

Gungormusler et al. (2011) immobilized *C. beijerinckii* in ceramic rings and pumice stones, achieving 2.5-fold higher productivity in comparison to the suspended cell system of *C. beijerinckii*.

Based on the results and the literature reviews, the use of immobilized systems brings numerous advantages, although they have hardly been used in the literature.

The molar yields were around 0.55 mol/mol when used with the entrapment technique, and 0.61 mol/mol when used with attachment cells (Gungormusler et al. 2011). These values are similar to those with free cell cultures, with the advantage of robustness, bioparticle reuse, diverse reactor configurations and culture system possibilities, handling high cell density that allows high volumetric productivity and a simpler downstream processing.

The absence of acetic acid formation in batch fermentation is another advantage observed in the present work, and this was also observed by Zhao et al. (2006) in batch immobilized experiments.

4. Conclusion

The production of 1,3-propanediol by immobilized *C. butyricum* in alginate bio-beads demonstrated desirable characteristics, including repeated cultivations with high density of biomass, enhanced substrate tolerance of cells, stable cell activity, and the removal of cells from fermentation broth.

In addition, it could create an anaerobic and stable environment in the bioreactor that is beneficial for the growth of *C. butyricum* and 1,3-propanediol formation.

The use of bioparticles could also reduce the costs of the downstream process as the spherical beads can easily be separated from the fermentation broth. Further, the immobilization decreases the potential pathogenic risk of *C. butyricum* DSMZ 10702 (recently regrouped into the risk group type 2).

The batch fermentation results with immobilized *C. butyricum* provided a higher concentration of 1,3-propanediol and substantially higher biomass concentrations. Furthermore, the acetic acid route was affected and this acid was not detected in the effluent.

In continuous fermentations, in terms of 1,3-PD production, the results were similar to those obtained in chapter 6 with free cell continuous fermentations of crude glycerol. Acetic acid was considerably below the concentrations obtained in continuous free cell cultures for all dilution rates.

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CHAPTER 8. GENERAL CONCLUSIONS

General Conclusions

Several conventional and innovative biotechnologies for the optimization of 1,3-propanediol biosynthesis from crude glycerol were herein studied, focusing on the maximization of 1,3-propanediol concentration and yield and the minimization of by-product formation.

Model assays

1. The first purpose of this work was to determine the operation conditions that provide higher concentrations of 1,3-propanediol and low concentrations of by-product formation. In assays carried out in batch systems with pure glycerol, 40 g/L of 1,3-propanediol, with a corresponding mass yield of 0.57 g/g, was achieved at 37ºC, pH 6.5 and 70 g/L of initial substrate concentration.

2. The tolerance of *C. butyricum* at higher initial pure glycerol concentrations was evaluated. The microorganism presented inhibition to values of initial pure glycerol higher than 100g/L. A good agreement was found between the experimental and predicted values of a kinetic Contois type-model.

3. Different fed-batch strategies were evaluated. The strategy of continuously feeding 1.4 g/h of glycerol provided the best results and was set as the optimal feed rate for the further fed-batch experimentations.

4. The fed-batch fermentations of pure glycerol provide 37.3 g/L of 1,3-propanediol and a mass yield of 0.53 g/g. Despite the relatively low concentration and yield (when compared with the batch system values), a total amount of 49 g of 1.3-propanediol was achieved after 80 h of fermentation.

5. In continuous cultures of pure glycerol, the highest concentration of 1.3-propanediol was 23.5 g/L and this was obtained for 70 g/L of feed glycerol at $D=0.05\ h^{-1}$. Low values of by-products were produced at these conditions: 3.93 g/L of butyric acid and 0.86 g/L of acetic acid. Furthermore, a lower production

of by-products, when compared with the batch and fed-batch systems, was observed in all the continuous fermentations.

The lower production of by-products represents an advantage of the continuous strategy in comparison with the batch and fed-batch systems. Moreover, higher productivity was obtained in continuous cultures.

Crude glycerol assays

1. The batch fermentation of crude glycerol without further purification produces significant amounts of 1,3-propanediol (35.9 g 1.3PD, $Y_{PD/S}$= 0. 0.51gg^{-1}) at 37°C, pH = 6.5 and 70 g/L glycerol. The maximum tolerance of *C. butyricum* DSM 10702 to the inhibitory effect of the crude substrate was 70 g/L, lower than the maximum tolerance for the pure substrate, but still allowing fermentation at the conditions that provide the higher 1,3-propanediol concentration and yield (37ºC, pH 6.5 and 70 g/L of initial substrate concentration).

   The results were found to be very satisfactory, when compared with bibliographic reports where some *Clostridium* strains were reported to be strongly inhibited, or to not grow at all, by the use of untreated crude glycerol.

2. The kinetic Contois type-model also successfully simulates the batch fermentation of crude glycerol. The maximum specific estimated growth rate was similar for both substrates, although slightly higher for pure glycerol, indicating a low inhibition of the microorganism by crude glycerol.

3. In fed-batch fermentations, the maximum 1,3-propanediol concentration was 36.1 g/L, close to the 37.3 g/L reached in fed-batch pure glycerol experiments. Acetic and butyric acid concentrations reached, respectively, 2.5 g/L and 9.0 g/L. These values were also similar to those reached in the model assays.

4. The continuous cultures with crude glycerol provided 27.5 g/L of 1.3-propanediol at a dilution rate of 0.05 h^{-1} and 70 g/L of glycerol in the fed stream. This value is higher than that obtained with pure substrate under the same

conditions. The presence of traces of methanol on crude glycerol from biodiesel manufacturing may produce a stimulating effect on microorganism cell growth, which could explain the high concentration and productivity achieved in crude glycerol fermentations.

5. Although the highest concentration of 1,3-propanediol has been reached for 0.05 h^{-1} and 70 g/L of glycerol, for 0.15 h^{-1} and S_0=50g/L, 21.5 g/L of 1,3-propanediol was reached with a lower production of butyrate and acetate, while at the same time tripling the productivity.

Pretreated crude glycerol assays

1. The production of 1,3-propanediol by using pretreated crude glycerol was improved for the two pretreatements tested. Furthermore, *C. butyricum* DSM 10702 demonstrated a higher tolerance to high concentrations of pretreated crude glycerol. The GF resins provided higher concentrations of 1,3-propanediol and a higher tolerance of the microorganism at high concentrations of substrate. Nevertheless, the pretreatment using LEWATIT S8528 represents a practical option with lower costs and a high production of 1,3-propanediol.

2. The serum bottle results reveal that the presence of salts between 4 and 5 wt% in crude glycerol does not cause *C. butyricum* DSM 10702 inhibition. The principal inhibitory agent seems to be in the non-glycerol organic matter, and is most likely the free fatty acids, such as oleic and linoleic acids.

3. The production of 1,3-propanediol using pretreated crude glycerol (PCG) as a sole carbon source was practical in the two pretreatments tested.

   In batch systems, the use of PCG increased the 1,3-propanediol concentration and yield (41.4 g/L and 0.56 g/g respectively), reaching values close to those of pure glycerol. Furthermore, the by-products, acetic and butyric acids, present similar concentrations in both cases. A considerable decrease in fermentation time was observed. An increase in the productivity (g/L/h) and the biomass concentration of pretreated glycerol, as compared to that of pure glycerol, can be

attributed to the presence of certain growth-promoting nutrients, such as the potassium and magnesium ions or methanol present in the crude glycerol that were not removed in the pretreatment step.

4. In fed-batch fermentation, 54.2 g of 1,3-propanediol was accumulated in the fermentation broth. Significant differences were also observed in the formation of by-products. The amount of butyric acid formed, when PCG was used as substrate, was higher than the amount found in the pure glycerol fed-batch fermentation. On the other hand, the formation of acetic acid was higher when pure glycerol was used as the substrate. Furthermore, volumetric productivity (Q_{PD}) was higher for the PCG fermentation.

Immobilized cell bioreactor

1. *C. butyricum* DSM 10703 was successfully immobilized in 2.5-3 mm spherical alginate bio-beads and demonstrated desirable characteristics, such as the possibility of repeated cultivations with a high density of biomass, enhanced substrate tolerance of cells, stable cell activity, and the removal of cells from the fermentation broth. The use of bioparticles could reduce the cost of the downstream process, as the bio-beads can easily be separated from the fermentation broth.

2. The batch fermentation results carried out in serum bottles using the bioparticles and crude glycerol as substrate provided a higher concentration of 1,3-propanediol (13.77 g/L in immobilized cells vs. 11.56 g/L in free cells cultures). Substantially higher biomass concentrations were achieved (3.80g /L), which was approximately 2 times higher than that of the free cell culture. Furthermore, the by-product, acetic acid, was not detected in the effluent.

3. In continuous fermentations of crude glycerol, 20.54 g/L of 1,3-propanediol was achieved at 0.05 h^{-1} and 50 g/L of feed substrate concentration. 1,3-propanediol concentrations and yields were similar to those obtained with free cell continuous fermentations of crude glycerol. However, once again, acetic acid

was considerably below the concentrations obtained in continuous free cell cultures for all dilution rates.