Directing Product Formation by Mixed Culture Fermentation

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Summary

Biobased industry generates huge amounts of waste streams that are very rich in organic compounds. The conversion of these streams into valuable products will not only reduce the amount of waste as it will also contribute to improve the sustainability of these processes. Mixed culture biotechnology may become an attractive option to convert agricultural waste streams into valuable products. Open mixed cultures consist of a mixture of microorganisms of high diversity, as found in nature, which is used as an inoculum to start a biological production process. Depending on the conditions imposed, the microorganisms that best adapt will dominate. Mixed culture biotechnology has specific advantages over pure culture biotechnology: does not require sterile conditions, there is no risk of strain degeneration and due to the high microbial diversity, the mixed culture has a higher ability to convert mixtures of substrates.

The process investigated in this thesis is the first stage of a proposed two steps process that aims at producing biodegradable polymers (polyhydroxyalkanoates, PHA) from organic waste streams. The first step consists of the anaerobic conversion of carbohydrate containing streams into a stable mixture of smaller molecules, as volatile fatty acids and alcohols. These small molecules are used as substrates for the second process, during which the substrate is accumulated as intracellular polymers (PHA).

The goal of this thesis is to investigate fermentation by an open mixed culture, aiming at controlling the conversion of organic compounds present in a waste stream into a known and stable range of fatty acids and/or solvents. Experiments were conducted in CSTR type bioreactors operated at various pH-values, with different substrates at various concentrations, under fully anaerobic conditions. The mixed microbial culture was fed with defined nutrients solution and the performance was characterized under different conditions.

First experiments were conducted with glucose as sole carbon and energy source. Glucose is the most abundant sugar and has been extensively studied in mixed culture fermentation processes under many different conditions. In chapter 2, the product spectrum of glucose fermentation by mixed culture was investigated under different pH values. All products in the experimental system were determined and a link between the product

spectrum as a function of the pH, and the thermodynamic state of the system was elaborated based on generalized biochemical principles.

In chapter 3, xylose fermentation by mixed culture was investigated. In agricultural waste streams, glucose is often accompanied by other sugars and xylose is one of the most abundant C5 sugars. Additionally, the two substrates were studied as the sole carbon source and as a mixture of the two substrates, in a chemostat at pH 8. The product spectrum obtained at each condition was compared. Short pulse experiments were also performed to evaluate the conversion under substrate non-limiting conditions. Despite the similar product spectrum, it was observed that the biomass yield on xylose was, in every condition, significantly lower than on glucose (80%). This result is associated to the xylose catabolism, which seems not to generate the same amount of ATP per product formed. Two possible explanations are suggested for the higher energy expended during xylose metabolism when compared to glucose: first, the xylose uptake system and its activation are not coupled, meaning one more ATP is consumed per mole of substrate consumed; or second, the isomerization reactions involved in the pentose phosphate pathway (conversion of C5 units into C3 units) may require some extra energy.

As a result of the increase of the biodiesel industry, the supply of glycerol is dramatically increasing. In chapter 4, glycerol fermentation by mixed culture was investigated at pH 8 and compared with glucose fermentation. Glycerol is more reduced than glucose, and this was reflected in the product spectrum. Under substrate limiting conditions, ethanol was the major catabolic product of glycerol fermentation. The conversion of 60% of the carbon into ethanol seems to be a very promising process for future research. However, when increasing influent substrate concentrations were tested the product spectrum changed. A metabolic shift into 1,3-propanediol and acetate was observed and seemed to be a consequence of the higher biomass pecific substrate conversion rates.

In mixed culture fermentation (MCF) systems, based on the operational conditions set, it is possible to define the thermodynamic state of the system. And in this way, to predict which catabolic product(s) allows the more efficient growth. Therefore, this product (spectrum) is suggested to dominate independent of the population present. One fundamental question that needed to be answered during this research, was whether the process

conditions were determining the product spectrum or was the product spectrum resulting of the established microbial community. In chapter 5, the microbial diversity established at each condition studied was assessed by PCR-DGGE analyses. From these analyses it was observed that: the operational pH and the substrate used had a clear impact on the population established. In some situations, however, a metabolic shift was observed (e.g. addition of a second substrate or increase of the influent substrate concentration), which was not always associated to a shift in the microbial population. Therefore, these results did not allow us to exclude the relevance of the microbial population present and of the operational conditions applied in the determination of the product spectrum.

In conclusion, this thesis presents a picture of the possibilities of products that can be obtained from fermentation with a mixed culture when operated under different (steady) conditions. A stable product composition can be established under substrate limiting conditions. At this stage, it could be directly used to feed the second reactor where the volatile fatty acids and alcohols are accumulated as PHA. Chapter 6 summarizes and integrates the main results and conclusions drawn through this research. A set of questions that remained after this study and recommendations for future research are also presented in this chapter.

Samenvatting

Agro-industrie produceert grote hoeveelheden afvalstromen, die veel organische stoffen bevatten. Omzetting van deze afvalstromen in hoogwaardige producten vermindert niet alleen de hoeveelheid afval, maar verbetert ook de duurzaamheid van de processen in deze sector. Mengcultuur biotechnologie kan een aantrekkelijk alternatief worden om agrarische afvalstromen om te zetten in hoogwaardige producten. Open mengculteren bestaan uit een zeer divers mengsel van micro-organismen net zoals in de natuur. Deze worden gebruikt als inoculum om een biologisch productie proces op te starten. Afhankelijk van de opgelegde condities zullen de micro-organismen domineren met de hoogste affiniteit voor het groeilimiterende substraat. Mengculturen hebben specifieke voordelen ten opzichte van reinculturen in de biotechnologie: steriele condities zijn niet nodig, er is geen risico op stam degeneratie en dankzij de grote microbiële diversiteit zijn mengculturen beter in staat om mengsels van substraten om te zetten.

Het onderzochte proces in dit proefschrift is de eerste stap van een twee-staps proces, dat gericht is op het produceren van biologisch afbreekbare polymeren (polyhydroxyalkanoaten, PHA) uit organische afvalstromen. De eerste stap bestaat uit de anaërobe omzetting van koolhydraatrijke stromen in een stabiel mengsel van kleine moleculen zoals vluchtige vetzuren en alcoholen. Deze kleine moleculen dienen als substraat voor het tweede proces waarin het substraat wordt omgezet in intracellulaire polymeren (PHA).

Dit onderzoek is gericht op controle van de conversie van organische componenten in een afvalstroom door fermentatie in open mengculteren teneinde te komen tot een bekende en stabiele reeks producten, zoals vetzuren, alcoholen en/of ketonen. Experimenten werden uitgevoerd in CSTR type reactoren bij verschillende pH-waarden, met verschillende substraten in verschillende concentraties, onder volledig anaërobe omstandigheden. Gemengde microbiële culturen werden gevoed met een gedefinieerde nutriënten oplossing en de productiviteit werd gekarakteriseerd voor verschillende condities.

Eerst werden experimenten uitgevoerd met glucose als de enige koolstof- en energiebron. Glucose is de meest voorkomende suiker en is al veelvuldig bestudeerd onder verschillende condities in fermentaties met een mengcultuur. In hoofdstuk 2 is het productspectrum onderzocht van

fermentatie van glucose met een mengcultuur bij verschillende pH waarden. Alle producten van het experimentele systeem werden bepaald en een verband tussen het product spectrum als functie van de pH en de thermodynamische staat van het systeem werd verder uitgewerkt op basis van algemene biochemische principes.

In hoofdstuk 3 wordt het onderzoek naar de fermentatie van xylose met een mengcultuur beschreven. In agrarische afvalstromen bevinden zich naast glucose andere suikers en xylose is een van de meest voorkomende C5-suikers. Daarnaast werden de twee suikers bestudeerd apart als enige koolstofbron en als een mengsel van de twee substraten in een chemostaat bij pH 8. De bij verschillende condities verkregen productspectra werden met elkaar vergeleken. Ook werden korte puls experimenten uitgevoerd om de conversie onder niet-limiterende substraat concentraties te bepalen. Ondanks een vergelijkbaar productspectrum werd voor elke conditie een lagere biomassa opbrengst op xylose (80%) dan op glucose gemeten. Dit resultaat lijkt gerelateerd aan het xylose katabolisme waarin niet dezelfde hoeveelheid ATP per gevormd product wordt gegenereerd. Twee mogelijke verklaringen worden voorgesteld voor het hogere energie verbruik in xylose metabolisme ten opzichte van glucose. Ten eerste zijn het xylose opname systeem en de activatie hier van niet gekoppeld wat betekent dat één ATP meer geconsumeerd wordt per mol geconsumeerd substraat. Ten tweede hebben de isomerisatie reacties in de pentose fosfaat route (conversie van C5 eenheden in C3 eenheden) mogelijk extra energie nodig.

Als een gevolg van de uitbreiding van de biodiesel industrie is het aanbod van glycerol enorm toegenomen. In hoofdstuk 4 is glycerol fermentatie met mengculturen bij pH 8 onderzocht en vergeleken met glucose fermentatie. Glycerol is meer gereduceerd dan glucose en dit was terug te zien in het product spectrum. Onder substraat gelimiteerde condities was ethanol het belangrijkste product van de glycerol fermentatie. De omzetting van 60% van de koolstof in ethanol lijkt een veelbelovend proces voor de toekomst. Echter wanneer hogere substraat concentraties werden getest veranderde het productspectrum. Een metabole verschuiving naar 1,3-propaandiol en acetaat werd waargenomen en leek een gevolg te zijn van de hogere specifieke substraat omzettingssnelheden.

In mengcultuur fermentatie (MCF) systemen, die gebaseerd zijn op de operationele condities, is het mogelijk om de thermodynamische toestand van het systeem te definiëren. En op deze manier te voorspellen welk katabool product een efficiëntere groei mogelijk maakt. Daarom wordt voorgesteld dat dit productspectrum domineert onafhankelijk van de aanwezige populatie. Een fundamentele vraag die beantwoord moest worden tijdens dit onderzoek was of dat de procescondities het productspectrum bepaalden of dat het product spectrum een resultaat was van de dominante microbiële populatie. In hoofdstuk 5 werd de microbiële diversiteit van elke onderzochte conditie bepaald met behulp van PCR-DGGE analyse. Uit deze analyses kwam naar voren dat de ingestelde pH-waarde en het gebruikte substraat een duidelijke invloed hadden op de ontstane populatie. In sommige situaties (bijvoorbeeld toevoeging van een tweede substraat of invloed van de substraatconcentratie) werd echter een metabole verschuiving vastgesteld welke niet was gekoppeld aan een verandering in de microbiële populatie. Deze resultaten sluiten de relevantie van de aanwezige microbiële populatie en de opgelegde operationele condities voor het bepalen van het productspectrum echter niet uit.

Concluderend toont dit proefschrift de mogelijke producten die verkregen kunnen worden met fermentatie met een mengcultuur onder verschillende (stabiele) procescondities. Een stabiele product samenstelling kan bereikt worden onder substraat gelimiteerde condities. Op dit moment kan het direct als substraat dienen voor de tweede reactor waarin de vluchtige vetzuren en alcoholen worden geaccumuleerd als PHA. Hoofdstuk 6 worden de belangrijkste resultaten en de uit dit onderzoek getrokken conclusies geïntegreerd en samengevat. Ook worden een aantal vragen, die overblijven na dit onderzoek, en aanbevelingen voor toekomstig onderzoek in dit hoofdstuk aangedragen.

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Chapter 1

General Introduction

General Introduction

Microbial production of chemicals and biofuels

By 2010, it is estimated that biotechnological industry may contribute up to 10% of the global chemical production market (US $125 billion) (www.europabio.com). This market includes all sorts of organic chemicals that are currently mostly produced from non renewable feedstocks: petroleum, natural gas, and coal feedstocks.

Our society is dealing with the shortage of fossil fuels and chemical feedstocks and, on the other hand, with the increase of wastes generated by municipalities, agriculture and industries. Biobased industry, the conversion of renewable resources or wastes to chemicals and fuels, by microbial fermentations or enzymes has been receiving increasingly attention (Angenent et al. 2004; Gross and Kalra 2003; Ragauskas et al. 2006). The aim is to develop new technologies, increase efficiencies and reduce the costs in fermentation, bioconversion, and in downstream processing.

Anaerobic microbial fermentations form the basis for microbial production of chemicals. In the absence of an external electron acceptor, the catabolism of organic matter results in a variety of organic compounds; while catabolism in aerobic conditions mainly generates CO_2 and H_2O. Fermentations are therefore of interest: higher substrate-to-product conversions, lower biomass yields and no need for aeration.

Carbohydrates can be converted to a variety of different chemicals and byproducts during anaerobic fermentations. These include several organic acids (acetic, propionic, butyric and lactic acids), solvents (ethanol and butanol) and gases (molecular hydrogen and methane). One of the main limitations of the biological production of chemicals is that except for lactate and ethanol, the main products are always accompanied by significant amounts of secondary product. This not only decreases the process efficacy but is also associated to high downstream processing costs. Currently, the most relevant in terms of industrial production, are the ethanol and lactic acid fermentations (Hahn-Hagerdal et al. 2006; John et al. 2007).

The annual biological production of lactic acid is 130-150 thousand tones (John et al. 2007). Microbial production of lactic acid is used for industrial purposes like food additive or polylactic acid polymers (PLA) production. One of advantages of the biological process is the stereospecificity of the lactic acid formed. Which of the isomers is produced depends on the microorganism applied in the process (John et al. 2007). In this process,

homolactic bacteria are used as they show a high product specificity and high productivity rate.

For ethanol production, mainly driven by the growing demand for energy for transportation and the limited resources of fossil fuels, yeasts are generally preferred due to the significant higher tolerance towards ethanol compared to bacteria (Hahn-Hagerdal et al. 2006). Ethanol can be used blended with petrol or used directly as car fuel. Currently, it is mainly produced from sugarcane (Brazil) or corn (USA). In 2005, the world production was 33 GL ((Agarwal 2007), http://www.ren21.net/), this amount is far below the global potential 490 GL $year^{-1}$ if wasted crops and crop residues could also be converted into ethanol (Kim 2004).

Conversion of waste streams

A great challenge is to develop fermentation technology that employs the underutilized biomass residues, which are often considered as wastes. Treatment of these streams typically requires net energy input without generation of a useful product. Worldwide, about 1.5 Pg $year^{-1}$ of dry lignocellulosic biomass becomes available, consisting of wasted crops and crop residues, 5 and 95 % respectively (Kim 2004). Despite being rich in carbohydrates, the utilization of agricultural wastes is still limited due to the low protein content or poor digestibility (Pandey et al. 2000). Of the major biomass polymers, α-cellulose, hemicellulose and amylopectin are directly fermented by anaerobic bacteria, whereas lignin is totally recalcitrant (Zeikus 1980).

The use of open mixed culture can be an option to convert these waste streams. Open mixed cultures, based on natural inocula with a high microbial diversity allow operating bioprocesses under non-sterile conditions with no risk of strain degeneration. As opposed to pure culture processes, mixed culture are furthermore capable of dealing with substrate mixtures of variable composition. Although a mixed culture involves a higher microbial diversity, selection occurs for a limited number of microorganisms that are best capable to adapt to the imposed conditions.

Anaerobic digestion (AD) is at present a successful process to convert dissolved, suspended or solid organic wastes into methane containing biogas by mixed culture (van Andel and Breure 1984; Verstraete et al. 1996). AD presents important advantages compared to aerobic processes like the generation of energy as methane, instead of energy consumption. Since the process runs in the absence of an electron acceptor, the energy generated for the microorganisms is much lower. This results in a significant lower volume

of biomass that should be regarded as an unwanted sideproduct in this kind of processes. In methanogenic environments, organic compounds are sequentially converted by different microbial populations: fermentative, acetogenic and methanogenic bacteria (Gujer and Zehnder 1983; Stams 1994). During fermentation, organic matter is converted into volatile fatty acids, lactic acid, alcohols, carbon dioxide and molecular hydrogen. The acetogenic bacteria convert further the shorter organic acids into acetate. At last, the methanogenic population converts part of these products into methane. These archaea are specialized microorganisms that can only use a limited range of substrates: acetate, formate, or CO_2 and H_2. The end product of the anaerobic digestion process is biogas, composed by similar amounts of CO_2 and methane. After purification, biogas can be burned and converted to electricity and heat in a combined heat and power plant.

Another product that has been receiving increasing interest by researchers is molecular hydrogen (Hallenbeck and Benemann 2002). Hydrogen combustion results in only water, which is a very exergonic reaction, reason why molecular hydrogen is such an interesting fuel. Despite the advantage of being a gas and easily separated from the fermentation broth, the conversion of organic substrates into molecular hydrogen by microbial still shows very low product yields (Angenent et al. 2004). A different option is the production of hydrogen by photosynthetic microorganisms, but such processes also present low efficiencies and would require large surfaces for light capture (Hallenbeck and Benemann 2002).

A new process that combines organic substrate degradation with energy production, in the form of electricity, is the microbial fuel cell (MFC) (Cheng and Logan 2007; Rabaey and Verstraete 2005). MFCs use an insoluble electron acceptor (MFC anode) for electron transfer. The electron transfer can either occur via membrane-associated components, or soluble electron shuttles (mediators). The electrons, by flowing from the anode to the cathode, where an electron acceptor is reduced, create an electrical current and the off-gas is mainly carbon dioxide. Currently, MFCs are not competitive due to the high material costs and to the low power densities reached so far.

Another process to convert agricultural wastes is through gasification. By introducing a limited amount of oxygen to the combustion, the carbon compounds are mainly converted into CO and H_2. Both, molecular hydrogen and CO can be used in a chemical process, e.g. Fischer-Tropsch, to produce liquid hydrocarbons of various forms. This process involves condensation reaction of variable amounts of H_2 and CO, with chemical catalysts at high

temperatures and pressures. The CO is one of the main components of Syngas, but can also be obtained from methane.

An alternative to the production of energy, as methane or hydrogen, could be the production of intermediates of the methanogenesis. This can be established by inhibiting methane production using operational measures like lowering the pH or decreasing the biomass growth rate. Examples of intermediates are organic acids (acetate, butyrate, lactate and succinate), solvents (ethanol, butanol and acetone) and energy carriers like molecular hydrogen. Which mixture of products are formed will depend on the substrate treated, the microbial population present and the operational conditions (Lengeler et al. 1998; Rodriguez et al. 2006; Zoetemeyer et al. 1982a; Zoetemeyer et al. 1982c). These bulk chemicals can be either used in other chemical processes or directed into other bioprocesses.

One application of the fermentation products is to use as feeding medium for the biological production of polyhydroxyalkanoates (PHA) by open mixed culture. During this process, the organic compounds are assimilated as intracellular storage polymers, as PHA. Polyhydroxyalkanoates are a group of polyesters that are biodegradable and made from renewable resources (Byrom 1987; Reis et al. 2003). They have properties similar to petrochemical plastics. PHAs are naturally produced by many bacteria as an internal storage material for substrate and energy from organic feed stocks (sugars, fatty acids, alcohols and fats) (Byrom 1987). Accumulation of PHA occurs when microorganisms are submitted to discontinuous feeding. By immediately accumulating the substrate when it is present, the bacteria are then able to grow when it becomes absent, providing a competitive advantage over the other microorganisms that can only grow while substrate is present (Reis et al. 2003; van Loosdrecht et al. 1997). An advantage of producing storage polymers compared to dissolved products is the easier separation from the liquid phase. During extraction of PHA from the cells, the PHA can be hydrolyzed into the main monomers (e.g hydroxybutyrate, hydroxyvalerate, etc), which are economically more valuable than the substrates converted.

A summary of the possibilities to convert agricultural wastes is illustrated in figure1.1.

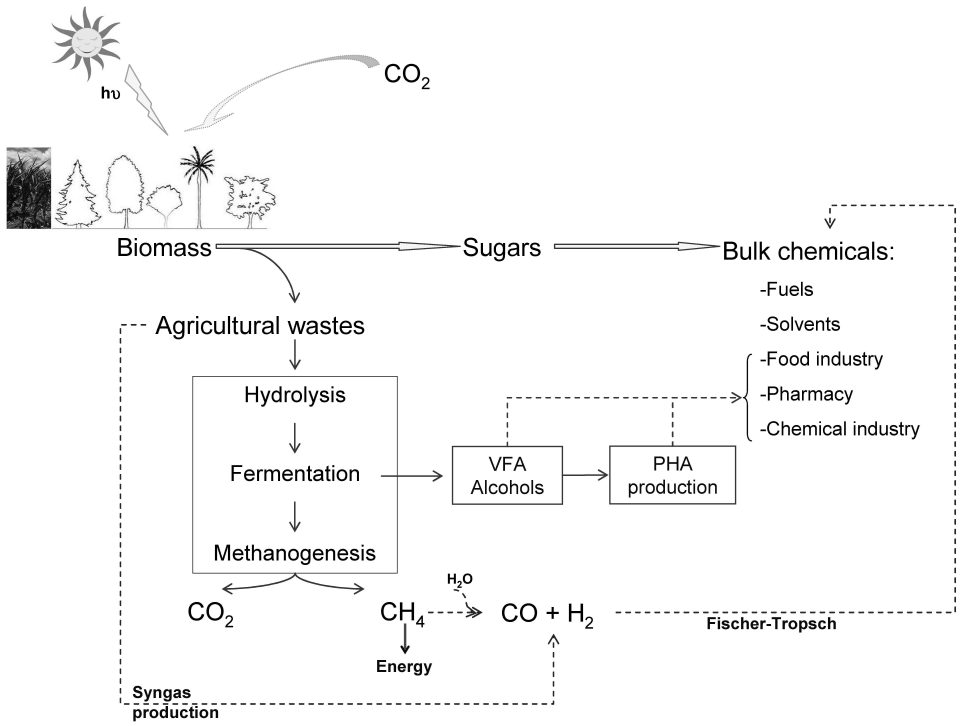

Figure 1.1-Alternatives for converting agricultural wastes. Biological conversions are indicated with solid lines and physico-chemical conversions with dashed lines.

In order to implement mixed culture fermentation process it is required to be able to predict the product spectrum as a function of the operational conditions imposed. This thesis aims at gaining insight into the fermentation process with open mixed cultures, by answering to two important questions: first, which products are obtained under which conditions (and why). And second, to which extent a stable product spectrum can be established. To this end, mixed culture fermentation was studied in a CSTR with different carbon sources under different conditions (pH, different substrates, substrate concentration, mixture of substrates) and the microbial population established at each condition was characterized.

Approach to study mixed culture fermentation

Glucose is used as the reference substrate in this work. Glucose is the most dominant carbohydrate in nature and has a central role in the whole metabolic chain. It is one of the main products of photosynthesis and starts cellular respiration (aerobic/anaerobic) in both prokaryotes and eukaryotes. In industrial biotechnology, glucose is the main substrate for the production of chemicals by microorganisms (enzymes, antibiotics, etc). Due to its high fraction in agricultural products, glucose fermentation by mixed microbial culture has been widely studied with different aims such as at the production of molecular hydrogen, a specific organic acid, or ethanol production (Fang and Liu 2002; Horiuchi et al. 2002; Ren et al. 1997; Willke and Vorlop 2004). Experimental results have shown the dependency of the ratios between different products formed on the operational conditions (pH, substrate concentration, growth rate, temperature, etc) (Fang and Liu 2002; Horiuchi et al. 2002; Zoetemeyer et al. 1982a; Zoetemeyer et al. 1982b; Zoetemeyer et al. 1982c). Despite the sometimes comparable trends observed in the different studies, a clear relation between the metabolic and thermodynamic state of the system has not been established.

In this thesis, we will try to link the process conditions with the product spectrum obtained, the thermodynamic state of the system and the metabolic diversity in the system, for a mixed culture fermentation process.

Thermodynamic characterization of a process

Thermodynamics study the flow of energy in a system. This energy can be exchanged as heat or work. The thermodynamic laws have been applied to chemical and biochemical processes providing a basis for analysis of experimental results (Hanselmann 1991; Heijnen and Vandijken 1992; von Stockar et al. 2006). The thermodynamic characterization of mixed culture fermentation systems suggests methods for analysing the possibilities to manipulate the product profiles, and which branches may control their production (Kohn and Boston 2000; Rodriguez et al. 2006). At constant temperature and pressure, the Gibbs free energy function of a chemical reaction at standard conditions, ΔG^0, can be written as

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \qquad \text{(eq. 1.1)}$$

Where ΔH^0 and ΔS^0 are the enthalpy and entropy variation, and T the temperature. These are state functions that only depend on the reactants and products and not how they are formed. Additionally, under a constant pressure, the change in free energy of a reaction is a function of the activity of

the products and reactants

$$\Delta G = \Delta G^0 + RT \ln \frac{\prod_j \{\mathrm{Pr}\, oducts\}^{vj}}{\prod_i \{\mathrm{Re}\, ac\, \mathrm{tan}\, ts\}^{vi}} \qquad \text{(eq. 1.2)}$$

The vj,i are the stoichiometric coefficients, R is the gas constant, 8.314 $J.K^{-1}mol^{-1}$ and T the temperature in Kelvin. Although thermodynamics tells which direction the reaction will go, it says nothing about the reaction rate.

Metabolic diversity

In most microbial systems, carbohydrate monomers are converted into pyruvate, ATP and NADH. This process is called glycolysis and there are three main pathways of enzymatic reactions that can take place in heterotrophic microorganisms. The alternatives are the Embden-Meyerhof pathway (EMP), the Entner-Doudoroff pathway (EDP) and the phosphoketolase pathway (PKP). In case of glucose as carbon source, the pathways can be summarized as in the following equations:

$$1Glu\,\mathrm{cos}\,e- > 2\,pyruvate + 2\,NADH + 2\,ATP \qquad \text{EMP}$$

$$1Glu\,\mathrm{cos}\,e- > 2\,pyruvate + NADH + NADPH + ATP \qquad \text{EDP}$$

$$1Glu\,\mathrm{cos}\,e- > pyruvate + Acetyl - Pi + CO_2 + 2\,NADH + ATP \qquad \text{PKP}$$

Pyruvate is a key metabolite, it stands in the center of the carbon metabolism. ATP stands for adenosine 5'-triphosphate and is the main molecular carrier for metabolic energy. NAD(H) and NADP(H) (nicotinamide adenine dinucleotide (phosphate)) are two important electron mediators involved not only in substrate conversion but also in the biomass synthesis. Both ATP and NAD(P)H are conserved moieties, exist in limited amounts inside the cell and their production rate equals their consumption rate in the cell. In fermentative microorganisms the ATP is mainly produced via substrate level phosphorylation (SLP). This process consists of the transfer of a phosphate group from a reactive intermediate (energy rich compound, contains either a phosphate group or a coenzyme-A group, the hydrolysis is highly exergonic); ATP production can also be coupled electron transport (ETP) when in the presence of a certain electron acceptor (Thauer et al. 1977). In fermentation processes these electron acceptors are absent and, therefore, ATP production mainly occurs via SLP.

In energetic terms, the glycolysis can be split in two stages. During the first stage, there is energy investment; ATP is consumed for glucose uptake and to activate the process. In the second phase, highly exergonic reactions are

involved and a net production of two ATP occurs. The overall glycolysis process is exergonic ($\Delta G^{0'}$=-112 kJ/mol), being part of the energy generated conserved with the formation of two ATP.

During glycolysis also NADH is generated that must be reoxidized to allow glycolysis to continue. In the absence of an external electron acceptor, the main way to reoxidize NADH is to transfer its electrons to part of the carbon source present. So, in fermentation processes part of the carbon source is oxidized and part is usually reduced. Whereas glycolysis is an irreversible process, the catabolic reactions from pyruvate are very close to equilibrium. There are several catabolic pathways that allow the microorganism to fulfill its metabolic requirements. In Figure 1.2, the main reactions deriving from pyruvate into the main catabolic products are illustrated. Concerning the free energy associated to each catabolic reaction (Table 1.1), under standard conditions every reaction is favourable, but only some are coupled to ATP formation (acetate and butyrate). This is an important factor for the cell metabolism, because if the cell is not able to conserve the energy as e.g. ATP, it might be lost as heat without performing work (McCarty 1965; Thauer et al. 1977). Furthermore, because intracellular concentrations of reactants are low compared to the products, according to the second law of thermodynamics, some of the possible reactions may no longer proceed if the free energy associated becomes positive. Under these circumstances thermodynamics rather than kinetics control which pathways are active.

Table 1.1- Free energy associated of the main catabolic reactions derived from pyruvate (Thauer et al. 1977).

Metabolic reaction	**ATP**	**$\Delta G^{0'}$ (kJ/mol)**
Pyruvate + $2H_2O$ → Acetate + H_2 + HCO_3^- + H^+	1	-94.2
Pyruvate + $2H_2$ → Propionate + H_2O	-	-123.0
2Pyruvate + $2H_2O$ → Butyrate + 2 HCO_3^- + H^+	1	-142.7
Pyruvate + H_2 → Lactate	-	-43.1
Pyruvate + HCO_3^- + $2H_2$ → Succinate + H_2O	-	-102.9
Pyruvate + H_2O + H_2 → Ethanol + HCO_3	-	-56.9
2Pyruvate + $3H_2O$ → Isopropanol + H_2 + 3 HCO_3^- + H^+	-	-182.5
2Pyruvate + H_2O + $2H_2$ → Butanol + 2 HCO_3^-	-	-159.0
2 Pyruvate + H_2O + H_2 → 2,3-Butanediol + 2 HCO_3^-	-	-84.5

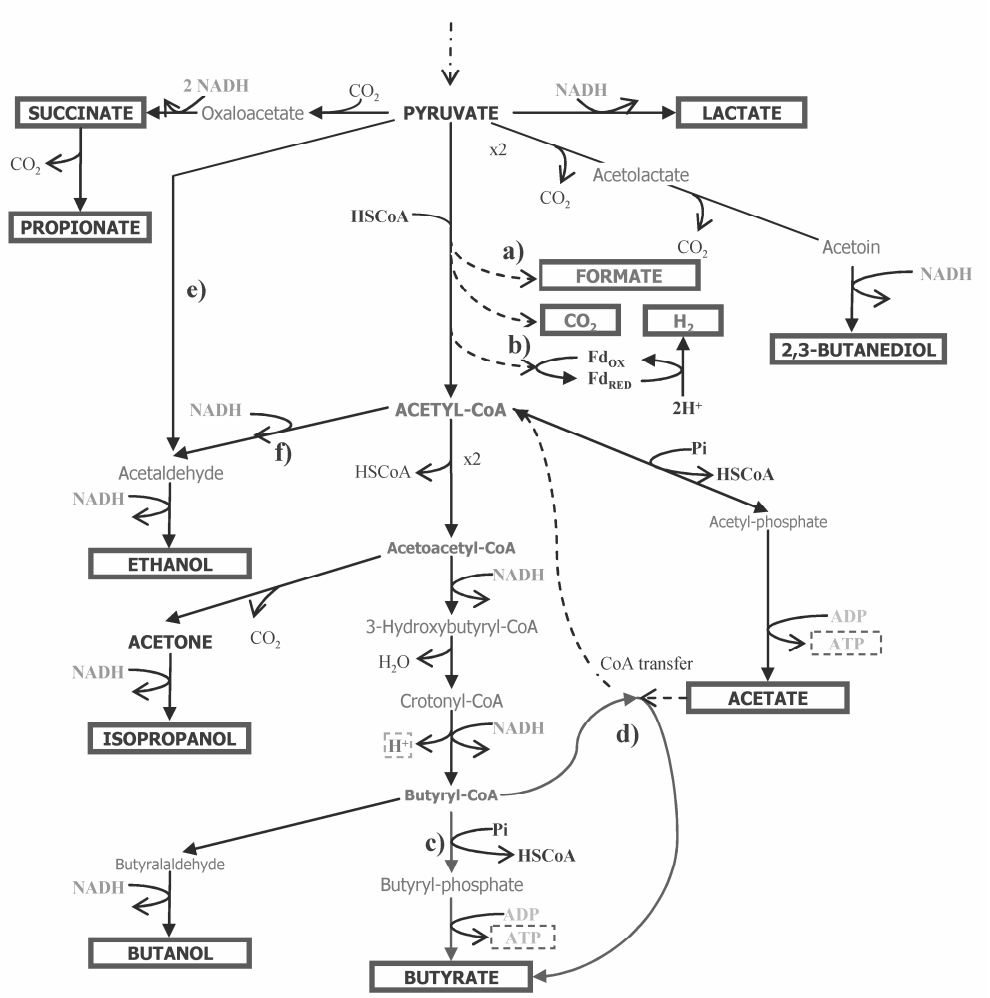

Figure 1.2- Adapted scheme with the most important and expected metabolic pathways during anaerobic fermentation by mixed culture. The reactions indicated with (a) and (b), (c) and (d) and (e) and (f) are alternative pathways for the same product, depending mainly on the type of microorganism (Lengeler et al. 1998; Papoutsakis 2000; Papoutsakis and Meyer 1985; Sawers 2005).

In principle, no microorganism has all the metabolic pathways shown, but in general has more than one possibility to catabolize the central intermediate pyruvate. This way the ATP and thermodynamic efficiency of the ATP synthesis are variable and adjustable to the growth conditions.

Another way of conserving energy is via membrane transport processes. During this process the free energy associated with transport processes can be converted into energy by coupling to translocation of cations or protons across the cytoplasmic membrane (Konings et al. 1997). The

electrochemical gradient of a proton exerts a force on these ions named proton motive force (pmf) and is described by:

$$pmf = \Delta\Psi - \frac{\ln 10 RT}{F}\Delta pH\,(mv) \qquad \text{(eq.1.3)}$$

The pmf is composed of two components: an electrical potential ($\Delta\Psi=\Psi_{IN}-\Psi_{OUT}$) and a chemical potential of protons ($\ln(10)RT\Delta pH/F$) across the membrane. The pmf generated can drive energy requiring processes, such as ATP synthesis. One well studied example is the extrusion of lactic acid at different pH values. At low pH values lactate is exported by a proton symport system and does not generate membrane potential. At high pH values, however, the same transporter excretes lactate together with two or more protons, generating pmf (Konings and Booth 1981). The two main factors determining the potential of pmf generated are the pK_a of the organic acid and the pH gradient across the membrane (van Maris et al. 2004). To which extent other organic acids (like acetate or butyrate) transporters have the same mechanism remains unknown.

Microbial growth

Microbial metabolism is a set of chemical reactions that occur in living cells and is composed of two main processes: anabolism and catabolism. The anabolism consists on the assembly of smaller molecules into biomass, while catabolism involves the chemical reactions that will provide the energy (ATP) needed for the anabolism.

Compared to aerobic growth, the energetic yield of anaerobic processes is significantly lower. The aerobic conversion of one mole of glucose generates between 36-38 moles of ATP. Two moles of ATP are generated during glycolysis by substrate level phosphorylation (SLP) and the rest are formed in the Krebbs cycle and electron transport chain (oxidation of the NADH and FADH generated during the metabolism). In the absence of an electron acceptor, however, ATP production is only possible via SLP and in same situations via pmf. This means that only two moles of ATP are produced per mole of glucose during glycolysis and, possibly, one more ATP if acetate or butyrate is produced. As a consequence, in order to generate the same amount of ATP as required for growth or maintenance, a considerable amount of carbon is "wasted" as catabolic products needed to generate this ATP and reoxidize the NADH generated in the meantime, resulting in a lower amount of biomass per carbon converted. Nevertheless, the total energy dissipated per C mole of biomass formed under aerobic and anaerobic conditions is highly comparable. Whereas in aerobic processes most of the Gibbs energy is

dissipated as heat; in anaerobic conditions, the Gibbs energy dissipated is due to the increase of the chemical entropy related to the high number of molecules formed during biomass formation (Heijnen and Vandijken 1992).

Scope of this thesis

The aim of this thesis is the understanding of the fermentation process by mixed culture. In order to apply the fermentation products on other processes, it is needed to be able to control the product composition and to predict the impact of the operational conditions on the overall performance. Hence, in the second chapter, glucose fermentation by mixed culture at different pH values is evaluated. In addition, a link between the product spectrum as a function of the pH and the thermodynamic state of the system is elaborated based on generalized biochemical principles.

During the pre-treatment of agricultural wastes, the cellulose (35-45%) can be separated from the hemicellulose (25-40%). Hemicellulose is a heteropolymer of hexose and pentose sugars, with D-xylose as the major constituent. Xylose is actually the second most abundant carbohydrate monomer after glucose in agricultural wastes. In the third chapter, xylose fermentation was studied in the presence and absence of glucose.

Another potential waste stream to be used is derived from the biodiesel industry. This industry is growing very fast and the effluents generated possess a high content in glycerol. In chapter four, anaerobic glycerol fermentation is studied and compared to glucose fermentation by open mixed culture.

The question that always remains in mixed culture fermentation is whether the operational conditions determine which metabolism is most adequate, or select for the microorganism present that performs best. Therefore, in the fifth chapter the dominant microbial population established was studied under the different conditions (pH and carbon source). In chapter 6, the main results and conclusions are summarized. In addition, some questions that remained after this study and suggestions for future research are given.

Chapter 2

Influence of the pH on open mixed culture fermentation: a chemostat study

(This chapter was published as: Margarida F Temudo, Robbert Kleerebezem, Mark C.M. van Loosdrecht. 2007. Influence of the pH on (open) mixed culture fermentation of glucose, a Chemostat study. Biotechnology and Bioengineering, 98:69-79)

Abstract

Catabolic products from anaerobic fermentation processes are potentially of industrial interest. The volatile fatty acids and alcohols produced can be used as building blocks in chemical processes or applied directly as substrates in a mixed culture process to produce bioplastics. Development of such applications requires a predictable and controllable product spectrum of the fermentation process. The aim of the research described in this paper was (i) to investigate the product spectrum of an open mixed culture fermentation (MCF) process as a function of the pH, using glucose as the substrate, and (ii) to relate the product spectrum obtained to generalized biochemical and thermodynamic considerations.

A chemostat was operated under carbon and energy limitation in order to investigate the pH effect on the product spectrum in a mixed culture fermentation process. A transition from CO_2/H_2 production at lower pH values to formate production at higher pH values was observed. The ratio of CO_2/H_2 versus formate production was found to be related to the thermodynamics of formate dehydrogenation to CO_2/H_2. This transition was associated with a shift in the catabolic products, from butyrate and acetate to ethanol and acetate, likely due to a decrease in the oxidation state of the electron carriers in the cell. The product spectrum of the MCF process as a function of the pH could largely be explained using general biochemical considerations.

Introduction

The anaerobic fermentation is a widely applied process for treatment of wastewater and organic solid waste; generally methane is the end product. An alternative anaerobic process could aim for the production of intermediates in the methanogenic process from complex substrates. This can be established by specific inhibition of methane production using operational measures like lowering the pH or the biomass growth rate. Potentially interesting products that can be produced in this way include organic acids (acetate, butyrate, lactate, succinate), solvents (ethanol, butanol, acetone) and energy carriers like molecular hydrogen. Which mixture of products are formed will depend on the substrate treated, the microbial population present and the operational conditions (Lengeler et al. 1998). An extra advantage of anaerobic processes is its association with a low biomass yield, i.e. low sludge production.

Open mixed culture biotechnology based on natural inocula with a high microbial diversity allows operation of bioprocesses under non-sterile conditions with no risk of strain degeneration. As opposed to pure culture processes, mixed culture furthermore allow the use of continuous processing, and are capable of dealing with mixtures of substrates of variable composition. All these aspects significantly reduce the costs of MCF processes compared to pure culture based industrial fermentations.

Glucose fermentation can provide a variety of end products (Figure 2.1). Substrates first need to be transported into the cell, phosphorylated and enter the hexose monophosphate pool. The latter enters the glycolytic pathway, producing pyruvate, energy (ATP) and reducing equivalents (NADH) (Lengeler et al. 1998). The fate of pyruvate will be controlled by the cellular redox balance and the kinetic properties of the catalytic capacities available. Table 2.1 summarizes the possible products from glucose fermentation (Figure 2.1) together with ATP and NADH generated during the whole conversions.

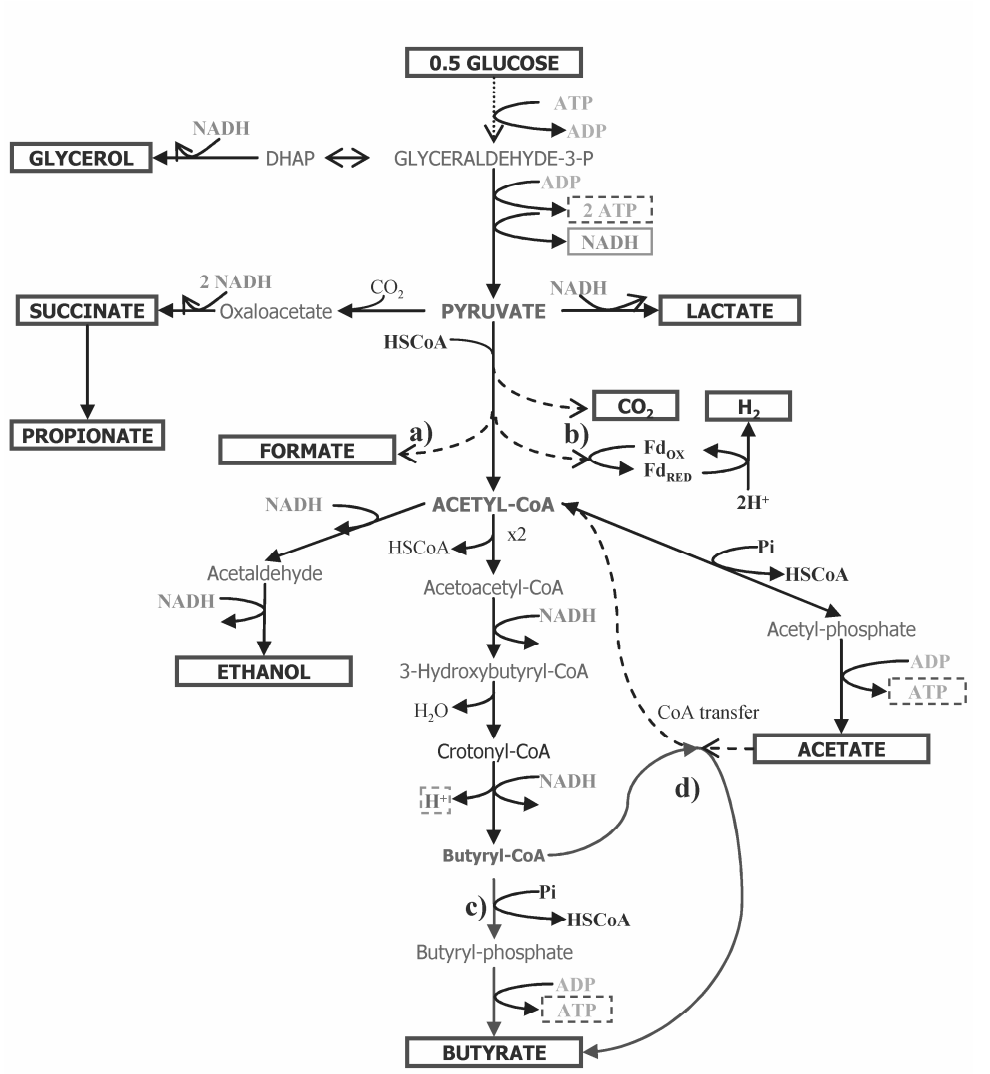

Figure 2.1- Adapted scheme with the most important and expected metabolic pathways during anaerobic fermentation by mixed culture. The reactions indicated with (a) and (b), (c) and (d) and (e) and (f) are alternative pathways for the same product, depending mainly on the type of microorganism (Lengeler et al. 1998; Papoutsakis 2000; Papoutsakis and Meyer 1985; Sawers 2005).

Table 2.1- Possible products from the MCF process and respective relevant intermediaries and cofactors involved. The number of NADH and ATP involved are in agreement with figure 1 and were determined based on glycolysis through the Embden Meyerhof Parnas Pathway (EMP) and substrate level phosphorylation (SLP) involved in each product formation pathway (Lengeler et al. 1998; Vandijken and Scheffers 1986).

Product	$Y_{prod/Glucose}$	$Y_{NADH/Glucose}$	$Y_{FdH/Glucose}$	$Y_{AcCoA/Glucose}$	$Y_{ATP/Glucose}$
Acetate	2	+2	2	2	4
Ethanol	2	-2	2	2	2
Butyrate	1	0	2	2	3
Lactate	2	0	0	0	2
Propionate	2	-2	0	0	2
Succinate	2	-2	0	0	2
glycerol	2	-2	0	0	-2

Fluxes in a metabolic network are controlled by kinetic and/or thermodynamic limitations. Thermodynamic control of a certain branch in the metabolism is more likely to occur if the overall Gibbs energy change of the reaction is close to thermodynamic equilibrium (Kohn and Boston 2000). On the other hand, if the rate of substrate utilization controls the pathways for product formation, and all the reactions are thermodynamically favourable, then the reaction pathway is kinetically controlled. The range of products formed depends only on substrate concentrations and on the enzyme activities, which control the rates of the competing pathways (Chang 1977; Kohn and Boston 2000). Under carbon and energy limiting conditions thermodynamics have been suggested to control which pathway branches are feasible and to define the final concentration of the products (Chang 1977; Kohn and Boston 2000; Rodriguez et al. 2006). The conversion of pyruvate proceeds very close to thermodynamic equilibrium and thermodynamic control may play a role, by making the operational conditions in favour of certain reactions.

Glucose fermentation by mixed microbial culture has been studied with different aims: production of molecular hydrogen, specific organic acid, or ethanol production (Fang and Liu 2002; Horiuchi et al. 2002; Ren et al. 1997; Willke and Vorlop 2004; Zoetemeyer et al. 1982b; Zoetemeyer et al. 1982c). Experimental results have shown the dependency of the ratios between different products formed on the operational conditions (pH, substrate

concentration, growth rate, temperature, etc) (Fang and Liu 2002; Horiuchi et al. 2002; Zoetemeyer et al. 1982c). Despite the sometimes comparable trends observed in the different studies, a relation with the metabolic and thermodynamic state of the system has not been established. Furthermore, the operational conditions are often poorly defined and the measurements conducted do not allow for straightforward comparison of the results reported by different authors. This knowledge would allow understanding and predicting the behaviour at different operational conditions.

The aim of this research was to investigate the product spectrum of glucose fermentation as a function of the pH in a MCF process. By determination of all substrates and products in the experimental system a thermodynamic and kinetic state analysis can be conducted. If possible, a link between the product spectrum as a function of the pH, and the thermodynamic and kinetic state of the system will be elaborated based on generalized biochemical principles. If we understand why certain products are prevailing in mixed culture fermentation, we may be able to predict the product spectrum as a function of the operational conditions independent of the specific population present.

Materials and Methods

Reactor operation. A continuous stirred reactor of 3 L capacity (2 L working volume) was used (mechanical stirring 300 rpm). Water was recirculated through a water jacket to maintain the temperature constant at 30 ±1 °C. The reactor was sparged with nitrogen gas at a flow rate of 120 ml/min, to maintain anaerobic conditions. The pH was controlled (pH±0.1) by automatic titration (ADI 1030 Bio controller) with 4M NaOH and HCl solutions. The hydraulic retention time (HRT) was 8 h for pH ≥ 5.5 and 20 h for pH ≤ 5.5. To prevent excessive foaming, 3 ml/h of a 3% solution of silicone antifoaming agent (BDH, England) was continuously added.

Inoculum. The anaerobic mixed culture used as inoculum consisted of a mixture of two sludges obtained from two different sources. The first inoculum was from a distillery wastewater treatment plant, Biothane in Delft, The Netherlands. An UASB operated at 30°C, pH 6.8-7.2, COD=2500 mg/L. The second inoculum was a sludge solution from a potato starch processing acidification tank in Broek op Langendijk, Smiths Food, The Netherlands. Before each experiment the reactor was freshly inoculated, with approximately 20 g of each inoculum, and operated in batch conditions until biomass growth was observed.

Medium. The cultivation medium contained the following (in g/L): glucose 4.0; NH_4Cl 1.34; KH_2PO_4 0.78; NaCl 0.292; $Na_2SO_4.10H_2O$ 0.130; $MgCl_2.6H_2O$ 0.120; $FeSO_4.7H_2O$ 0.0031; $CaCl_2$ 0.0006; H_3BO_4 0.0001; $Na_2MoO_4.2H_2O$ 0.0001; $ZnSO_4.7H_2O$ 0.0032; $CoCl_2.H_2O$ 0.0006; $CuCl_2.2H_2O$ 0.0022; $MnCl_2.4H_2O$ 0.0025; $Ni.Cl_2.6H_2O$ 0.0005; EDTA 0.050. The glucose and mineral solutions were prepared and fed separately. The glucose solution was sterilized in an autoclave at 110 °C for 20 min.

Steady State Characterization. To characterize the product spectrum at each pH, the reactor was run in continuous mode until a stable product composition and a biomass concentration was established. It took always approximately three weeks to reach stable operation, which means more than 25 generation times at low pH, and more than 60 generation times at pH higher than 5.5. The gas productivities (H_2 and CO_2) and the base added for pH control were monitored on-line, allowing direct analysis of the system stability. When these three rate measurements were stable or varied within a limited range (±10%), not tending to increase or decrease, a set of samples was taken during the following week. Concentrations of soluble organic fermentation products and biomass concentration in the reactor volume were determined.

Analytical methods. Reactor broth samples were immediately filtered (Millipore membrane of 0.45 μm), substrate and end products were determined and quantified. Glucose, volatile fatty acids (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and caproate), lactate, succinic acid, formic acid were determined by HPLC, using an Aminex HPX-87H column from Bio Rad (T=60ºC) coupled to an UV and a RI detector, while phosphoric acid 0.01M was used as eluent. The alcohols analyzed were ethanol, propanol and butanol. These analyses were performed by gas chromatography, Chromopack 9001, equipped with a flame ionization detector (FID) and a fused-silica capillary column 15 m x 0.53 mm HP-INNOWAX. The column temperature was 80 ºC for alcohols. The temperature of the injector and detector were 180 and 200 ºC, respectively. Helium was used as carrier gas.

Measurements of H_2 and CO_2 were performed on-line. A gas detection system was coupled to the bioreactor outlet and a Rosemount Analytical NGA 2000 MLT 1 Multi-component analyzer (infrared detector). Data acquisition (pH and gas) was made with SCADA software, Sartorius BBI systems MFCS/win 2.1.

Total dissolved and suspended COD were measured in the influent and in the reactor liquid with the Dr Lange kit for the range 1000-10000 mgCOD/L. Total

organic carbon (TOC) and total inorganic carbon (TIC) were measured using a Shimadzu TOC 5050A analyzer. For total TOC and COD in samples with suspended solids, the samples were first homogenized through cell disruption during 15 sec in an ultrasound bath.

The inorganic carbon was also estimated from the offgas concentration of CO_2 based, the mass transfer coefficient (k_La) of the system and the acid-base equilibrium constants for inorganic carbon dissociation. The k_La was measured for oxygen (Riet and Tramper 1991) and corrected for molecular hydrogen and carbon dioxide based on the relative diffusivity coefficients. The k_La determination for oxygen was conducted dynamically in the same reactor filled with water and minerals at 30^0C. The diffusion coefficient for oxygen, molecular hydrogen and carbon dioxide used were 2.1, 5.0 and $1.92*10^{-9}$ m^2s^{-1} (Kirwan and Rousseau 1987) and corrected for temperature according to the Stokes-Einstein relation (Li and Gregory 1974).

The biomass dry weight was determined after filtration according to the standard methods (APHA et al. 1992).

The Yields of the fermentation products were calculated per glucose consumed, corrected for the dilution factor of the base added to control pH. COD and carbon balances were made based on the number of electrons and carbon atoms per mole, assuming a standard biomass composition of $CH_{1.8}O_{0.5}N_{0.2}$ (Roels 1983).

The Gibbs free energy of a reaction in the conditions studied was calculated using the following equation (Hanselmann 1991):

$$\Delta G = \Delta G^{0'} + RT \ln Q \qquad (1)$$

Results

Steady state characterization

To evaluate the impact of the pH on the product distribution during glucose fermentation by an undefined mixed microbial culture, experiments were performed in chemostat reactors at pH values ranging from 4 to 8.5. An example of the steady state reactor product distribution measurements is given in figures 2.2 and 2.3. The online measurements of the system shown were conducted at pH 5.5 and retention time of 8 h. The production of H_2 and CO_2, as well the base addition rate (a measure of the acid productivity) were stable despite small variations during the days (Figure 2.2). Offline measurements showed glucose limitation (>99.5% consumption) and a stable product spectrum. The product yields are given in figure 2.3. One of the

reasons for the small day-to-day variations could result from wall growth. This wall growth was regularly removed from the system but resulted in small instabilities in operational performance.

The CO_2 and H_2 partial pressure lines are highly congruent. This is logic since their production is expected to be coupled based on the general microbial metabolism (Figure 1 and Table I). The dominant products at this pH were butyrate and acetate, corresponding to respectively 45 and 14 % of the COD converted. Biomass and H_2 had COD based yields of 18 and 12 %, respectively. The sum of these COD yields indicates a closed electron balance and confirms that the main products in the process have been identified. COD stands for chemical oxygen demand and is a measurement of the oxidation state of the chemicals present, proportional to the number of electrons that can still be transferred to oxygen producing water and oxidizing the carbon compound into inorganic carbon.

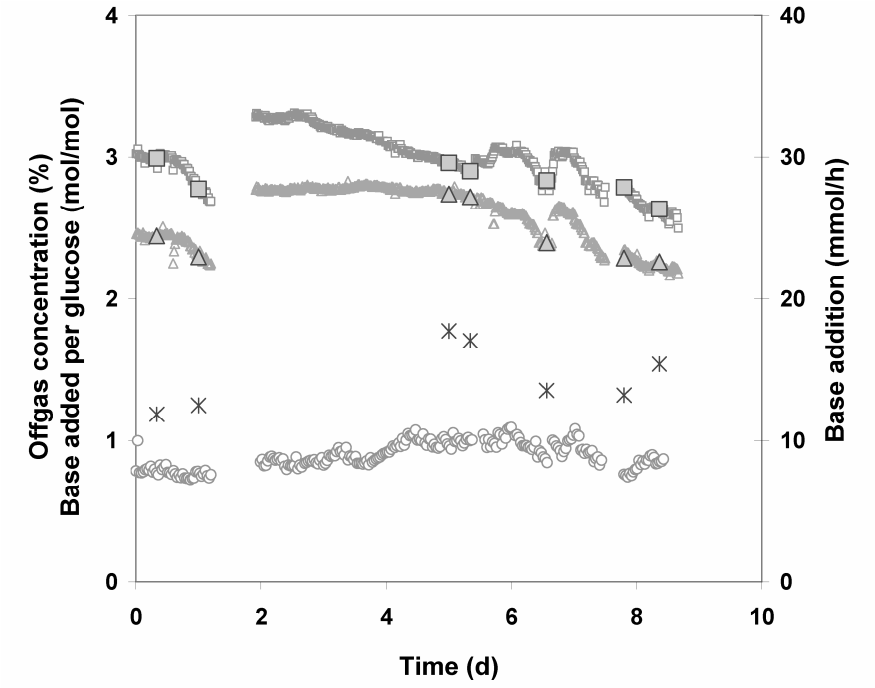

Figure 2.2- Online measurements when pseudo steady state is reached. Data from the reactor running at pH 5.5 (D=0.12 h^{-1}, S_0=4g/L, T=30 °C, sparged with nitrogen 0.06 vvm). The offgas concentrations (%) are represented on the left side scale: □ H_2 and △ CO2; ○ base addition rate (mmol/h), right side scale. The symbols correspond to the data at the sampling time: ▩ H_2 partial pressure, ▲ CO_2 partial pressure; ✳ moles of based added per mole of glucose consumed (mol/mol), on the left side.

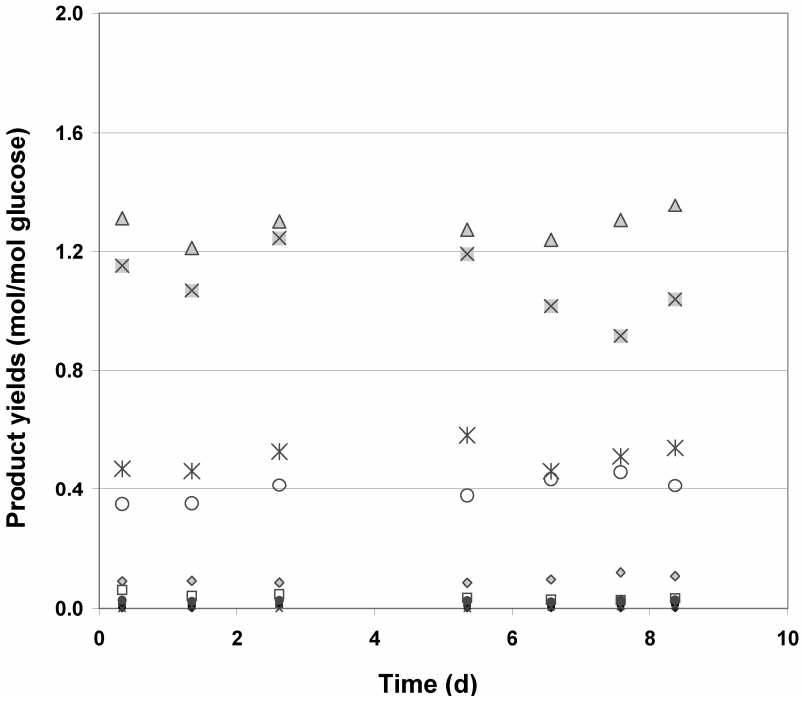

Figure 2.3- Product yields (mol product/mol glucose) calculated from the offline measurements at steady state: ✳ butyrate, ○ acetate, ◇ glycerol, ● ethanol, ◆ lactate, ■ formate, □ propionate, × succinate, △ hydrogen and ⊠ biomass (in Cmol/mol glucose). The reactor was operated at 30 °C, pH 5.5, dilution rate 0.125 h^{-1} and 4g/L glucose in the influent.

Glucose fermentation by mixed culture at different pH values

From a set of data as shown above, average product concentrations and standard deviations were calculated for each pH experiment. The dilution rate, COD and carbon balances for all experiments are presented in table 2.2. In general these balances close between 90-110% recoveries. Variations are probably due to sideproducts that could not be detected or by measurement errors. Other possible fermentation products such as pyruvate, malate, fumarate, citrate, propanol, butanol, valerate, iso-valerate, caproate that could be measured by HPLC analysis were not detected in our samples. However, in samples taken at pH 6.25 and 7.0 an unidentified peak appeared in the HPLC chromatogram at 10 minutes retention time. According to the other product concentrations and mass balances this product was not very significant.

Table 2.2- The COD and carbon balances for each experiment at different pH values and respective dilution rate.

pH	D (h^{-1})	COD balance ± standard deviation	Carbon balance ± standard deviation
4	0.04	96 ±3	91 ±2
4.75	0.05	96 ±5	101 ±5
5	0.05	99 ±4	96 ±2
5.5	0.05	107 ±5	103 ±5
5.5	0.13	98 ±4	96 ±5
6.25	0.12	94 ±5	93 ±5
7	0.13	93 ±5	90 ±4
7.75	0.12	101 ±4	101 ±4
8.5	0.12	94 ±5	94 ±6

The effect of the pH value on the steady state product spectrum is shown in figures 2.4 and 2.5. The substrate concentration was maintained constant at 4 g/L throughout the entire experimental period. Given the difficulties of reaching a steady state at pH-values of 5 or lower, the reactor was operated at a lower dilution rate in order to establish substrate limitation and prevent oscillatory behaviour. Thus, during the experiments at pH 4-5.5 the dilution rate was 0.05 h^{-1} while at 5.5-8.5 it was 0.12 h^{-1}.

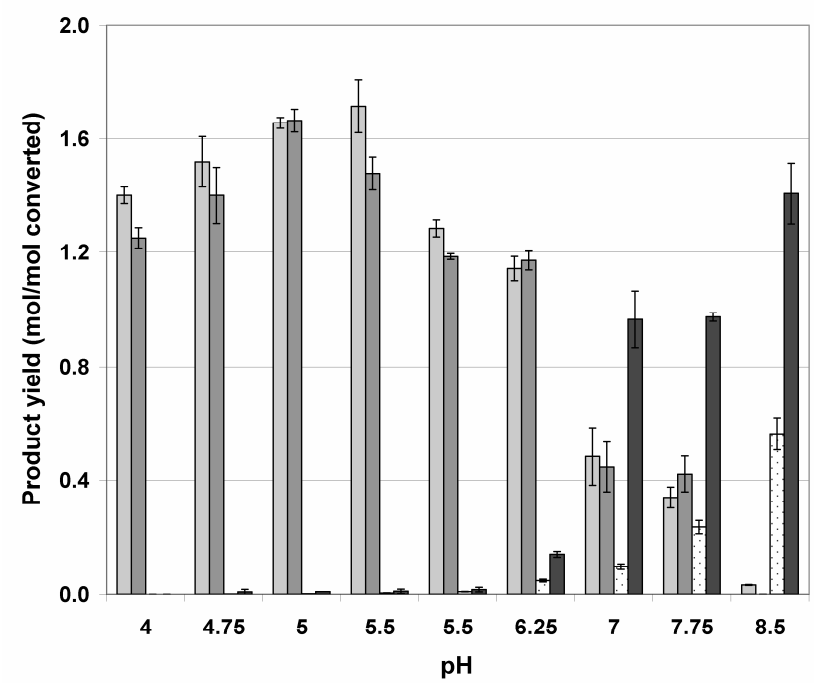

Figure 2.4- Influence of pH on the yields of glucose fermentation by mixed culture at different pH: ▫ H_2, ▪ CO_2, □ HCO_3^- (estimation) and ■ formate. The calculated k_La of CO_2 was $1.32x10^{-3}$ s^{-1}. The results of the first four pH values results were operated at a dilution rate of $0.05h^{-1}$ and the other five at 0.12 h^{-1}.

The yield of gaseous products (H_2 and CO_2) and the formate yield are given in figure 2.4. Also the estimated concentrations of bicarbonate are indicated. At high pH values, a clear decrease in the hydrogen production occurs associated with an increase in formate production. The sum of hydrogen and formate yield, however, is not really influenced by the pH (Figure 2.6).

As can be seen in figure 2.5, the environmental pH also had an effect on the distribution of the organic acids and alcohols produced in the MCF process. At low pH values the major dissolved organic products detected were butyrate and acetate, whereas at higher pH-values butyrate production decreased and ethanol and acetate become the main products.

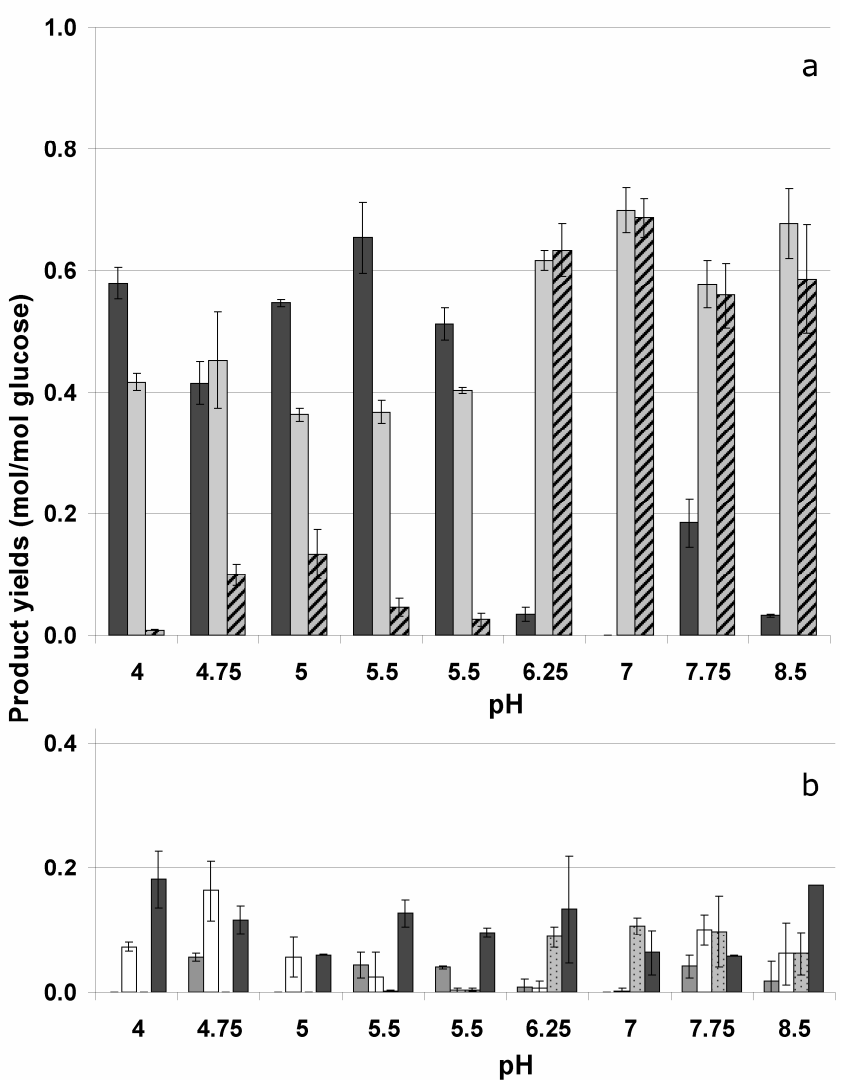

Figure 2.5- (a) Influence of pH on the dissolved products yields of glucose fermentation by mixed culture at different pH: ■ butyrate, □ acetate and ▨ ethanol. (b) Influence of pH on the minor products yields of glucose fermentation by mixed culture at steady state at different pH values: ▩ propionate, □ lactate, ▩ succinate and ■ glycerol. The first four pH values results were operated at a dilution rate of $0.05h^{-1}$ and the other five at $0.12\ h^{-1}$.

Besides these main products, other compounds were produced at minor concentrations (Figure 2.5 b). Glycerol was produced over the whole range of pH-values tested, while succinate was produced only at pH > 6. Lactate production occurred mainly at low pH. Lactate was detected in higher concentrations during transient states, such as a sudden increase of substrate concentration, changes in dilution rate, or pH changes (data not shown).

Glucose is converted via pyruvate into acetyl-CoA. The latter conversion is associated with the production of one mole of hydrogen or formate per mole acetyl-CoA produced (Figure 2.1). Figure 2.6 compares the yields of H_2 and formate versus acetyl-CoA derived products and shows the biomass yield under each condition. As expected, the amount of acetyl-CoA derivatives matches with the production of H_2 and formate.

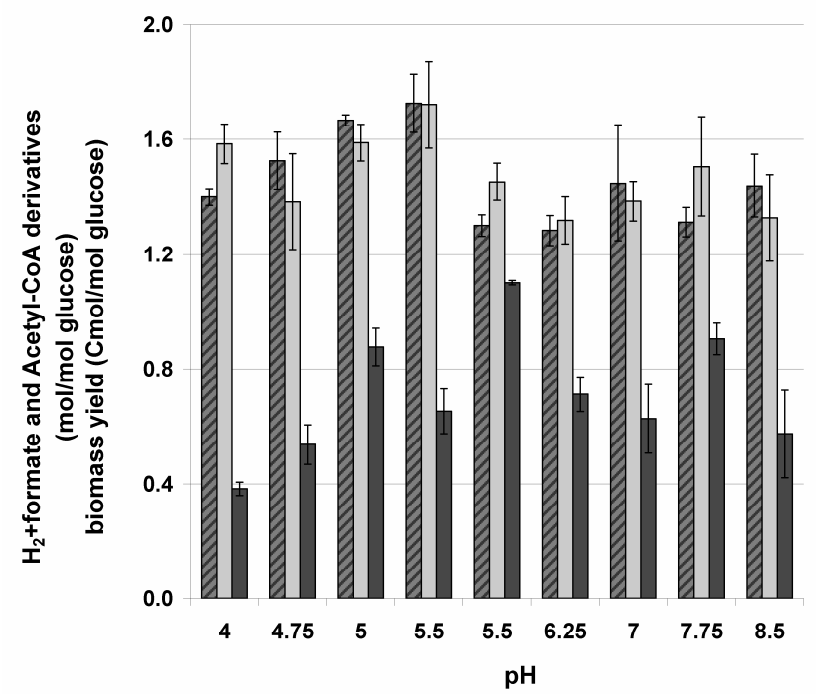

Figure 2.6- Influence of pH on the H_2 and formate yield, acetyl-CoA derivatives yield and biomass yield. Symbols: ▨ sum of the molecular hydrogen and formate yields; ▫ sum of acetate, butyrate (x2) and ethanol (Acetyl-CoA derivatives) yields; ■ biomass yield.

Discussion

Previous studies on glucose fermentation at different pH values have shown the importance of this parameter on the product distribution in continuous bioreactors (Fang and Liu 2002; Horiuchi et al. 2002; Zoetemeyer et al. 1982c). Horiuchi *et al.* (2002) and Fang *et al.* (2002) observed a decrease in molecular hydrogen and butyrate production as pH increased. At high pH values (≥7) acetate production increased, and propionate and ethanol were produced as well. However, in both studies there was no full substrate conversion. Zoetemeyer *et al.* (1982) worked in substrate limiting conditions, in a pH range from 4.5 to 7.9 and at higher growth rates (>0.3 h^{-1} instead of 0.12 h^{-1} for pH values higher than 5.5). This author observed the same trends in product composition as a function of the pH as found in this work (Figure 2.4 and 2.5): at increasing pH values the H_2, CO_2 and butyrate yields decreased, while formate, acetate and ethanol yields increased. All these results indicate that the product spectrum in a MCF is reproducible and independent of the inoculum.

Formate vs. CO_2/H_2

One of the main results of this study is concerned to the shift in the production of molecular hydrogen into formate related to the increase of the operational pH value. According to the results obtained, the yield of hydrogen and formate is approximately the same as the yield of acetyl-CoA derivatives (Figure 2.6). This suggests that under the conditions studied the only reaction that generates H_2 or formate is the conversion of pyruvate into acetyl-CoA. In anaerobic conditions, the electrons involved in the pyruvate oxidation into acetyl-CoA are either transferred to ferredoxin or formate (Figure 2.1). These two are both capable of inducing hydrogen production. Thermodynamically, reduced ferredoxin is a stronger electron donor compared to NADH and the reduction of protons by a hydrogenase into molecular hydrogen is more favourable (Collet et al. 2005; Stams 1994):

$$NADH + H^+ \rightarrow NAD + H_2 \qquad \Delta G^{0'} = +18.1\ kJmol^{-1} \quad (2.2)$$

$$2\ Fd\ (red) + 2\ H^+ \rightarrow 2\ Fd\ (ox) + H_2 \qquad \Delta G^{0'} = +3.1\ kJmol^{-1} \quad (2.3)$$

Reduced ferredoxin can also transfer its electrons to bicarbonate, producing formate instead or, in case CO_2 and H_2 are produced (catalyzed by pyruvate ferredoxin oxidoreductase-PFOR), the CO_2 can be further reduced by CO_2

reductase into formate, taking the electrons from reduced ferredoxin as shown above (Jungerma et al. 1970; Scherer and Thauer 1978).

$2\ Fd\ (red) + HCO_3^- \rightarrow 2\ Fd\ (ox) + formate + H^+ \qquad \Delta G^{0'} = +1.7\ kJmol^{-1}$ (2.4)

$H_2 + HCO_3^- \rightarrow formate + H_2O \qquad \Delta G^{0'} = -1.3\ kJmol^{-1}$ (2.5)

Formate can at low pH be cleaved into H_2 and CO_2 by the formate hydrogenlyase (Sawers 2005) or formate dehydrogenase (Ferry 1990; Liu and Mortenson 1984; Voolapalli and Stuckey 2001). Formate can also derive from the reaction catalyzed by the enzyme pyruvate formate lyase (PFL) (Thauer et al. 1977):

$Pyruvate + CoA \rightarrow Acetyl\text{-}CoA + Formate \qquad \Delta G^{0'} = -13\ kJmol^{-1}$ (2.6)

This suggests that the molecular hydrogen produced is independent from the cellular NADH generated during glycolysis, given that it comes exclusively from the pyruvate oxidation into acetyl-CoA.

In equilibrium the ratio [formate]/[H_2] depends mainly on the pH, shifting to formate at increasing pH-values. This result can be clearly seen in figure 2.7, which besides the fractions of formate and hydrogen presents also the free energy of the reaction HCO_3^--H_2/$HCOO^-$. The calculated values, free energy at each given condition, are always very low (<10 kJ/mol) and approximately constant along the whole range of pH values.

According to the estimated value (dashed lines) the trend is in agreement with the observed results. However from the data obtained no conclusive answer can be given to the question which microorganism or enzyme was present in the mixed anaerobic culture (whether ferredoxin or formate induces molecular hydrogen production) because the shift (hydrogen into formate) is the same as pH changes.

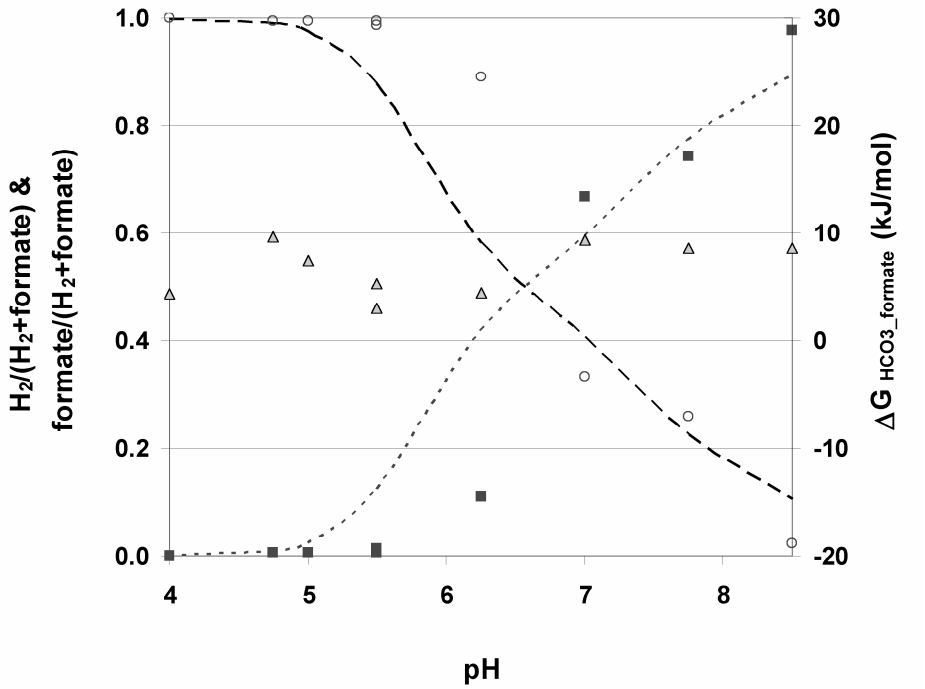

Figure 2.7- Hydrogen and formate fraction produced as a function of the pH (open circles and black squares, respectively); Gibbs free energy of the reaction of bicarbonate and molecular hydrogen into formic acid (grey triangles). The lines are the estimated fractions calculated from the average free energy of the conversion of bicarbonate into formate (6.5 $kJmol^{-1}$), considering the actual bicarbonate concentration and the total amount of H_2 and formate produced.

Redox Balance

To understand which products can be produced at each condition it is important to look at the different possible metabolic pathways and at the thermodynamic constrains. For this purpose a general scheme with the most common metabolic pathways for glucose fermentation is shown in Figure 2.1. According to this scheme glucose is converted through the glycolysis into pyruvate. Besides pyruvate, glycolysis supplies the cell with energy (ATP) and reducing equivalents (NADH). In the absence of an external electron acceptor the organic substrate is used as electron donor and receptor. This means that part of the substrate will be reduced and part oxidized. The sum of the degree of reduction of the products formed during catabolism and anabolism has to be equal to that of the substrate. Additionally, NAD/NADH is a cofactor and exists in limited amounts inside the cell, the NAD reduced into NADH during glycolysis has to be regenerated, which involves the reoxidation of NADH through reduction of organic intermediates (Neijssel et al. 1997). All products that derive from pyruvate involve NADH generation, but only some allow its

reoxidation namely butyrate, ethanol, succinate, glycerol, lactate and propionate, figure 2.1 and table 2.1 (Lengeler et al. 1998). Lactate and butyrate production are in this case the redox-neutral conversions (consume the number of NADH produced in the glycolysis, see figure 2.1 and table 2.1). Whereas during acetate production net NADH is formed, as it is derived from pyruvate. The NADH that is left can not be reoxidized into H_2 unless the hydrogen partial pressure is very low. Thus, this NADH must be reoxidized by reducing other organic compounds (acetyl-CoA, dihydroxiacetone phosphate, oxaloacetate, etc), producing more reduced compounds (ethanol, glycerol, succinate, etc). Glycerol production is one way of restoring the redox balance under anaerobic conditions when acetate is produced, besides succinate and ethanol. Glycerol production did not show pH dependency (Figure 2.5b). Succinate, on the other hand, is mainly produced at neutral pH values (>5.5), suggesting its dependency on the bicarbonate concentration since it involves a carboxylation reaction.

This phenomenon is also known in yeast, in Saccharomyces cerevisiae, glycerol is a by-product of the fermentation of sugar to ethanol in a redox-neutral process. The role of NADH-consuming glycerol formation is to maintain the cytosolic redox balance especially under anaerobic conditions, compensating for cellular reactions that produce NADH e.g. growth or acetate that is produced in order to get more ATP, for this the double amount of glycerol is generated (Vandijken and Scheffers 1986). Analogously, in Anammox microorganisms that convert ammonia and nitrite into molecular nitrogen in a neutral-redox process. This microorganisms need, in order to grow, to produce also nitrate. The oxidation of nitrite into nitrate will provide the reducing equivalents to reduce the carbon source (deGraaf et al. 1996).

Figure 2.8 represents the NADH and NAD yields on glucose obtained at different pH values. Both values are between 1.5 and 2.0, which is a reasonable value if it is assumed that most of glucose is converted via glycolysis into pyruvate (maximum yield of 2 mol/mol), and part is used for biosynthesis. Another observation is that at low pH the NADH produced (pyruvate derivatives) is slightly higher than the NADH consumed (NAD yield). This is acceptable since anaerobic microorganisms are used to dealing with different NADH/NAD ratios at different conditions as long as the turnover remains approximately constant, but it is also possible that the scheme presented in figure 2.1 is incomplete (Snoep et al. 1994). At high pH, however, the yield on products consuming NADH is considerably higher than the amount produced and therefore the ratio is in these conditions higher than one. It is suggested that a small fraction of reduced ferredoxin (derived from

the excess of formate) may be used as a reducing mediator by the cell and might therefore explain these observations.

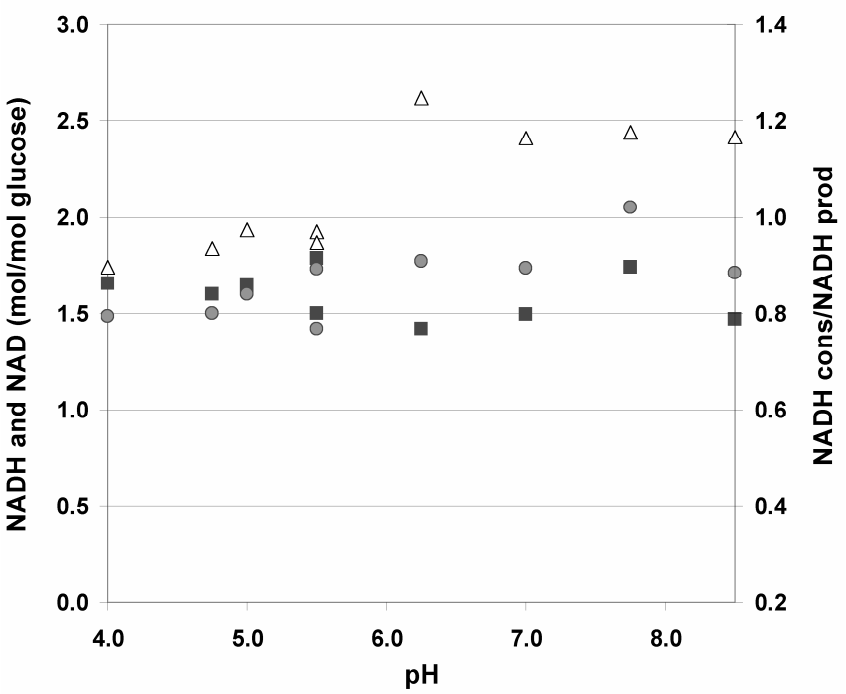

Figure 2.8- The NAD yield based on the products that involve NADH reoxidation: biomass, 0.146 NADH/Cmol and on the catabolic products: butyrate, lactate, ethanol, glycerol, propionate, succinate, according to table 2.1); and NADH yield (pyruvate derivatives) at different pH values, circles and squares, respectively. The ratio between both specific yields is on the right side of the graph (triangles).

Anaerobic growth on glucose needs extra reducing equivalents as biomass is more reduced than glucose. It can be estimated that approximately 0.146 NADH is needed per carbon mole of biomass formed (Papoutsakis 2000). Based on what was stated above it is possible that bacteria have different strategies in order to derive this amount of reducing equivalents for growth. At low pH bacteria produce mainly butyrate and acetate, and acetate will account for the NADH production required for the anabolism. Acetate production does not involve any NADH regeneration, but this can be balanced through the production of other catabolic products and biosynthesis. While at high pH values, due to the increase in formate, some of this can be oxidized into bicarbonate. When this happens, the electrons are transferred to the oxidized ferredoxin (Scherer and Thauer 1978), which becomes reduced. Reduced ferredoxin can donate the electrons to different biosynthetic pathways in

fermentative bacteria or it can activate the ferredoxin-NAD oxidoreductase, producing the required NADH (Buchanan and Arnon 1970; Hugo et al. 1972; Petitdemange et al. 1976).

At low pH

$$2\ Fd\ (red) + 2H^+ \rightarrow 2\ Fd\ (ox) + H_2(g) \uparrow \qquad (2.7)$$

At high pH

$$2\ Fd\ (red) + HCO_3^- \leftrightarrow 2\ Fd\ (ox) + formate + H^+ \qquad (2.8)$$

$$2\ Fd\ (red) + NAD(P) \leftrightarrow 2\ Fd\ (ox) + NAD(P)H \qquad (2.9)$$

Furthermore, the reduced ferredoxin can also transfer its electrons to NADP, producing the needed NADPH for biosynthesis (Biesterveld et al. 1994b; Lengeler et al. 1998). In many clostridia species ferredoxin-NAPH oxidoreductase is the only source of NADPH (Biesterveld et al. 1994b; Jungerma et al. 1973).

Shift in the catabolic products

Another product shift observed in this study and in Zoetemeyer's research, concerns the dissolved products. At experiments performed at higher pH values the butyrate yield decreased and acetate and ethanol yields increased. The conversion of glucose into butyrate or acetate-ethanol is in electron and in carbon unit equivalent, and the free energy involved in both reactions is very close and negative (Table 2.3). This conversion involves the consumption of two pyruvate molecules and two NADH and the generation of 3 ATP (two from glycolysis and one from substrate level phosphorylation). Thus, it is unclear why this shift occurs with the pH increase.

The conversion of glucose into butyrate is the most exergonic (Table 2.3, equation A), and butyrate is therefore expected to be the major product. In some microorganisms butyrate production implies the presence of acetate (Figure 2.1). Acetate production is also very exergonic and allows the adjustment of the intracellular NADH levels by producing other products independently. So, the equilibrium observed at low pH is better described as written in equation B (Table 2.3) and equation C represents the conversion that occur at high pH. The difference of the free energy seems not to be enough to justify such a shift in products as a function of the pH. The number of protons involved in reaction B is lower compared with reaction C. Although it is a very small difference, it might gain some significance when the pH values rise for two reasons: to help maintaining the intracellular pH more acidic than

the extracellular at higher pH values (and vice versa), and eventually to gain more energy by transport via proton motive force. Still, glycerol production has not always been mentioned in the literature and there might be other reason for this shift in products.

Table 2.3- Free energy of the main reactions observed at different pH values. Formate was considered, instead of CO_2+H_2, for simplicity and as it has been observed to be very close to equilibrium (above). Calculations were made based on the standard free energy of formation and corrected to pH 7 (Hanselmann 1991). The ATP yield on glucose converted was calculated for each reaction based on table 2.1.

	Reaction	**$\Delta G^{0'}$**	**ATP**
A	Glucose → Butyrate + 2formate + $3H^+$	-266.6	3
B	Glucose + $0.53H_2O$ → 0.6Butyrate + 0.53Acetate + 0.27glycerol + $0.27H_2$ + 1.73formate + $2.87H^+$	-207.7	2.6
C	Glucose + H_2O → Ethanol + Acetate + 2formate + $3\ H^+$	-216.1	3

The increase in the ethanol yield as a result of the pH increase might have a different explanation, such as biochemical reasons. It has been observed higher ethanol yields associated to higher formate fractions, but the exact mechanism that triggers this shift is rather not clear and difficult to prove. An increase of the relative amount of reduced ferredoxin at high pH, as a result of the formate present, might be therefore a cause as it has been shown that ethanol production is greatly influenced by the regulation of the electron flow at the ferredoxin level (Lamed and Zeikus 1980). The reduction of oxidized ferredoxin by acetaldehyde has been observed (Brill and Wolfe 1965); and experiments under elevated hydrogen pressure or with neutral red, a dye that decreases the electron flow to the hydrogenase and therefore increases the fraction of reduced ferredoxin, had a similar effect: a decrease in acids produced and increase in alcohols (Girbal et al. 1995; Hugo et al. 1972). Still, the exact mechanism of how the increase in formate influences ethanol metabolism remains uncertain since the sum of formate and hydrogen is about the same as the amount of acetyl-CoA derivatives independently of the pH.

The shift from butyrate to acetate/ethanol production in these open mixed culture occurred in the pH range of 6-8. In this range the outcomes were variable: in the reactor running at pH values of 6.25 and 7.0 ethanol was a predominant product, while at 7.75 there was significant butyrate and higher

hydrogen yields than expected. This suggests that minor operational variations can induce a shift from production of butyrate and ethanol/acetate in these conditions. As explained above the equilibrium between formate/hydrogen production likely determines this product spectrum. There are other factors that can have a similar impact in this shift namely: a decrease in the temperature (gas solubility is higher, bicarbonate concentration increases shifting the equilibrium towards formate); increase of the substrate concentration, increases total amount of formate. In both cases we observed an increase on the formate and ethanol yields coupled with a decrease on butyrate yield (data not shown).

Comparison with theoretical predictions

To summarize this investigation a comparison between the obtained data and one of the more recent theoretical models was made. Rodriguez et al. developed a theoretical model that describes the predicted behavior of a mixed culture fermentation at different operational conditions (pH and hydrogen partial pressure) (Rodriguez et al. 2006). In this model, the product formation is assumed to be determined by the maximum energy yield (expressed in ATP units) of the different pathways as a function of environmental conditions. Processes considered in the model include besides the biomass formation, the costs for product transport out of the cell and redox balance, aiming at the maximization of ATP production. Only NAD/NADH is considered as electron carrier and the oxidation state of NAD/NADH is assumed to be in equilibrium with molecular hydrogen in the gas phase.

In this work we have observed experimentally that the total amount of H_2 and formate produced was linearly related to the number of acetyl-CoA molecules produced. The activity of the hydrogenase system seems therefore to be coupled directly to the pyruvate oxidation. The model developed assumes that all the net NADH produced (in order to maximize ATP production) can be converted into molecular hydrogen. This assumption is unlikely to be correct because at the actual hydrogen partial pressures hydrogen production from NADH is thermodynamically unfavourable ($\Delta G^{0'}$ is +18.1 $kJmol^{-1}$) (Collet et al. 2005). Also the experimental evidence points out that hydrogen is in fact only produced in the conversion of pyruvate to acetyl-CoA. The model should therefore take both electron carriers into account separately.

As opposed to the experimentally observed butyrate production described here, the mathematical model developed by Rodriguez *et al.* (2006) predicts that at low pH-values ethanol is produced. Under the conditions

studied ethanol was not a considerable product at low pH. This can be because acid toxicity against the gain of ATP of the produced acids is not yet influential at the pH values studied. Furthermore, in bacteria ethanol production seems to be rather triggered by other aspects such as a higher amount of reduced ferredoxin. Due to the coupling of the H_2 production with pyruvate oxidation, only one NADH is supplied for ethanol to be produced. Consequently, ethanol production should be accompanied by acetate production to fulfil the NADH balance.

Implementation in the model of two different electron carriers will therefore also solve this discrepancy between the model and the experimental observations as mentioned above. Another possibility is to directly couple H_2/formate to pyruvate oxidation.

Conclusion

It is predictable from thermodynamic and general metabolic reasoning that, independent of the inoculum, a reactor fed with glucose running under substrate limitation will shift its product distribution from butyrate, acetate and molecular hydrogen at low pH (4-6.5) into acetate, ethanol and formate at high pH (6.5-8.5). The shift in product formation seems to be directly related to the pH-dependent change thermodynamics of the Formate/H_2-HCO^-_3 reaction. However, it remains unclear which is the main cause of the product shift between butyrate and ethanol, since both conversions are in electron and carbon terms equivalent; two hypothesis have been suggested.

Chapter 3

Xylose fermentation by open mixed culture

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Abstract

Xylose is after glucose the dominant sugar in agricultural wastes. In anaerobic environments, carbohydrates are converted into volatile fatty acids and alcohols. These can be used as building blocks in biotechnological or chemical processes, e.g. to produce bioplastics. In this study, xylose fermentation by mixed microbial culture was investigated and compared with glucose fermentation. The product spectrum obtained with both substrates was comparable. It was observed that, in the case of xylose, a higher fraction of the carbon was converted into catabolic products (butyrate, acetate and ethanol) and the biomass yield was approximately 20% lower than on glucose. When submitted to a pulse of glucose, the population cultivated on xylose could instantaneously convert the glucose. No substrate preference was observed when glucose and xylose were fed simultaneously to the continuously operated reactor.

Introduction

Lignocellulosic materials, such as wheat- or rice-straw, corn stovers or sugar cane bagasse, are available in large amounts at a reasonable cost. Every year, about 73.9 Tg of dry wasted crops are generated in the world (Kim 2004). During the pre-treatment, the cellulose (35-45%) can be separated from the hemicellulose (25-40%). Hemicellulose is a heteropolymer of hexose and pentose sugars, with D-xylose as the major constituent. Xylose is the second most abundant carbohydrate monomer after glucose in agricultural wastes. Whereas glucose can be efficiently converted to ethanol and fine chemicals, an economical application for xylose needs to be developed.

Anaerobic fermentation is a widely applied process for treatment of wastewater and organic solid waste; generally, methane is the end product. An alternative anaerobic process could aim for the production of intermediates in the methanogenic process from complex substrates. This can be established by specific inhibition of methane production using operational measures like non-neutral pH or increasing the biomass dilution rate. Potentially interesting products that can be produced in this way include organic acids (acetate, butyrate, lactate, succinate), solvents (ethanol, butanol, acetone) and energy carriers like molecular hydrogen. Which mixture of products are formed will depend on the substrate treated, the microbial population present and the operational conditions (Kleerebezem and van Loosdrecht 2007; Lengeler et al. 1998). An extra advantage of anaerobic processes is its association with a low biomass yield, i.e. low waste production. Fermentation of hemicellulose and hemicellulose hydrolysates is a major subject of research, mostly associated with the production of ethanol (Sommer et al. 2004; van Maris et al. 2004). One of the problems in this fermentation is the inability of many microorganisms to metabolize efficiently all the carbohydrates derived from hemicellulose. Moreover, the presence of mixtures of sugars may lead to a preferential utilization of one of the substrates, diauxic growth.

Open mixed culture biotechnology based on natural inocula with a high microbial diversity allows for operation of bioprocesses under non-sterile conditions with no risk of strain degeneration (Kleerebezem and van Loosdrecht 2007). As opposed to pure culture processes, mixed culture furthermore allow for the use of continuous processing, and are capable of dealing with mixtures of substrates of variable composition. All these aspects significantly reduce the costs of mixed culture fermentation processes compared to pure culture based industrial fermentations. The impact of the operational conditions on glucose fermentation by mixed culture has been

extensively studied (Chapter-I; Hawkes et al. 2007; Zoetemeyer et al. 1982a; Zoetemeyer et al. 1982c). To xylose fermentation, however, almost no attention has been given yet. Recently, xylose has been reported as a suitable substrate for biohydrogen production by open mixed culture (Lin and Cheng 2006; Lo Yung-Chung et al. 2007).

D-xylose is not so readily utilized as D-glucose for the production of chemicals by microorganisms (Dien et al. 2003). The reason may lie on the biochemical pathways used for pentose and hexose metabolism. In most fermentative anaerobic bacteria, D-xylose has to be converted into D-xylulose-5-phosphate (X5P). This metabolite is further metabolized through either pentose phosphate pathway (PPP), or through the phosphoketolase pathway (PKP) (Figure 3.1). The PPP concerns a series of reactions where D-xylulose-5-phosphate is rearranged and converted into glyceraldehyde-3-phosphate (GAP) (eq. 3.1), an intermediate of the glucose metabolism. The phosphoketolase cleaves X5P into GAP and acetyl phosphate, which is further converted into acetate and generates one ATP (eq. 3.2)(Tanaka et al. 2002). Despite the fact that the PPP is the most common pathway, the enzyme phosphoketolase is quite spread over different genera.

PPP: 3 Xylose → 5 Pyruvate + 5 NADH + 5 ATP (3. 1)

PKP: 3 Xylose → 3 Pyruvate + 3 NADH + 3 ATP + 3 Acetyl-P (3. 2)

The aim of this research is to investigate the product spectrum of xylose fermentation by mixed culture process and compare it to glucose fermentation under the same process conditions. A link between the product spectrum and the type of substrate is made, based on generalized biochemical principles.

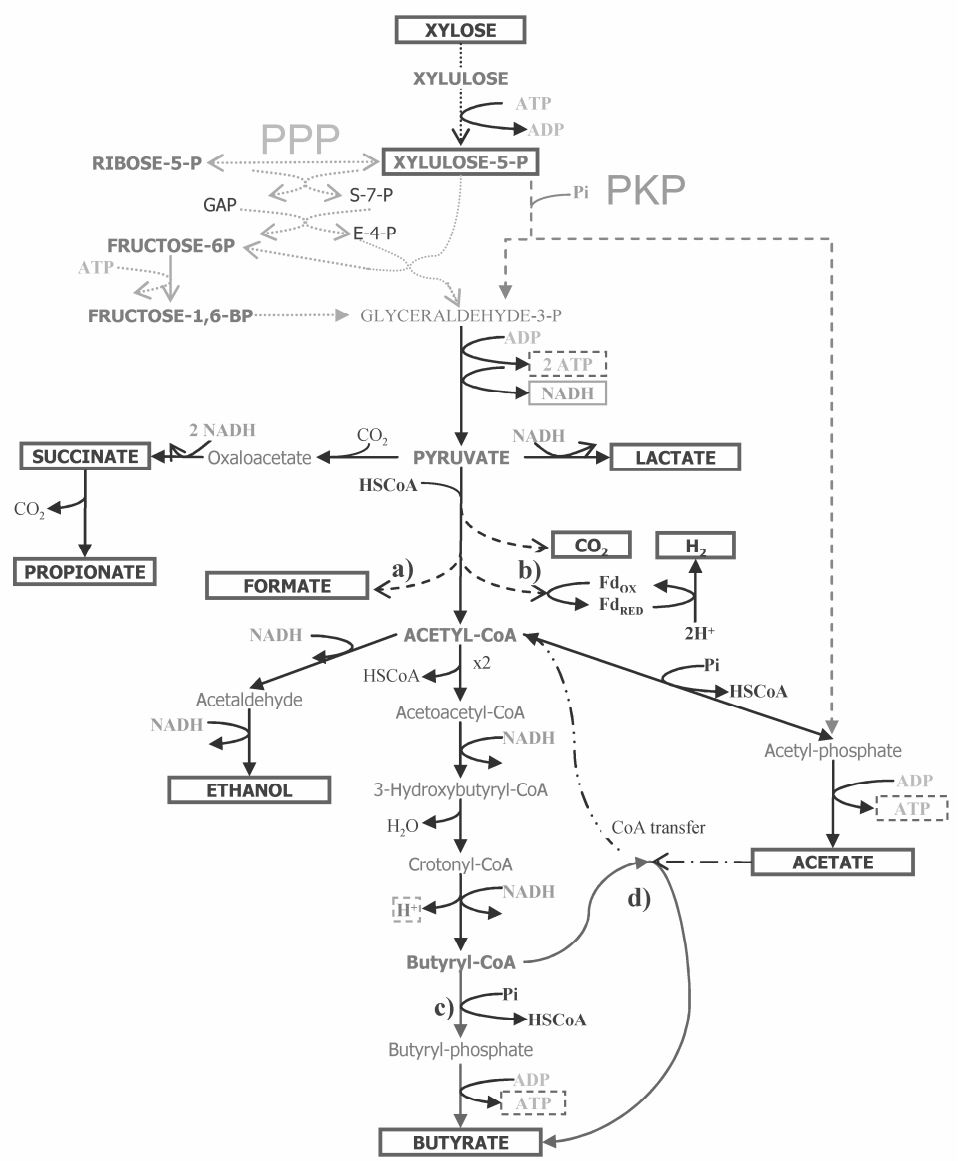

Figure 3.1- Adapted scheme with the most important and expected metabolic pathways during anaerobic fermentation of xylose by mixed culture. The differences between the pentose phosphate pathway (PPP) and the phosphoketolase pathway (PKP) are indicated. The reactions indicated with (a) and (b), (c) and (d) are alternative pathways for the same product, depending mainly on the type of microorganism (Biesterveld et al. 1994b; Tanaka et al. 2002).

Materials and Methods

Inoculum. The anaerobic mixed culture used as inoculum consisted of a mixture of two sludges, obtained from two different sources. The first inoculum came from a distillery wastewater treatment plant, Biothane in Delft, The Netherlands. This plant consists on an UASB operated at 30°C, pH 6.8-7.2, COD=2500 mg/L. The second inoculum was a sludge solution from a potato starch processing acidification tank in Broek op Langendijk, Smiths Food, The Netherlands. Approximately 20 ml of each inoculum was added to the two reactors at the beginning of the experiment, and operated in batch conditions until biomass growth was observed.

Medium. Initially, the cultivation medium contained the following (in g/L): xylose 4.0; NH_4Cl 1.34; KH_2PO_4 0.78; NaCl 0.292; $Na_2SO_4.10H_2O$ 0.130; $MgCl_2.6H_2O$ 0.120; $FeSO_4.7H_2O$ 0.0031; $CaCl_2$ 0.0006; H_3BO_4 0.0001; $Na_2MoO_4.2H_2O$ 0.0001; $ZnSO_4.7H_2O$ 0.0032; $CoCl_2.H_2O$ 0.0006; $CuCl_2.2H_2O$ 0.0022; $MnCl_2.4H_2O$ 0.0025; $Ni.Cl_2.6H_2O$ 0.0005; EDTA 0.050. It was also tested a substrate concentration of 10 g/l and co-fermentation of glucose and xylose (each at a final concentration of 2 g/l). Before starting the last experiment, the reactor was reset to the initial concentration (4 g/l). The ratio carbon/mineral solution was maintained during all the experiments. The carbon and mineral solutions were prepared and fed separately. The carbon containing solution was sterilized in an autoclave at 110 °C for 20 min.

Reactor operation. A continuously stirred reactor with a 3 L capacity (2 L working volume) was used (mechanical stirring 300 rpm). Water was recirculated through a water jacket to maintain a constant temperature of 30 ±1 °C. The reactor was sparged with nitrogen gas at a flow rate of 0.06 vvm in order to maintain anaerobic conditions. The pH was controlled (pH±0.1) by automatic titration (ADI 1030 Bio controller) with 4M NaOH and HCl solutions. To prevent excessive foaming, 3 ml/h of a 3% solution of silicone antifoaming agent (BDH, England) was dosed continuously. The dilution rate was 0.12 h^{-1}.

Steady State Characterization. To characterize the product spectrum obtained the reactor was run in continuous mode until a stable product composition and biomass concentration were established. The gases productivities (H_2 and CO_2) and the base added for pH control were monitored on-line, allowing for direct analysis of the system stability. When these three rate measurements were stable or varied within a limited range (±10%), not tending to increase or decrease, a set of samples was taken during the

following week. The samples were analyzed for the concentrations of substrate and soluble organic fermentation products and the biomass concentration.

Pulse experiments. These experiments were performed in the chemostat reactor while it was running stably. During each experiment, the inflow pumps were switched off and a pulse of a substrate (xylose, glucose and a mixture of the two) and minerals was given; samples were taken and the base addition to control the pH was monitored. A period of at least 10 days was waited before giving another pulse to assure full recovery of the reactor performance.

Calculations. The specific substrate uptake rate (-qS in Cmol-S/Cmol-X.h) was determined by adjusting a linear function to the experimental data of the substrate concentration plotted over time, and dividing the value obtained by the active biomass concentration at that point.

Analytical methods. Reactor broth samples were immediately filtered (Millipore membrane of 0.45 μm). The substrate and end products were determined and quantified. Xylose, glucose, xylitol, volatile fatty acids (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and caproate), lactate, succinic acid, formic acid were determined by HPLC, using an Aminex HPX-87H column from Bio Rad (T=60ºC) coupled to an UV and a RI detector, while phosphoric acid 0.01M was used as an eluent. The alcohols analyzed were ethanol, propanol and butanol. These analyses were performed by gas chromatography, Chromopack 9001, equipped with a flame ionization detector (FID) and a fused-silica capillary column 15 m x 0.53 mm HP-INNOWAX. The column temperature was 80 ºC for alcohols. The temperature of the injector and detector were 180 and 200 ºC, respectively. Furthermore, helium was used as a carrier gas. Measurements of H_2 and CO_2 in the off gas were performed on-line. A gas detection system was coupled to the bioreactor outlet and a Rosemount Analytical NGA 2000 MLT 1 Multi-component analyzer (infrared detector). Data acquisition (pH and gas) was achieved with SCADA software (Sartorius BBI systems MFCS/win 2.1).

The total soluble and total chemical oxygen demand (COD) was measured in the influent and in the reactor liquid using the Dr Lange kit (1000-10000 mgCOD/L). The total organic carbon (TOC) and total inorganic carbon (TIC) were measured using a Shimadzu TOC 5050A analyzer. For the total TOC and COD in samples with suspended solids, the samples were first homogenized through cell disruption in an ultrasound bath during 15 seconds. The inorganic carbon was also estimated based on the Henry coefficient of CO_2, the mass transfer coefficient (kLa) of the system and carbonate

equilibrium constants (see Chapter I). The biomass dry weight was determined after filtration, according to standard methods (Greenberg et al. 1992).

The yields of the fermentation products were calculated per substrate consumed, and were corrected for the dilution factor due to dosage of base for pH-correction. The COD and carbon balances were established based on the number of electrons and carbon atoms per mole, while assuming a standard biomass composition of $CH_{1.8}O_{0.5}N_{0.2}$ (Roels 1983).

Results

Comparison of glucose and xylose fermentation by mixed culture at pH 8

Two bioreactors were freshly inoculated with 20ml of each inoculum, and were left in batch mode with either glucose or xylose until growth and microbial activity was observed. Microbial activity could be detected by the online acquisition of the gas production (CO_2 and H_2) and the base added to maintain a constant pH. After this adaptation period, continuous feeding was initiated. The operational performance of the reactors was characterized when a pseudo steady state was achieved. The pseudo steady state as characterized by a constant product spectrum and biomass concentration was established after approximately four weeks of operation (eighty volume changes).

Under substrate limiting conditions, the product spectrum was largely comparable to glucose in the same conditions (Table 3.1). Compared to glucose, xylose fermentation produced more butyrate and less ethanol and the biomass yield per C-mol substrate was 20% lower. Succinate and propionate yields are not shown in Table 3.1, because they were always lower than 0.02 Cmol Cmol^{-1}. The carbon and electron balances showed 100±10 % recovery. Other possible fermentation products such as pyruvate, malate, fumarate, valerate, iso-valerate, caproate, propanol, butanol, 2,3-butanediol, acetoin and methylglyoxal were below the detection limit.

The yield of the acetyl-CoA derived products (sum of acetate, ethanol and butyrate) in both reactors was close to the combined yield of formate and molecular hydrogen. This indicates that formate and hydrogen are generated upon cleavage of pyruvate into acetyl-CoA. This suggests that both substrates should have been metabolized through glycolysis, Embden Meyerhof Parnas Pathway (EMP), and pentose phosphate pathway in the case of xylose (eq. 3.1). In case of a significant contribution of the phosphoketolase pathway (PKP), a substantial amount of acetate not accompanied by hydrogen or formate production (eq. 3.2) should have been detected.

The ATP generated through catabolism was estimated by assuming that the ATP is only generated by substrate level phosphorylation (SLP) involved in each product formation pathway (Decker et al. 1970). This means, each pyruvate-derived product involves a net formation of one ATP from glycolysis; additionally, per mole of acetate and butyrate formed one more ATP is generated. The estimated catabolic ATP yields per carbon mole of substrate converted are also given in Table 3.1. These values suggested that xylose catabolism was more efficient than glucose because more ATP was generated per carbon of xylose converted. However, the measured biomass yield indicated that xylose grown biomass had a significantly lower efficiency in ATP-utilization for biomass production compared to growth on glucose.

Table 3.1- Product yields of xylose and glucose fermentation in a chemostat by mixed culture, at pH 8.

	Xylose, 4 g/L	**Glucose, 4 g/L**	**Xylose, 10 g/L**
Dilution rate (h^{-1})	0.128 ± 0.004	0.128 ± 0.004	0.134 ± 0.004
Biomass yield (Cmol/Cmol)	0.16 ± 0.01	0.21 ± 0.01	0.13 ± 0.01
Product Yields (Cmol/Cmol-S)			
H_2 (mol/Cmol-S)	0.13 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
Formate	0.20 ± 0.02	0.22 ± 0.01	0.18 ± 0.01
Acetate	0.18 ± 0.01	0.16 ± 0.02	0.22 ± 0.01
Butyrate	0.46 ± 0.02	0.30 ± 0.04	0.07 ± 0.01
Ethanol	0.017 ± 0.004	0.08 ± 0.01	0.23± 0.01
H_2 + Formate	0.33 ± 0.02	0.27 ± 0.01	0.24 ± 0.01
Acetyl-CoA derivatives [a)]	0.66 ± 0.03	0.54 ± 0.02	0.53 ± 0.02
ATP/Cmol-S [b)]	0.54 ± 0.01	0.42 ± 0.01	0.41 ± 0.01
X/ATP (g/mol ATP)	7.6	12.3	8.0

a) Sum of the acetyl-CoA derivatives: acetate, butyrate and ethanol in carbon units.

b) The yield of ATP was estimated based on the catabolic products: one ATP per ethanol produced, two ATP per acetate and 3 ATP per butyrate.

When the xylose concentration was increased to 10 g/L, the product spectrum shifted to more ethanol and acetate at the expense of butyrate (Table 3.1). Additionally, the catabolic ATP yield decreased, but the biomass yield on ATP remained in the same range of the yield at lower substrate concentration. Analogous effect of increased substrate/product concentration has been observed when glucose was the substrate (chapter IV).

Characterization under substrate non-limiting conditions

To make a clearer comparison between xylose and glucose metabolism by the selected population three pulse-experiments were conducted in the xylose fed chemostat. In the first experiment, two pulses of xylose were supplied. The second pulse was supplied after xylose from the first pulse was fully converted. Additional pulse experiments were performed with glucose, and with a mixture of xylose and glucose. During each experiment, the bioreactor was put in discontinuous mode, samples were taken and the base addition to control the pH was monitored. Results are shown in Figure 3.2. While in the chemostat reactor the rates (growth and substrate uptake) were set by dilution rate, during these pulse experiments, substrate non-limiting conditions were established. The calculated kinetic values and the biomass yield are given in Table 3.2. The initial substrate uptake rates were comparable with the chemostat reactor (1.01, 0.78 and 0.97 compared with 0.89 Cmol/Cmol-X h). The catabolic products were the same as obtained during continuous operation: mainly butyrate and acetate.

The operational performance of the bioreactor was largely unaffected by the first xylose pulse. However, when a second pulse with the double amount of substrate was supplied, the uptake rate decreased 26%. This suggests either substrate or product inhibition, since these were the only differences between the two pulses. In terms of product concentrations, the total amount of acids increased more than 30 %.

When a glucose pulse was supplied, the culture immediately started taking up the glucose. Despite the lower substrate uptake rate, the culture could make a more efficient use of this substrate as indicated by the increase in the biomass yield (0.14 Cmol/Cmol and 0.08 Cmol/Cmol for glucose and Xylose, respectively). Considering that the catabolic products were comparable, the difference in the biomass yield should be attributed to the substrate.

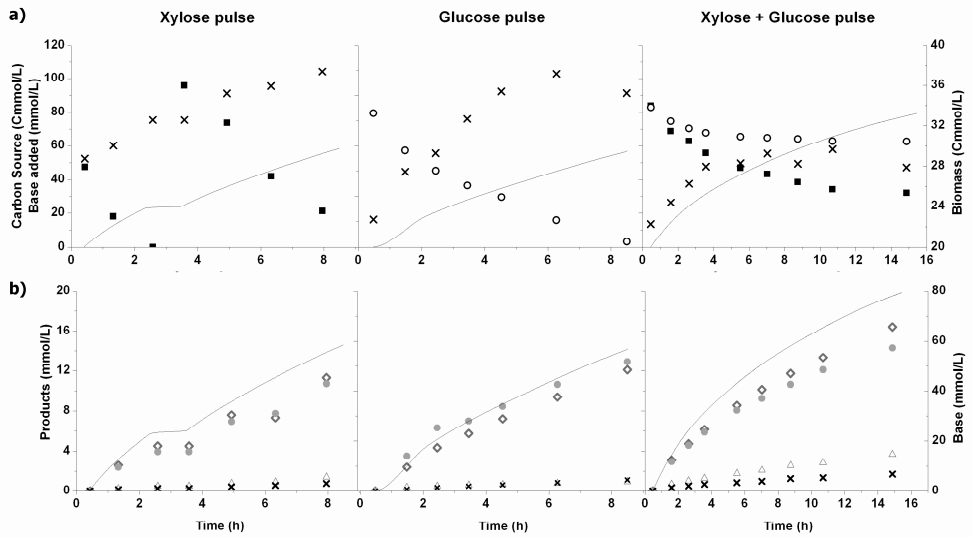

Figure 3.2- **a)** Substrate consumption, biomass growth and base added after Xylose and glucose pulses to the chemostat fed with xylose (4 g/L, pH 8.0, dilution rate of 0.12 h^{-1} and temperature 30 C): ■ xylose, O glucose, ✕ biomass (Cmmol/L) and – sodium hydroxide added to adjust the pH (mmol/L). **b)** Catabolic products and base added after Xylose and glucose pulses to the chemostat fed with xylose (4 g/L, pH 8.0, dilution rate of 0.12 h^{-1} and temperature 30 C)): ● acetate, △ propionate, ◇ butyrate, ✕ ethanol (Cmmol/L) and – sodium hydroxide added to adjust the pH (mmol/L).

During the pulse with the two substrates, it can be seen in figure 3.2 that initially (first two hours) both substrates were simultaneously consumed. The glucose uptake rate was approximately 20% lower than the xylose uptake rate; this difference was also observed between the single substrate pulse experiments. However, after this period, glucose consumption almost ceased and xylose conversion decelerated. The biomass yield was also affected and decreased by 20%. The product spectrum was not affected.

From these pulse experiments in the chemostat, it was confirmed that: although no significant difference was observed in the catabolic products, the growth yield was enhanced when glucose was the substrate (Figure 3.2 and table 3.2). Additionally, glucose conversion capacity was constitutively present in the microbial community growing on xylose.

Table 3.2- Kinetic parameters of the mixed microbial culture operated in continuous (substrate concentration of 4 g/L, pH 8.0, dilution rate of 0.12 h^{-1} and temperature 30 C) and when subjected to a pulse of a substrate (xylose, glucose and a mixture of the two substrates).

Experiment	**Substrate**		**Specific Substrate Uptake Rate ($-q_s$, Cmol/Cmol-X L h)**	**Biomass yield (Cmol/Cmol)**
Reactor	Xylose[a]		0.89	0.16
	Glucose		0.62	0.21
Pulse Experiments	Xylose	1st addition (2g/L)	1.01	0.081
		2nd addition (5g/L)	0.55	0.064
	Glucose	Glucose (2.5g/L)	0.78	0.143
	Xylose and glucose	Phase 1 Xylose + Glucose	0.97	0.111
		Xylose (2.5 g/L)	0.56	
		Glucose (2.5 g/L)	0.41	

a) Average of the values determined during three days before these experiments.

Co-fermentation of glucose and xylose by mixed culture

In agricultural waste streams a mixture of sugars is present. Since a slight different product spectrum was obtained when glucose and xylose were fermented in separate, co-fermentation of xylose and glucose by the culture grown on xylose was studied. The two substrates were fed together in equal carbon amounts (2 g carbon/L each) (Figure 3.3). During the first 24 hours (three retention times) little difference could be detected. However, after three days, the ethanol yield increased and the butyrate yield decreased. This product spectrum seems to be an intermediate to the one obtained when the two substrates were fed separately.

Despite the fact that the biomass yield on the carbon source was not significantly affected, the biomass yield on the ATP generated increased approximately 10%. The residual concentrations of the two substrates were below the detection limit (0.05 mM) and no substrate preference could be detected when a xylose-glucose mixture was used.

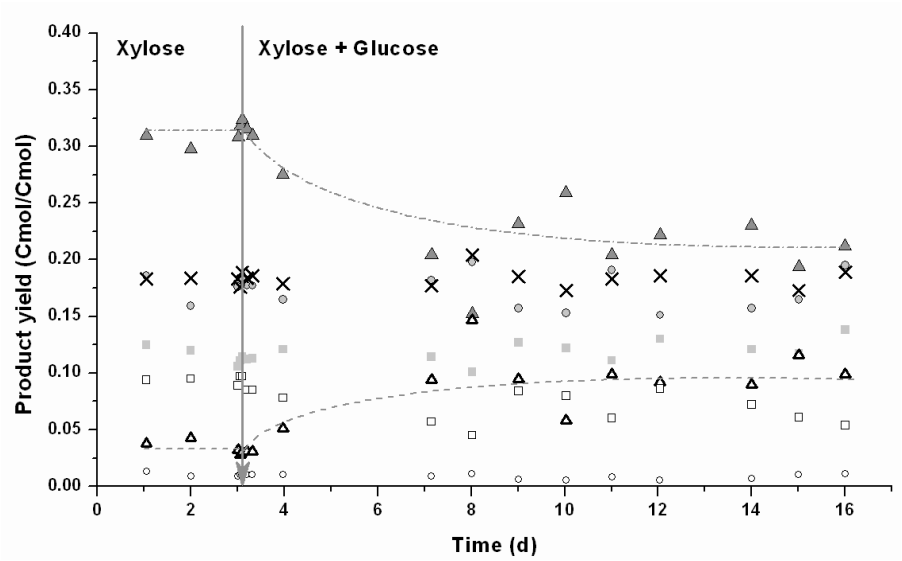

Figure 3.3- Co-fermentation of glucose and xylose by a mixed microbial culture cultivated on xylose. The arrow indicates the moment when the substrate in the medium (xylose 4 g/l) was replaced by a mixture of two substrates (glucose and xylose, both 2 g/l): □ molecular hydrogen, ▒ formate, ● acetate, ○ propionate, ▲ butyrate, △ethanol and × biomass.

Discussion

A mixed microbial population has been selected from a rich inoculum, in a CSTR fed with xylose under substrate limiting conditions. This culture was capable of xylose conversion to concentrations lower than the detection limit of 50 μm, suggesting a high affinity for the substrate. The xylose grown chemostat culture was also able to degrade glucose, not showing a preference for glucose or xylose when point doses of the two substrates are supplied to the bioreactor.

One of the major differences observed between mixed culture grown on xylose and on glucose is the biomass yield on ATP ($Y_{X/ATP}$). The yield of catabolic products is higher for xylose, which would suggest a higher generation of ATP ($Y_{ATP/S}$); however, the biomass yield is in fact higher when glucose is the substrate ($Y_{X/S}$). This difference has also been observed in studies conducted with pure bacterial cultures, where the biomass yield on the catabolic ATP-yield estimated ($Y_{ATP/S}$) is significantly higher for glucose than for xylose (Biesterveld et al. 1994a; Heyndrickx et al. 1991; Ounine et al. 1985). Additionally, in solvent producing bacteria not only growth is inhibited but also the xylose uptake is affected by the solvents (Ounine et al. 1985; Qureshi and Blaschek 2000), this is in agreement with the lower energy provided during xylose catabolism. Such a difference has not been reported for modified yeast cells that were able to ferment xylose. In this study, the $Y_{ATP/S}$ remained in the same range for growth on the two substrates (Kuyper et al. 2005a). Xylose transport can occur by different mechanisms (Dien et al. 2003). Facilitated diffusion is the most common mechanism in yeast, the glucose transporters can also catalyze xylose uptake, but have a much lower affinity (Hamacher et al. 2002). In bacteria, however, specific transporters with different affinities have been identified (Bothun et al. 2004). Of particular interest is the high affinity transporter that consists of an ABC protein and therefore involves one ATP consumption for each xylose transported (Ahlem et al. 1982; Hasona et al. 2004). This transporter might be widespread over bacterial species able to metabolize xylose. According to genome databases (www.genome.jp), the gene is present in fermentative (gram-positive) microorganisms like *Cl. acetobutylicum*, but whether it is expressed remains unknown. In case this is the transporter most active in the microbial population selected, the catabolic ATP yield can be recalculated by considering the need of one ATP for xylose transport and one for activation inside the cell. This implies an ATP-yield per mole pyruvate of 0.4 moles ATP/ mol pyruvate. Whereas glucose requires only one ATP for transport and activation, being the

glycolytic efficiency 1 mole ATP/ mol pyruvate. Including these assumptions in our calculations the $Y_{X/ATP}$ becomes 12.4 g-VSS/mol-ATP, which is in the same range as glucose (12.2 g-VSS/mol-ATP). We therefore suggest that the lower biomass yield ($Y_{X/S}$) for growth on xylose compared to glucose, is related to the ATP-consumption required for active xylose uptake. As a consequence, in case the products become toxic, the growth will stop earlier, and lower amounts of substrate are converted.

Xylose, as one of the main constituents of hemicellulose, has been a major subject of research, mostly in connection with the production of ethanol (van Maris et al. 2004). One of the problems in this fermentation was the inability of many organisms to metabolize efficiently all the carbohydrates derived from hemicellulose. In this study, a mixed microbial population was selected especially for xylose conversion. It was shown that even a culture that was cultivated only on xylose, could immediately convert glucose. Under non-limiting conditions, however, the conversion of the two substrates showed some limitations. Mixed substrate utilization in batch mode could be further improved by operating in a sequencing batch reactor on the two substrates (Kuyper et al. 2005b).

Conclusions

In line with the hypotheses in the introduction, the results of this study show that despite the similar chemical structure of xylose and glucose, the fermentation of these two substrates is different. The biomass yield on xylose is approximately 20% lower, resulting in the production of more catabolic products. This lower biomass yield is likely associated with the ATP needed for the xylose uptake by the bacteria.

Chapter 4

Glycerol fermentation by (open) mixed culture: a chemostat study

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Abstract

Glycerol is an important byproduct of bioethanol and biodiesel production processes. This study aims to evaluate its potential application in mixed culture fermentation processes to produce bulk chemicals. Two chemostat reactors were operated in parallel, one fed with glycerol and the other with glucose. Both reactors operated at a pH of 8 and a dilution rate of 0.1 h^{-1}. Glycerol was mainly converted into ethanol and formate. When operated under substrate limiting conditions, 60% of the substrate carbon was converted into ethanol and formate in a 1:1 ratio. This product spectrum showed sensitivity to the substrate concentration and partly shifted towards 1,3-propanediol and acetate in a 2:1 ratio at increasing substrate concentrations. Glucose fermentation mainly generated acetate, ethanol and butyrate. At higher substrate concentrations, acetate and ethanol were the dominant products.

Co-fermentations of glucose-glycerol were performed with both mixed cultures, previously cultivated on glucose and on glycerol. The product spectrum of the two experiments was very similar: the main products were ethanol and butyrate (38 and 34% of the COD converted, respectively).

The product spectrum obtained for glucose and glycerol fermentation could be explained based on the general metabolic pathways found for fermentative microorganisms and on the metabolic constraints: maximization of the ATP production rate and balancing the reducing equivalents involved.

Introduction

Glycerol is now widely recognized as an important waste stream. The enormous increase in biodiesel production is accompanied by a rapidly increasing availability of glycerol containing waste streams. The rule of thumb is that 1 kg of glycerol emerges as a by-product per 10 kg of biodiesel produced (http://www.biodiesel.org), reaching concentrations of more than 60 % in the waste streams (Gonzalez-Pajuelo et al. 2004). In 2004, 1900 kton biodiesel were produced together with 190 kton of glycerol. By 2005, the biodiesel production had already increased to 3200 kton (http://europa.eu.int). Bioethanol, another biofuel, is widely produced from sugar fermentation by yeast. During this process, glycerol is also a significant byproduct (Vandijken and Scheffers 1986), representing up to 10% of the total substrate converted at the end of a fermentation process (Borzani 2006). Despite the many efforts made to decrease the glycerol yield, reduction of glycerol production also leads to decreased ethanol productivity (Bideaux et al. 2006).

Glycerol has many uses in different industries (pharmaceutical, cosmetic, paint, automotive, food, etc), but its surplus is dramatically increasing and, therefore, new applications should be developed. The biological conversion of glycerol from wastestreams into raw materials or biofuels could be an attractive option. Glycerol can be fermented by a number of microorganisms and can be converted into various interesting products (Pachauri and He 2006). Examples of catabolic products from anaerobic fermentation are organic acids (acetate, propionate, and butyrate), solvents (ethanol, butanol) and 1,3-propanediol (Lengeler et al. 1998). A particularly attractive product of glycerol fermentation is 1,3-propanediol, because it can potentially be produced in large amounts during this process and, more importantly, it can be used as a monomer in the synthesis of bioplastics (polyesters and polyurethanes) (Biebl et al. 1992; Cheng et al. 2006). To investigate glycerol fermentation, most previous studies have applied pure culture; *Klebsiella aerogenes* or *Clostridium butyricum* are the best known examples (Papanikolaou et al. 2000; Zeng et al. 1993). In a study with C. butyricum, 1,3-propanediol emerged when the culture was fed with glycerol instead of glucose, whilst the production of organic acids (butyrate and acetate) decreased (AbbadAndaloussi et al. 1996). Ethanol production from glycerol has also been investigated, because for certain bacterial strains under similar operational conditions, the ethanol yields obtained on glycerol are higher than those obtained on glucose (Dharmadi et al. 2006; Ito et al. 2005; Jarvis et al. 1997). For this purpose, strains unable to produce 1,3-propanediol

are preferred in order to decrease the sideproduct formation. Examples of such strains are *E. coli* and *Enterobacter aerogenes* (Dharmadi et al. 2006; Homann et al. 1990; Sakai and Yagishita in Press).

To profit from these bulk products, the processing costs should be low. One way of decreasing the costs is working under non-sterile conditions, using open mixed culture (Kleerebezem and van Loosdrecht 2007). These cultures are based on natural inocula with a high microbial diversity that allow for an operation of bioprocesses under non-sterile conditions with no risk of strain degeneration. Furthermore, due to the higher microbial diversity, these cultures are better capable of dealing with substrate mixtures of variable composition. Although a mixed culture involves a higher microbial diversity, selection occurs for a limited number of microorganisms that are best capable to adapt to the imposed conditions. In a previous study, it has been observed that, at high pH, glucose fermented by mixed culture is mainly converted into acetate and ethanol in equimolar amounts. This result was largely explained on the basis of the metabolic constraints and on the maximization of energy generation (chapter II). Due to the higher degree of reduction of glycerol compared to carbohydrates, it is hypothesized that glycerol fermentation may result in the generation of more reduced compounds like ethanol, butanol or 1,3-propanediol, and less oxidized fatty acids like acetate (Table 4.1).

Glucose and glycerol share the same metabolic pathway, from glyceraldehyde-3-phophate until pyruvate and the respective derived products (Figure 4.1)(Lin 1976; Neijssel et al. 1975). Figure 4.1 presents the most common metabolic pathways of glucose and glycerol fermentation that can be expected from a mixed culture. The place where each of the substrates enters the glycolysis is also indicated.

An important difference between the two substrates, however, is the number of electrons per carbon. Thus, the conversion of glycerol into pyruvate generates two moles of NADH per pyruvate produced, while only one mole is involved in the case of glucose. An additional difference is that another product can be formed from glycerol: 1,3-propanediol, which has not been observed when glucose is the substrate. Table 4.1 summarizes the main product formation routes derived from glycerol and glucose as shown in Figure 4.1. The acetyl-CoA yield represents the acetyl-CoA derived products, namely acetate, butyrate and ethanol. Each mole of acetyl-CoA produced is associated with the formation of one mole of molecular hydrogen or formate.

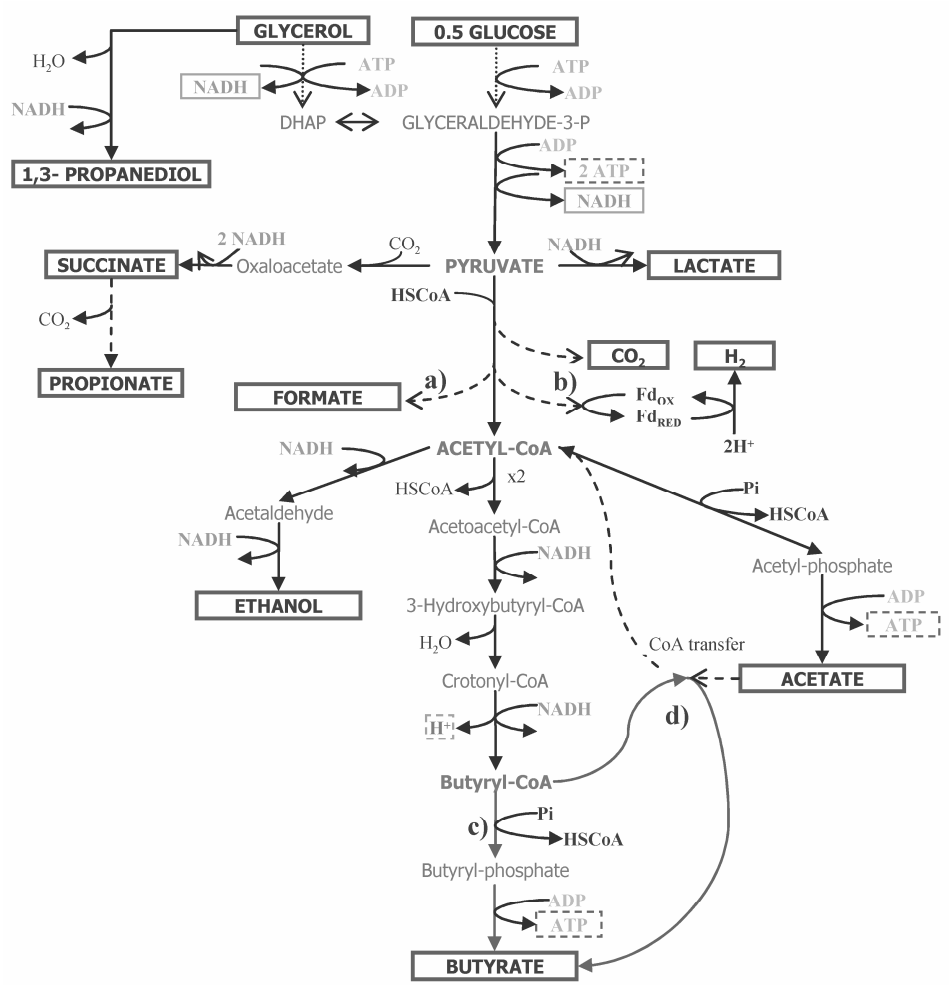


Figure 4.1- Scheme with the most important metabolic pathways during anaerobic fermentation of glucose and glycerol by mixed culture. The reactions indicated by (a) and (b), (c) and (d) and (e) and (f) are alternative pathways for the same product, depending mainly on the type of microorganism (Barbirato et al. 1997; Gonzalez-Pajuelo et al. 2006; Lengeler et al. 1998; Lin et al. 1960; Neijssel et al. 1975; Papoutsakis 2000; Papoutsakis and Meyer 1985; Saint-Amans et al. 2001; Sawers 2005).

Table 4.1 - Possible products from the glucose and glycerol fermentation process and the respective relevant intermediates and cofactors involved in the metabolic pathways. The number of NADH and ATP molecules involved is in agreement with figure 1 and was based on the assumption that glycolysis occurs through the Embden Meyerhof Parnas Pathway (EMP) and substrate level phosphorylation (SLP) involved in each product formation pathway. The two substrates were expressed in C_3 units, three moles of substrate carbon converted.

	Substrate (C_3 unit) → product			$Y_{NADH/Product}$	
Product	$Y_{prod/\ C3\ unit}$	$Y_{Ac\text{-}CoA/\ C3\ unit}$	$Y_{ATP/\ C3\ unit}$	**0.5 Glucose**	**Glycerol**
Acetate	1	1	2	+1	+2
Ethanol	1	1	1	-1	0
Butyrate	0.5	1	1.5	0	+2
Lactate	1	0	1	0	+1
Propionate	1	0	1	-1	0
Succinate	1	0	1	-1	0
1,3 -Propanediol	1	0	0	-	-1

Note: Each mole of acetyl-CoA produced is associated with the formation of one mole of molecular hydrogen or formate.

The aim of this research is to investigate the product spectrum of glycerol fermentation at high pH in a mixed culture fermentation process and compare it to glucose fermentation under the same process conditions. A link between the product spectrum as a function of the type of substrate, and the thermodynamic and kinetic state of the system is elaborated, based on generalized biochemical principles.

Materials and Methods

Reactor operation. A continuously stirred reactor with a 3 L capacity (2 L working volume) was used (mechanical stirring 300 rpm). Water was recirculated through a water jacket to maintain a constant temperature of 30 ±1 °C. The reactor was sparged with nitrogen gas at a flow rate of 0.06 vvm in order to maintain anaerobic conditions. The pH was controlled (pH±0.1) by automatic titration (ADI 1030 Bio controller) with 4M NaOH and HCl solutions. To prevent excessive foaming, 3 ml/h of a 3% solution of silicone antifoaming agent (BDH, England) was dosed continuously. The dilution rate was 0.12 h^{-1}. To test the impact of the substrate concentration, the influent concentration was step wisely increased (10, 15, 20 and 24 g/l). The reactors were reset to the initial condition (4 g/l) before the study of mixed substrates fermentation, glucose and glycerol, each at a final concentration of 2 g/l).

Inoculum. The anaerobic mixed culture used as inoculum consisted of a mixture of two sludges, obtained from two different sources. The first inoculum came from a distillery wastewater treatment plant, Biothane in Delft, The Netherlands. This plant consists of an UASB operated at 30°C, pH 6.8-7.2, COD=2500 mg/L. The second inoculum was a sludge solution from a potato starch processing acidification tank in Broek op Langendijk, Smiths Food, The Netherlands. Approximately 20 g of each inoculum was added to the two reactors at the beginning of the experiment, and operated in batch conditions until biomass growth was observed.

Medium. The cultivation medium contained the following (in g/L): glucose or glycerol 4.0; NH_4Cl 1.34; KH_2PO_4 0.78; NaCl 0.292; $Na_2SO_4.10H_2O$ 0.130; $MgCl_2.6H_2O$ 0.120; $FeSO_4.7H_2O$ 0.0031; $CaCl_2$ 0.0006; H_3BO_4 0.0001; $Na_2MoO_4.2H_2O$ 0.0001; $ZnSO_4.7H_2O$ 0.0032; $CoCl_2.H_2O$ 0.0006; $CuCl_2.2H_2O$ 0.0022; $MnCl_2.4H_2O$ 0.0025; $Ni.Cl_2.6H_2O$ 0.0005; EDTA 0.050. The ratio of carbon/mineral solution was maintained during all the experiments. The carbon and mineral solutions were prepared and fed separately. The carbon containing solution was sterilized in an autoclave at 110 °C for 20 min.

Steady State Characterization. To characterize the product spectrum obtained from each substrate, the two reactors were run in continuous mode until a stable product composition and biomass concentration were established. The gas productivities (H_2 and CO_2) and the base added for pH control were monitored on-line, allowing for direct analysis of the system stability. When these three rate measurements were stable or varied within a limited range (±10%), a set of samples was taken during the following week. The concentrations of soluble organic fermentation products and biomass concentration in the reactor volume were determined.

Analytical methods. Reactor broth samples were immediately filtered (Millipore membrane of 0.45 μm). The substrate and end products were determined and quantified. Glucose, glycerol, 1, 3-propanediol, volatile fatty acids (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and caproate), lactate, succinic acid, formic acid were determined by HPLC, using an Aminex HPX-87H column from Bio Rad (T=60ºC) coupled to an UV and a RI detector, while phosphoric acid 0.01M was used as an eluent. The alcohols analyzed were ethanol, propanol and butanol. These analyses were performed by gas chromatography, Chromopack 9001, equipped with a flame ionization detector (FID) and a fused-silica capillary column 15 m x 0.53 mm HP-INNOWAX. The column temperature was 80 ºC for alcohols. The

temperature of the injector and detector were 180 and 200 ºC, respectively. Furthermore, helium was used as a carrier gas.

Measurements of H_2 and CO_2 were performed on-line. A gas detection system was coupled to the bioreactor outlet and a Rosemount Analytical NGA 2000 MLT 1 Multi-component analyzer (infrared detector). A Sartorius BBI MUX 100 Multiplexer was used to analyze the off gas composition of the two reactors sequentially. Data acquisition (pH and gas) was achieved with SCADA software (Sartorius BBI systems MFCS/win 2.1).

The total soluble and total chemical oxygen demand (COD) was measured in the influent and in the reactor liquid using the Dr Lange kit (1000-10000 mgCOD/L). The total organic carbon (TOC) and total inorganic carbon (TIC) were measured using a Shimadzu TOC 5050A analyzer. For the total TOC and COD in samples with suspended solids, the samples were first homogenized cell disruption during 15 seconds in an ultrasound bath (Sonifier B12, Bransonic). The inorganic carbon was also estimated based on the Henry coefficient of CO_2, the mass transfer coefficient (kLa) of the system and carbonate equilibrium constants (see Chapter II).

The biomass dry weight was determined after filtration, according to standard methods (Greenberg et al. 1992).

The yields of the fermentation products were calculated per substrate consumed, and were corrected for the dilution factor due to dosage of base for pH-correction. The COD and carbon balances were established based on the number of electrons and carbon atoms per mole, while assuming a standard biomass composition of $CH_{1.8}O_{0.5}N_{0.2}$ (Roels 1983).

The Gibbs free energy change of a reaction in the actual process conditions was calculated using the following equation (Hanselmann 1991):

$$\Delta G = \Delta G^{0'} + RT \ln Q \qquad (1)$$

where R is the gas constant (8.31451 $J.mol^{-1}.K^{-1}$), T is the temperature (K) and Q is the ratio of the actual activity of the products and reactants.

Results

Comparison of glucose and glycerol fermentation by mixed culture at pH 8

To study the influence of the two substrates (glucose and glycerol) on the product spectrum of mixed culture fermentation, two reactors were operated in parallel under the same conditions, but fed with different substrates. Both reactors were freshly inoculated and were left in batch mode until growth was observed. After this adaptation period, continuous feeding was initiated. The operational performance of the reactors was characterized when a pseudo steady state was achieved (generally after 3-4 weeks, more than 60 volume changes). In Figure 4.2, the distribution of the major product yields is illustrated and the yields are given in Table 4.2. As can be seen, the main products were the same, except for 1,3-propanediol (only present in the reactor fed with glycerol (B)). Even though the products identified were comparable, the relative concentrations/yields were different. Acetate and butyrate, some of the major products of glucose fermentation, were almost negligible when glycerol was the substrate. Conversely, ethanol was produced in much bigger quantities with glycerol as a substrate. The biomass yield on the substrate was nearly twice as big when glucose was the substrate. The biomass yield on the ATP produced was 12.1 g-X/mol ATP and 9.5 g-X/mol ATP, for glucose and glycerol cultivated cultures. This is a difference of nearly 20%, lower in the case of the glycerol-grown culture. This can be related to the fact that for growth C6 units are required and these are provided when glucose is the substrate, while they have to be synthesized when glycerol is the substrate.

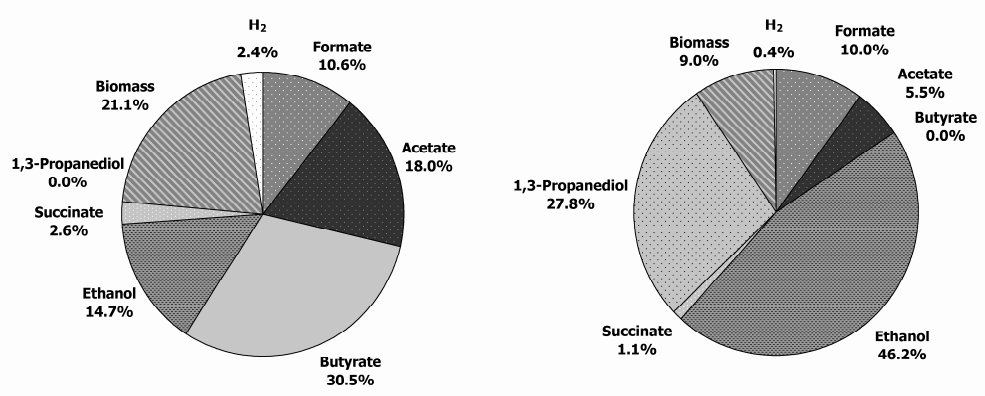


Figure 4.2- Product distribution from glucose (a) and glycerol (b) fermentation. The reactors were operated in continuous mode at pH 8, with a retention time of 8h. The substrate concentration was 4 g/l in both reactors.

Lactate and propionate are not given, because their yields on both substrates were lower than 2%. In both reactors, the carbon and electron balances were 100±10 % recovery. Other possible fermentation products such as pyruvate, malate, fumarate, valerate, iso-valerate, caproate, propanol, butanol, 2,3-butanediol, acetoin and methylglyoxal that could have been determined, were not detected in significant amounts.

With regard to NADH turnover, the redox neutral conversions for glucose are butyrate or acetate-ethanol (one-to-one) production (table 4.1). In the case of glycerol, the NADH production equals the consumption when ethanol or acetate-1,3-propanediol (one-to-two) are produced (see table 4.1). Biomass is assumed to have a degree of reduction of 33.6 gCOD/Cmol, which is different for both substrates (32 and 37.3 gCOD/Cmol for glucose and glycerol, respectively), and may therefore slightly altered the product ratios.

The two substrates studied have different compositions and degrees of reduction, but they share the same metabolic pathway from glyceraldehyde-3-phophate until pyruvate and the respective derived products pathways. Thus, for an easier comparison between the two types of metabolisms established in Table 4.2, the two substrates are referred to as C_3 units (3 moles of carbon converted (C_3mol). The same concentrations of C_3 moles were added to both reactors (approximately 45 mM). The yield of the acetyl-CoA derived products (sum of acetate, ethanol and butyrate) in the glucose fed reactor was comparable to the yield of formate and molecular hydrogen (0.81 and 0.78 mol/C_3mol, respectively). In the glycerol fed reactor the yield of the acetyl-CoA derived products (0.73 mol/C_3mol) was slightly lower, due to the production of 1,3-propanediol that does not derive from this metabolite.

Table 4.2- Product yields of glucose and glycerol fermentation by mixed culture in a chemostat reactor operated at pH 8 and with a dilution rate of $0.12h^{-1}$.

	Glucose	**Glycerol**
	Yield (mol/C_3 mol)	
Biomass (Cmol/C_3 mol)	0.59 ±0.02	0.30 ±0.07
H_2	0.17 ±0.00	0.05 ±0.01
Formate	0.64 ±0.00	0.75 ±0.02
Acetate	0.20 ±0.00	0.04 ±0.01
Butyrate	0.21 ±0.01	0.01 ±0.01
Ethanol	0.17 ±0.01	0.67 ±0.03
1,3-Propanediol	- -	0.14 ±0.02
H_2+formate	0.81 ±0.01	0.80 ±0.05
Acetyl-CoA derivatives	0.79 ±0.01	0.73 ±0.03

Effect of substrate concentration

In both reactors, the substrate concentration was increased from 4 to 25 g/L in a stepwise mode. The shift in the product yields is shown in Figure 4.3.

A shift in the product spectrum was observed in both reactors. In the reactor fed with glucose (a), butyrate, a significant product at low substrate concentrations, became less dominant as the substrate concentration increased, while ethanol and acetate became the major products in equal molar amounts. The amount of acetyl-CoA derived products was not affected by the changes in substrate concentrations, and was nearly the same as the sum of formate and molecular hydrogen yields.

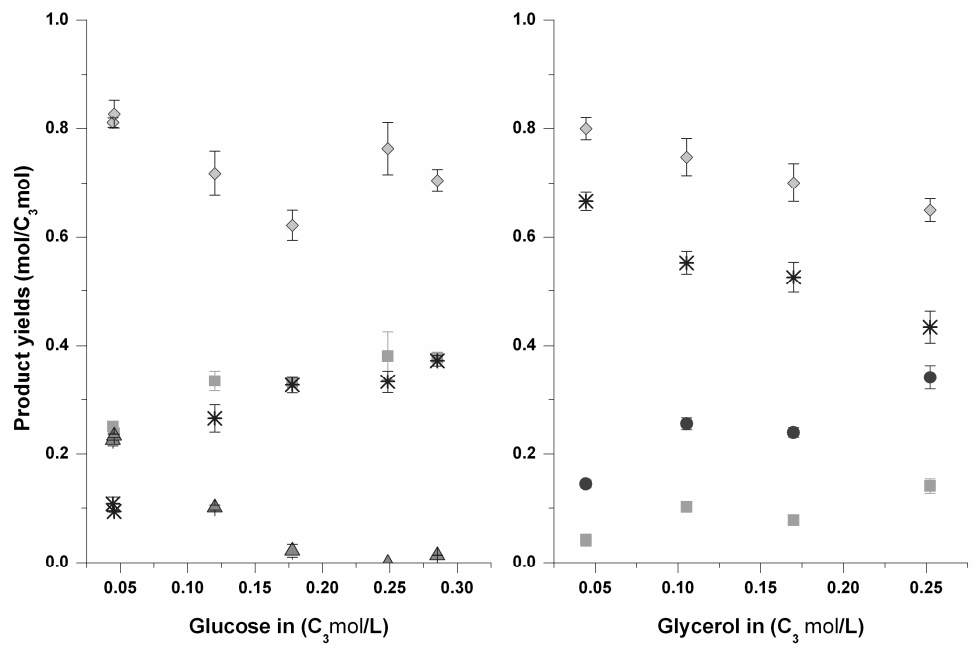


Figure 4.3- Impact of substrate concentration on the product spectrum of glucose (a) and glycerol (b) fermentation by mixed culture at pH 8: ◇ formate + H_2, ■ acetate, △ butyrate and ✳ ethanol and ● 1,3-propanediol.

A similar effect was observed in the reactor fed with glycerol (b). At increasing substrate concentrations, the yields of ethanol and formate decreased and 1, 3-propanediol and acetate increased. In this case, the biomass specific flux through acetyl-CoA decreased, as can also be seen by the decrease of the sum of formate and molecular hydrogen when the substrate concentration increased. This is due to the fact that 1,3-propanediol does not derive from acetyl-CoA the way acetate, butyrate and ethanol do.

Co-fermentation of glucose and glycerol by mixed culture

To study the capacity of adaptation of a mixed culture to the other substrate, the two reactors were subjected to a mixture of the two substrates after cultivation on either glucose or glycerol. This initial cultivation period on the individual substrates lasted three to four weeks, more than 60 biomass residence times. After this period, the feeding solution containing a single substrate was replaced by a mixture of the two substrates (glucose and glycerol), supplied in the same amount of C_3 units (11 mM of glucose and 22 of glycerol, totalizing 44 mM of C_3). The response of the systems during the transition was monitored and the product yields per C_3 unit of the different products during this period are presented in figure 4.4.

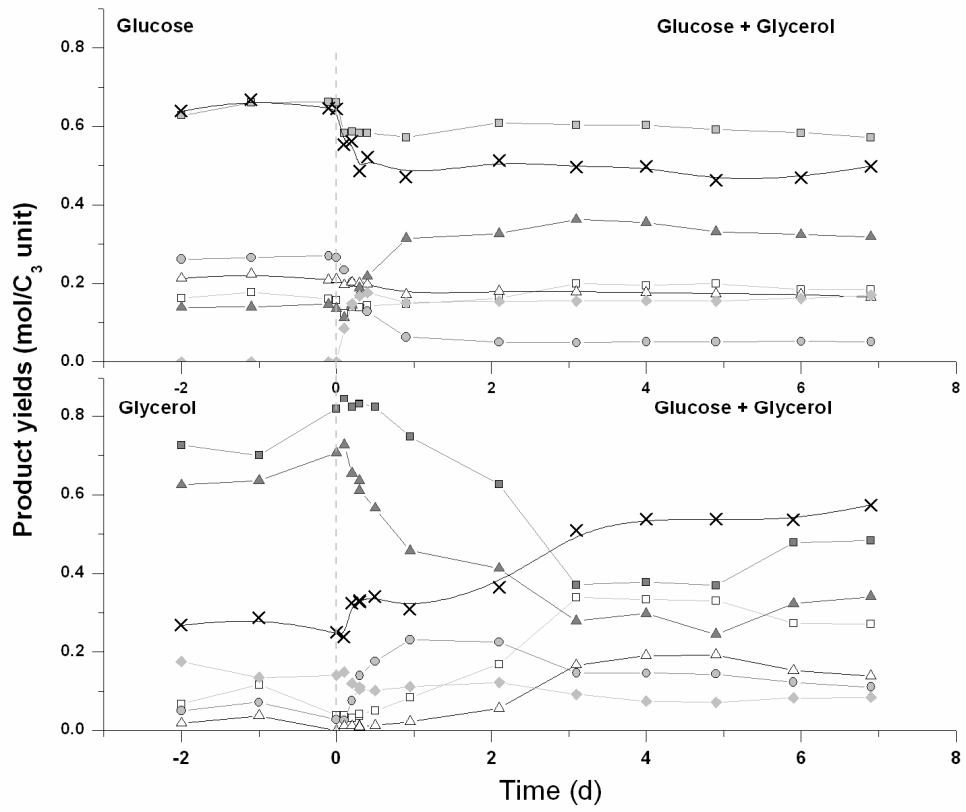


Figure 4.4- Product yields during the transition from a single substrate (a-glucose and b-glycerol) to a mixture of both substrates in a chemostat at pH 8 and a retention time of 8h: ■ formate, □ molecular hydrogen, ● acetate, Δ butyrate and ▲ ethanol, ◆ 1, 3-propanediol and × biomass.

Three days after the change, both reactors showed a stable product spectrum towards the newly imposed situation. Despite the differences in the

concentrations observed one week after the change in substrate supply, the relative yields were comparable in both reactors: ethanol accounted for approximately 30% of the converted C_3 units and butyrate for 34%. The main difference concerned acetate and 1,3-propanediol: in the reactor that was initially fed with glucose (a), acetate decreased drastically and 1,3-propanediol emerged. In the reactor fed with glycerol (b), acetate immediately increased after the switch and 1,3-propanediol decreased. As expected, the biomass yield decreased in reactor A and increased in reactor B, as a result of more or less energy associated products that started to form under the new condition.

Discussion

Glycerol fermentation at pH 8, comparison with glucose

Glucose fermentation by mixed culture provided a similar product spectrum as observed in previous experiments under the same condition (temperature, growth rate, substrate concentration), where the main products were as follows: formate, acetate, ethanol and butyrate (chapter II). Additionally, the product spectrum has been observed to be pH dependent in a mixed culture: at lower pH values the catabolic products are predominantly butyrate and acetate, while at higher pH values butyrate decreases and ethanol production increases. Whether butyrate production is inhibited or the ethanol production is enhanced as a consequence of the increase in the pH remains unclear (Chapter-II; Zoetemeyer et al. 1982c).

Glycerol fermentation resulted in the production of mainly formate and ethanol, and in minor amounts, 1,3-propanediol and some acetate. Most literature concerning anaerobic glycerol fermentation focuses on pure cultures, which has therefore become the starting point in understanding the diversity of metabolisms that were encountered in the system. *E. coli* and *K. pneumoniae* mainly ferment glucose into ethanol and acetate. When fermenting glycerol, these two strains mostly produce ethanol-formate, and some acetate and 1,3-propanediol in the latter case (Dharmadi et al. 2006; Zeng et al. 1993). The results obtained are comparable to those observed in the pure culture experiments. This product spectrum is also in agreement with the ATP-yield associated with the different pathways (see Table 4.3). Ethanol production, together with formate, is the redox neutral conversion that generates more ATP, which means a higher thermodynamic efficiency and therewith provides a competitive advantage of this product formation pathway.

Table 4.3- Free energy associated with the glycerol catabolism at standard conditions.

	Reaction	$\Delta G^{0,}$ (kJ/mol)	ATP
1	Glycerol → Ethanol + formate + H^+	-101.4	1
2	Glycerol → 0.3 Acetate + 0.3 formate + 0.6 H^+ + 0.6 1,3 Propanediol + 0.3 H_2O	-91.98	0.67
3	Glycerol → 0.25 Butyrate + 0.5 formate + 0.75 H^+ + 0.5 1,3 Propanediol + 0.5 H_2O	-104.0	0.75

The biomass growth on a certain substrate results from a series of reduction and oxidation reactions. The elemental composition of biomass has been established by Roels et al. (1983) ($CH_{1.8}O_{0.5}N_{0.2}$). This corresponds to a degree of reduction of 4.2 electrons per mole of carbon of biomass (Cmol-X) and can be compared to the electrons provided per Cmol of substrate, 4.7 per Cmol of glycerol. Zeng et al (1993) made an estimate of the minimal amount of 1,3-propanediol that must be produced in two extreme cases: first, when growth is optimized and ethanol is the only catabolic product; second, when growth is minimized and acetate is the only catabolic product. Additionally, it was assumed that only ethanol and acetate production provide ATP, and a value of 10.5 g of biomass is produced per mole of ATP generated. Such calculations were also done for the current study and the estimated values are given in Table 4.4. The results obtained indicate that the reactor operated close to limiting conditions, using the substrate efficiently (0.75 moles of ATP/glycerol). It should be noted that the difference in efficiency between producing only ethanol or ethanol plus some acetate is very small (0.80 and 0.75 ATP/glycerol converted), which means that ethanol varying from 0.58-0.68 and acetate from 0.03-0.10 provide the equivalent amount of ATP. Such a range of product yields was obtained during long term experiments.

Table 4.4- Comparison of the yields obtained in this study with a theoretical approach established by Zeng et al. 1993, considering catabolism and anabolism. The yields were calculated based on the ATP needed for growth and consequent NADH generation. The products that generate ATP are acetate or ethanol.

	Theoretical calculation		This study
	Maximal growth	Minimal growth	
Product	**Yields**		
Ethanol (mol/mol)	0.80	-	0.67 ±0.03
1,3-propanediol (mol/mol)	0.08	0.65	0.14 ±0.02
Acetate (mol/mol)	-	0.30	0.04 ±0.01
ATP (mol/mol) [a)]	0.80	0.59	0.75 ±0.04
Biomass yield (g/mol glycerol)[b)]	8.48	6.25	7.8 ±0.5
Biomass yield (g/mol ATP)	10.5	10.5	10 ±2

a) The yield of ATP was estimated based on the catabolic products (Table 4.1).
b) During this study the biomass composition was assumed as established by Roels *et al.* (1983).

During the conversion of glycerol into 1,3-propanediol, glycerol is normally first dehydrated and then reduced by 1,3-propanediol dehydrogenase (Gonzalez-Pajuelo et al. 2006). The possibility of generating such a product, more reduced than glycerol itself, allows the cell to produce more oxidized products (acetate, butyrate and biomass). Such a diversity in the metabolic pathways, other than solely the redox neutral ones, gives the cells flexibility to adjust to different conditions (Streekstra et al. 1987; Zeng et al. 1993). In fact, many bacterial species that are incapable of producing 1,3-propanediol have shown limited or non-existing anaerobic growth on glycerol, probably due to the lack of such a mechanism to dispose of the electrons generated during growth (Dharmadi et al. 2006; Sakai and Yagishita in Press).

Influence of the increase of substrate concentration

When the substrate concentration was increased, a considerable change in the product spectrum of glycerol fermentation was observed (figure 4.3). Furthermore, the biomass yield on the ATP generated decreased at elevated substrate concentrations (see figure 4.5). One possible cause is that product inhibition was occurring in the chemostat reactor. Product inhibition results in an increase of the energy spent for maintenance purposes and thereby decreases the biomass yield. To investigate if the ATP-based biomass yield was affected by the product concentration, batch experiments were performed in the absence and presence of the products, in higher amounts than present in the chemostat. The impact of these products on the substrate uptake rate, product yields, growth rates and productivities was undetectable (data not shown). Therefore, it was not possible to conclude which product and mechanism was specifically affecting the reactor performance and decreasing the yields.

As has been mentioned, glycerol and glucose are metabolized in a similar way, but the need to regenerate one more NADH has an effect on the catabolism. One of the consequences is the lower ATP generated per carbon during glycerol metabolism compared to glucose. Assuming that the maintenance requirements are independent of the substrate, the biomass yield is necessarily lower. However, if all the conditions are similar (including the substrate concentration in C_3 units), but the biomass yield is significantly lower, the substrate converted per unit of biomass is considerably higher in glycerol growing cells than in glucose growing cells. Figure 4.5 illustrates how the biomass yields on ATP and, consequently, the specific substrate uptake rate are affected by the increase of the substrate concentration, and how this is related to the shift in the catabolic products obtained. As can be seen in figure 4.5, for the same increase of substrate in the feeding medium, the increase of the biomass activity is more pronounced in the glycerol-fed reactor than in the reactor fed with glucose. This can be due to the lower biomass yield on glycerol, compared to glucose. Moreover, simultaneous to the increase of the substrate uptake rate, an increase of 1,3-propanediol yield and decrease of the ethanol yield is observed (see Figure 4.5). Such a shift in the product spectra intensifies the incapability of the biomass to balance the growth rate with the substrate uptake rate, given that the new product spectra is associated with even less ATP generation. Overflow metabolism has been described in the literature as a mechanism that occurs as a result of the uncoupling of catabolism from anabolism, when the growth rate cannot proceed as fast as

the substrate uptake rate (Streekstra et al. 1987). This product shift as a result of the increase of the substrate uptake rate has been reported in other studies (Ito et al. 2005; Reimann et al. 1996; Streekstra et al. 1987). Streekstra et al. (1987) observed a linear relation between the ratio of 1,3-propanediol-ethanol with increasing dilution rates, which also means higher substrate uptake rates.

Additionally, these observations are in agreement with other studies, which have reported that the glycolysis is controlled by the NADH/NAD ratio at the oxidation of glyceraldehyde-phosphate (GAP) (Girbal and Soucaille 1994). These authors have determined the impact of the NADH/NAD ratio on GAP dehydrogenase and on glycerol dehydrogenase activity. The activity of GAPDH considerably decreased at increasing ratios. Thus, a reaction that not only consumes NADH, but also relieves the whole metabolism (GAPDH activity), such as the formation of 1,3-propanediol, provides the microorganism with the necessary flexibility to adjust to different conditions imposed: increase the biomass specific substrate uptake rate even if growth remains slower.

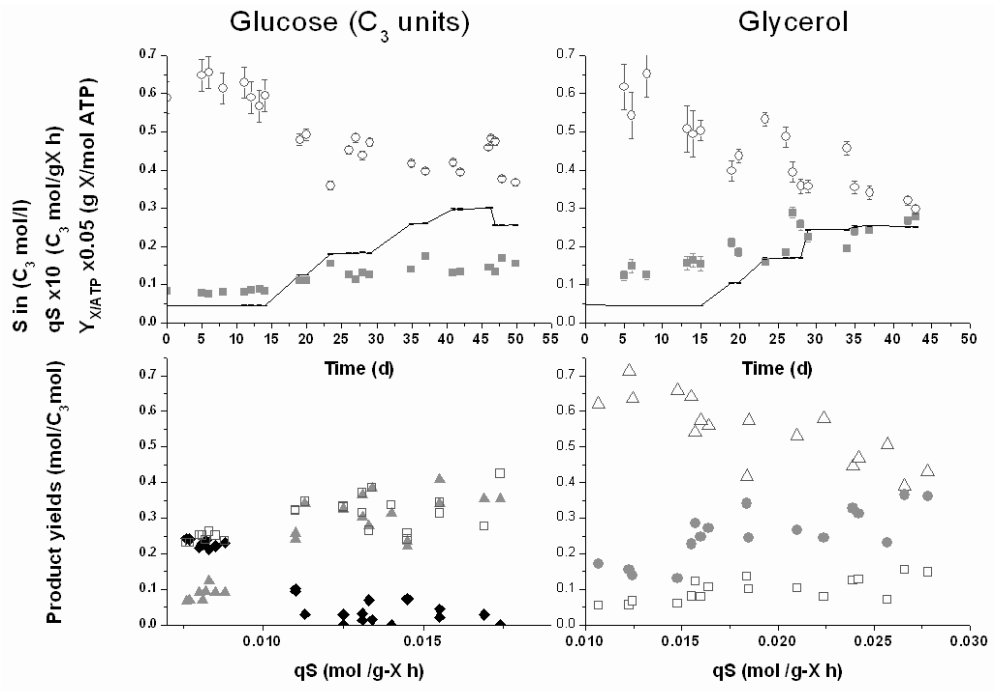


Figure 4.5- Impact of substrate concentration over time. Upper figures: substrate concentration (C_3moles), black line; ○ biomass yield per ATP-Y_{ATP} and ■ specific substrate uptake rate. Lower figures: product yields at different substrate uptake rates: □ acetate; ◆ butyrate; ▲ ethanol; △ ethanol; ● 1,3-propanediol.

The increase of glucose concentration in the reactor also caused a decrease in the biomass yield, and, simultaneously, a shift in the catabolic products. On the one hand, butyrate yield decreased from approximately 0.25 to less than 0.1 (mol/C_3mol). On the other hand, ethanol increased from 0.15 to 0.35 (on average). In addition, the acetate yield slightly increased too (from 0.25 to 0.35). As has already been mentioned, this shift does not involve a decrease of the total flow through glycolysis, and the ATP yield is the same for both conversions (3 ATP per butyrate and per acetate-ethanol). Thus, it remains unclear whether a slight increase in the maintenance requirements/substrate uptake rate, which does not disturb the overall flow through the glycolysis, is enough to cause such a shift in the catabolic products, unless it has an inhibitory effect on one of the enzymes in the butyrate pathway.

Glycerol and glucose co-fermentation, at pH 8

The co-fermentation of glucose and glycerol produced ethanol and butyrate as the major products, with 38 and 34% of the converted COD, respectively.

In both cases, the biomass yield, ATP and specific uptake rate were in the same range. The only difference concerns the amounts of acetate and 1,3-propanediol that were produced (Table 4.5). This difference appears to be related to the fact that one reactor was cultivated on glucose and the other on glycerol, and this condition should be reflected in the NADH flux inside the cell before the transition. The *NADH flux* was estimated on the basic assumption that each C_3 unit of substrate is converted into pyruvate and that this is associated with a certain amount of generated NADH, 1 or 2 for glucose and glycerol, respectively. This amount needs to be oxidized in a certain time period by the biomass present in the reactor. Before the transition, the cells are acclimatised to a different substrate, glucose or glycerol, which results in NADH fluxes of 0.009 and 0.032 mol-NADH/Cmol-X.h, respectively. Table 4.5 presents the main product yields and the estimated values for the ATP yield and for the NADH flux. In the reactor initially fed with glucose, the introduction of the two substrates caused an immediate increase of the NADH inside the cell that needs to be reoxidized, which has been reported to induce 1,3-propanediol production and decrease glycolysis flux (Girbal and Soucaille 1994; Malaoui and Marczak 2001). This explanation is compatible with the sudden decrease in the acetate, the only product that does not involve any NADH oxidation and, accordingly, an increase of 1,3-propanediol. In the reactor initially fed with glycerol, the opposite situation occurred: a decrease in

the total amount of NADH that had to be regenerated per unit of time, which resulted in a decline of the 1,3-propanediol and increase in the glycolysis flux.

Table 4.5- Average of the product yields obtained in Reactors A and B. In the first two columns (A and B), the average was calculated for the three days before being submitted to a change in the substrate solution composition (from single substrate to a mixture of the two substrates in equal C_3 amounts). In the last two columns (D and E), the average was calculated after four days of being changed (>10 volume changes), for the duration of three days. In the column in the middle (C), an average of A and B was calculated on the basis of what would be expected if both substrates would be equally consumed.

	A		B		C	D		E	
	Glucose		**Glycerol**		**Expected**	**Glucose-glycerol**		**Glycerol-glucose**	
Substrate fed (C_3-mmol/L)	45+0		0+49		22+22	22+23		24+24	
	Product yield (mol/C_3-mol)								
Acetate	0.27	±0.02	0.04	±0.01	0.15	0.05	±0.01	0.13	±0.01
Butyrate	0.21	±0.01	0.01	±0.01	0.11	0.17	±0.01	0.17	±0.02
Ethanol	0.14	±0.01	0.65	±0.03	0.39	0.33	±0.01	0.30	±0.03
1,3-propanediol	0	-	0.16	±0.02	0. 08	0.16	±0.01	0.08	±0.01
Biomass (Cmol/C_3-mol)	0.63	±0.02	0.39	±0.04	0.51	0.49	±0.02	0.54	±0.02
ATP (mol/-C_3 mol)	1.31	±0.02	0.78	±0.06	1.05	0.95	±0.01	1.07	±0.07
NADH flux (mol-NADH/Cmol-X.h)	0.009	±0.001	0.032	±0.001	0.020	0.016	±0.001	0.017	±0.002

It has been shown that the NADH flux is an important parameter, but the history of the culture also plays a role in the product spectrum during the transition phase. Furthermore, it may be concluded that higher NADH levels do enhance ethanol production and do not specifically inhibit butyrate or acetate production, as long as the NADH is oxidized at the necessary rate.

Perspectives of glycerol fermentation by mixed culture

In this study, glycerol fermentation by mixed culture at alkaline pH was investigated. The dominant catabolic product formation pathways identified were ethanol-formate and 1,3-propanediol-acetate. Which route is the dominant one depends on the conditions imposed. Both ethanol and 1,3-propanediol are interesting products that can be used as biofuels or building blocks in other chemical processes. Ethanol was mainly associated with substrate limiting conditions, whereas 1,3-propanediol production dominated when the substrate was in excess. In order to obtain one of the products in high concentrations as required for efficient down-stream processing, high substrate concentrations have to be converted. Processes should be designed in such a way that, depending on the desired product, the restrictions mentioned are imposed. Furthermore, 1,3-propanediol is only associated with acetate or butyrate production and growth, not with ethanol. Therefore, if 1,3-propanediol is the required product, ethanol should be avoided. Lowering the pH in glucose fermenting cultures was found to result in a decrease in ethanol production (Chapter-II). Whether the same holds for glycerol fermentation remains to be elucidated. Future work will be aimed at maximization of the individual product formation routes by varying the feeding regime of the bioreactor.

Conclusions

In line with the hypotheses in the introduction, the results of this study show that glycerol can indeed be converted by mixed microbial culture into a stable product spectrum with no requirement of sterile conditions. At alkaline pH and when operated under substrate limiting conditions the ethanol-formate yields are up to 60% of the carbon converted. However, this product spectrum proved to be sensitive to the substrate concentration, presumably as a consequence of the increase of the maintenance requirements that caused a decrease in the biomass yield, and an eventual increase of the intracellular NADH turnover. When the glycolysis derivatives cannot respond to the cell

demand of NADH oxidation, the production of 1,3-propanediol can allow this requirement, even though it is associated with a lower energy generation.

Glucose was mainly converted into acetate, ethanol and butyrate. When the substrate concentration was increased, the flow through the glycolysis was not affected, despite the shift in productivity to only acetate-ethanol.

Co-fermentations of a glucose-glycerol mixture were independently performed with mixed culture cultivated on glucose and on glycerol. The main difference observed was a higher yield of 1,3-propanediol in the glucose grown culture. This was likely a result of the sudden increase of the biomass specific NADH production rate upon glycerol addition, which triggered 1,3-propanediol production from glycerol.

Chapter 5

Diversity of microbial communities in open mixed culture fermentations, Impact of the pH and Carbon source

(This chapter was submitted for publication as: Margarida F Temudo, Gerard Muyzer, Robbert Kleerebezem, Mark C.M. van Loosdrecht. (2008). Diversity of microbial communities in open mixed culture fermentations. Impact of the pH and carbon source).

Abstract

Based on ecological selection principles, the operational conditions determine the product spectrum of mixed culture fermentation processes (MCF). The MCF process was investigated at different environmental pH values (4-8.5) and with three carbon sources (glucose, glycerol and xylose). Depending on the operational variations imposed, different fermentation products were formed. To evaluate whether the different operational results were related to changes in the microbial community, the population composition from each steady state was assessed by denaturating gradient gel electrophoresis (DGGE) analysis of the 16S rRNA gene fragments.

The environmental pH had a determinant effect on the microbial population at extreme values; whereas at intermediate-pH values the product spectrum seemed to be sensitive to variations in the microbial population. The fermentation of different substrates enabled different populations to establish, and in the simultaneous presence of two substrates mixotrophic microorganisms (capable of degrading both substrates) were found to overgrow the originally dominant specialists. The competitive advantage of mixotrophs is suggested to be due to the fact that growing on one substrate allowed a better conversion of the second substrate and vice-versa in the chemostat set-up of these experiments. Overall, the analyses performed do not provide a direct relationship between the process characteristics (associated to a metabolic response) and the microbial population present.

Introduction

Mixed culture biotechnology may become an attractive addition to traditional pure culture based biotechnology for the production of bulk chemicals from waste streams (Barbirato et al. 1997; Kleerebezem and van Loosdrecht 2007; Reis et al. 2003; Rodriguez et al. 2006). Open mixed cultures, based on natural inocula with a high microbial diversity, allow continuous operation of bioprocesses under non-sterile conditions with no risk of strain degeneration. The conditions in the process are chosen such that those bacteria that get an ecological advantage are enriched. It has been proposed that in mixed culture fermentation (MCF) systems the operational conditions will determine which catabolic product allows the more efficient growth, and will therefore dominate (Claassen et al. 1999; Hawkes et al. 2007; Rodriguez et al. 2006).

Ecosystem functioning often depends on the microbial composition. Recent studies on bacterial communities showed that changes in ecosystem functioning are associated with changes in the genetic structure of bacterial communities, suggesting that the magnitude and efficiency of ecosystem based processes depends on bacterial community composition (Dejonghe et al. 2001; Wittebolle et al. 2005). However, it has also been shown that bacterial communities are functionally redundant, i.e., communities with different compositions can perform similar functions such as catalysing metabolic reactions and primary production (Fernandez et al. 2000; Findlay and Sinsabaugh 2003; Langenheder et al. 2005).

The pH is a powerful parameter that can be used to control both the metabolism and to select the microorganisms that are best able to survive. The pH affects several microbial parameters such as the growth rate, the utilization of the carbon source, efficiency of the substrate conversion, etc (Russell 1992). Furthermore, the pH determines the fraction of undissociated acids in the broth, which are known to be able to permeate cell membranes. Indeed, it has been shown that the environmental pH has a strong impact on the product spectrum of mixed culture fermentation (Chapter II; Zoetemeyer et al. 1982c). Another important factor that shapes the community structure and product formation is the carbon source. The degree of reduction of the substrate and metabolic pathway used for degradation determine the product spectrum (chapter III and chapter IV). The most abundant sugar in nature, glucose, is always accompanied by other compounds e.g. xylose. The growth on such mixtures may not be controlled by only a single nutrient, and the kinetic properties of a cell may change due to adaptation (Kovarova-Kovar and Egli 1998). To which extent these factors have an impact on the microbial

community composition or on its functioning, is an important question in microbial ecology. Insight in these interactions between the environment and the microbial population may allow the prediction of the product spectrum formed in MCF systems. This is a prerequisite for successful implementation of mixed culture biotechnology based processes. The fundamental question to be answered is whether the process conditions are determining the product spectrum or is the product spectrum resulting of the established microbial community.

The aim of this research is to study the functional performance depending on the operational conditions (medium and pH) in several continuous culture fermentation systems, and to what extent this is related to the microbial community composition. The impacts of different substrates (glucose, glycerol and xylose) that involve different metabolic pathways or provide different amounts of energy per carbon fermented were investigated in this study.

Materials and Methods

Reactor operation and inoculum source

For the study of the impact of the pH on glucose fermentation by mixed culture, a continuously stirred tank reactor (CSTR) was operated at 30 ^{o}C at an influent glucose concentration of 4 g/l as described previously (see chapter II). The bioreactor had a working volume of 2 L and was sparged with nitrogen gas to maintain anaerobic conditions. The reactor was freshly inoculated for each pH-value studied. The pH values were investigated in the following order: 5.0, 4.0, 5.5, 8.5, 7.75, 6.25, 4.75, 7.0 and 8.0. The dilution rate was 0.05 h^{-1} for pH values equal or lower than 5.5 and 0.12 h^{-1} for pH values equal or higher than 5.5.

To study the influence of different substrates (glucose, glycerol and xylose) on the product spectrum and the composition of the microbial community, the CSTR was operated at pH 8.0, 30 ^{o}C, and D 0.12 h^{-1}. The influent substrate concentration initially was 4 g/l, and later increased to 10 g/l and to 25 g/l (chapter III and IV).

Fermentation of mixtures of two substrates was also tested with the biomass cultivated on a single substrate. A mixture of glucose and glycerol was added to the glucose and glycerol grown cultures, and a mixture of glucose and xylose was supplied to the reactor fed with xylose. In these experiments the total

amount of carbon supplied was kept constant and the two substrates were fed in equal amount of carbon moles. The mineral medium composition was described previously (chapter II) and, when needed, was adjusted to keep the C:N:P ratio constant.

The anaerobic mixed culture used as inoculum consisted of a mixture of two sludges, obtained from two different sources. The first inoculum came from a distillery wastewater treatment plant, Biothane in Delft, The Netherlands. This plant consists of an UASB operated at 30°C, pH 6.8-7.2, COD=2500 mg/L. The second inoculum was a sludge solution from a potato starch processing acidification tank in Broek op Langendijk, Smiths Food, The Netherlands. Approximately 20 ml of each inoculum was added to the reactor at the beginning of the experiment. The reactors were operated in batch conditions until biomass growth was observed, after which continuous operation was started.

Analytical techniques

Reactor broth samples were immediately filtered (Millipore membrane of 0.45 μm). The substrate and end products were determined and quantified. Xylose, glucose, glycerol, volatile fatty acids (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate and caproate), lactate, succinic acid, formic acid and 1,3-propanediol were determined. Measurements of H_2 and CO_2 in the off gas were performed on-line and the base added to maintain the pH constant was monitored. A detailed description of the analytical methods used can be found elsewhere (chapter II). The biomass dry weight was determined after filtration, according to standard methods (Greenberg et al. 1992).

Microbial diversity analysis

Nucleic acid extraction. Bioreactor community samples were concentrated by centrifugation. Genomic DNA was extracted directly from the concentrated biomass using the Ultra Clean Soil DNA extraction kit (MOBIO Laboratories, Inc. California, USA) according to the manufacturer's protocol. Extracted DNA was stored at -20°C, until further use.

PCR amplification. Amplification of 16S rRNA gene fragments was performed using the primer pairs, 341F-GC (5' CCT ACG GGA GGC AGC AG 3') and 907R(5' CCG TCA ATT CMT TTG AGT TT 3') (Muyzer et al. 1995). For the amplification reactions 1 μl of genomic DNA was used. The protocol used for the amplification of 16S rRNA gene fragments was as described previously (Muyzer et al. 1995). PCR amplification was performed in an automated thermal cycler. Initial denaturation was at 94 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C 1 min and

extension at 72 °C 20s, except for the last cycle, which was 15 min. A touchdown protocol was used to increase specificity of the amplification reaction. This consisted of a decrease of 0.5 °C in each cycle of the annealing temperature, 65 °C to 55 °C in 20 cycles. The quality of the PCR products was examined on 1% (wt./vol.) agarose gel and the yield was quantified by absorption spectrophotometry using the Nanodrop ND-1000 TM (NanoDrop Technologies, Delaware, USA).

DGGE of 16S rRNA gene fragments. DGGE was performed as described by Schäfer and Muyzer (Schafer and Muyzer 2001) using the D-Code system (Bio-Rad Laboratories, California, USA). Electrophoresis was performed with 1 mm-thick 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 40:1) submerged in 1X TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.5) at constant temperature of 60ºC. PCR product in an amount ranging from 300 to 500 ng was applied to the individual lanes on the gel. The electrophoresis conditions for 16S rDNA fragment were the same as described previously (Schafer and Muyzer 2001): 16 hr at 100 V in a linear 30 to 60% denaturant gradient (100% denaturants is a mixture of 7 M urea and 40% [vol./vol.] formamide). After electrophoresis, the gels were incubated for 30 min in Milli-Q water containing ethidium bromide (0.5 µg/ml), rinsed for 20 min in Milli-Q water, and photographed using a Bio-Rad GelDoc station (Bio-Rad, California, USA). Individual bands were excised, resuspended in 15 µl of Milli-Q water, and stored overnight at 4ºC. A volume of 3 to 5 µl of the supernatant was used for reamplification with the original primer sets. The reamplified PCR products were run again on a denaturing gradient gel to check their purity. Prior to sequencing, the PCR products were purified using the Qiaquick PCR purification kit (QIAGEN GmbH, Hilden, Germany).

Phylogenetic analysis. The obtained 16S rRNA gene sequences were first compared to sequences stored in GenBank using the BLAST algorithm. Subsequently, the sequences were imported into the ARB software program (Ludwig et al. 2004; Schafer and Muyzer 2001) and aligned using the automatic aligner function. The alignment was further corrected manually, and an optimized tree was calculated using the neighbour-joining algorithm with Felsenstein correction.

Results

Before each experiment, the bioreactor was freshly inoculated with 40ml of inoculum, and was left in batch mode until the substrate was depleted. The inoculum used was obtained from two different waste treatment plants and the microbial diversity present in each inoculum is shown in Figure 5.1. The two inocula seem to have a different and high microbial diversity as can be seen by the high number of bands spread over a wide range in the gel.

Figure 5.1- DGGE pattern of the 16S rRNA gene fragments of the DNA extracted from the two different inocula used in this study. I: inoculum from a distillery anaerobic wastewater treatment plant, the Netherlands; II: inoculum from a potato starch processing acidification tank in Broek op Langendijk, The Netherlands.

It took 3-5 weeks to establish stable operation. A stable operation was assumed when the measured product and biomass concentrations varied less than 20% within a time frame of at least a week. This corresponded to approximately 30 volume changes at a dilution rate of 0.05 h^{-1} ($pH \leq 5.5$), and about 70 volume changes when the dilution rate was 0.12 h^{-1} ($pH \geq 5.5$). The product spectrum of the system was characterized at each condition studied.

Impact of the pH on glucose fermentation

The pH not only had a significant effect on the product spectrum, but also on the most dominant microorganism as can be seen in Figure 5.2 (a and b). At low pH-values the product spectrum mainly consisted of butyrate and acetate, and at high pH it shifted to acetate and ethanol.

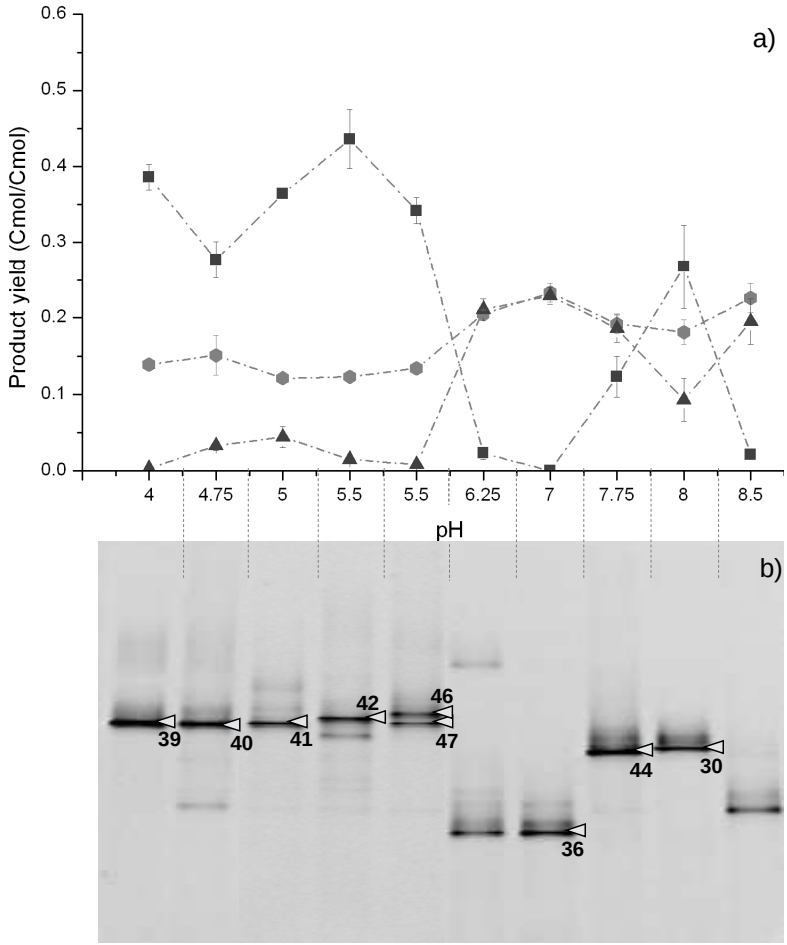


Figure 5.2- Impact of the operational pH on glucose fermentation: a) the product spectrum and b) DGGE analysis of the 16S rRNA gene fragment of DNA samples from the biomass present in the reactor at each condition. The dilution rate was 0.05 h^{-1} for the first pH values (4-5.5) and 0.12 h^{-1} for the last operational pH (5.5-8.5). Symbols correspond to the yields of the main products (Cmol/Cmol): ■ butyrate, ○ acetate and ▲ ethanol. Apart from biomass, the main other products were CO_2 and H_2 at low pH values and formate at high pH values.

This product shift could not be explained using bioenergetic considerations at different pH values, because both conversions are equally favourable in terms of Gibbs energy change and the estimated ATP yield on substrate (Y_{ATPS}) is comparable (Table 5.1). The measured biomass yield was lower at low pH values, which suggests an approximately 20% lower ATP based yield (Y_{XATP}). This observation can probably be attributed to higher biomass maintenance requirements at lower pH-values due to:

- the increasing fraction of non-dissociated acids in solution
- the lower dilution rate at lower pH-values (pH ≤ 5.5), and
- the higher toxicity of butyrate compared to acetate (Herrero et al. 1985).

Table 5.1- Free energy of the main reactions observed at different pH values. Calculations were made based on the standard free energy of formation and corrected to pH 7 (Hanselmann 1991). The yield of ATP was estimated based on the catabolic products, assuming glycolysis occurs through the Embden Meyerhof Parnas Pathway (EMP) and substrate level phosphorylation (SLP) involved in each product formation pathway.

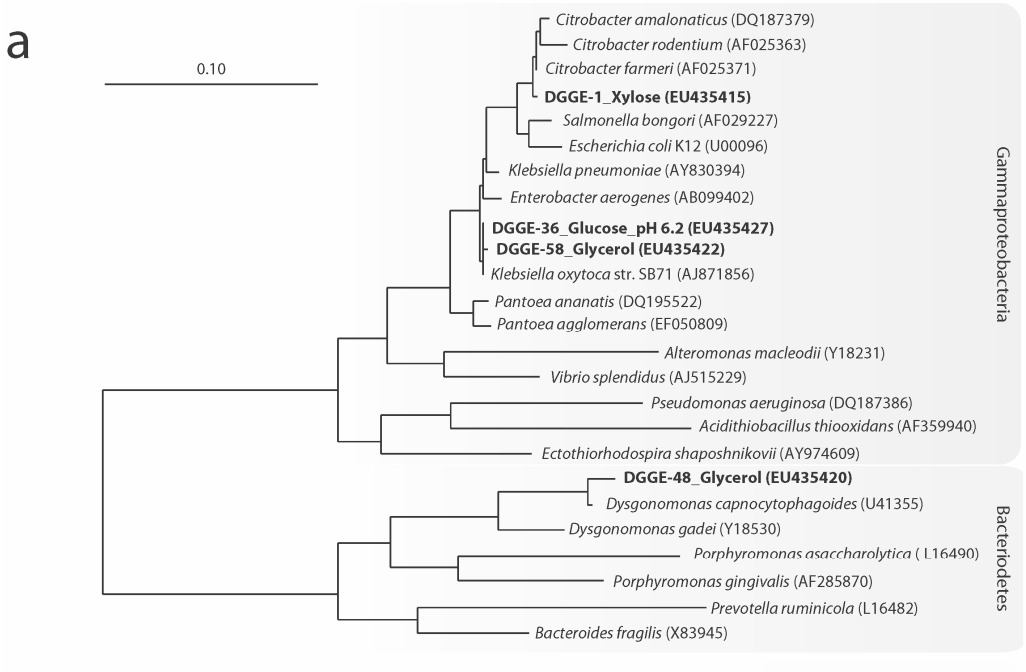
Reaction	**$\Delta G^{0\prime}$**	**Y_{ATPS}**	**Y_{XS}**	**Y_{XATP}**
Glucose + $0.53H_2O$ → 0.6 Butyrate + 0.53 Acetate + 0.27glycerol + $0.27H_2$ + 1.73formate + $2.87H^+$	-259.3	2.4	0.65	6.7
Glucose + H_2O → Ethanol + Acetate + 2formate + $3\ H^+$	-258.3	2.0	0.70	8.6

The operational pH also had an impact on the microbial population that was established at each condition as can be seen from the DGGE gel in figure 5.2. From the gel, the most dominant bands were excised and reamplified. The successfully sequenced DNA fragments are numbered in figure 5.2 and the respective phylogenetic affiliation is illustrated in the phylogenetic evolutionary tree (Figure 5.3).

We could distinguish three microbial communities at low (4-5.5), middle (6-7) and high (7.5-8) pH values. The dominant microorganisms at each of these conditions were quite related and different from the other conditions. The most dominant microorganisms at low and at high pH were located at two different subclusters of cluster I of the genus clostridium. The presence of clostridiaceae has been widely reported in similar operational conditions: fermentation of carbohydrates, mesophylic temperatures, anaerobic conditions,

dilution rate between 0.04-0.2 h^{-1}, and pH from 4-8 (Fang et al. 2002; Iyer et al. 2004; Morales et al. 2006; Ren et al. 2007; Ueno et al. 2001). The cluster I of the genus clostridium is composed of strictly anaerobic gram positive microorganisms, not able of sulfate reduction, and capable of endospore formation (Collins et al. 1994). Little is known about the difference within this cluster in terms of acid tolerance. The microorganisms found at low pH were most closely related to *C. acidisoli* and *C. pasteuranium*, that can grow at pH 3.6-7.0 (Wiegel et al. 2006). Most clostridia cannot grow at pH values lower than 6.2 or need to change their metabolism by producing solvents instead of fatty acids (Svensson 1992; Wiegel et al. 2006). Here, however, growth occurred with formation of acids at low pH.

At middle pH values, the principal microorganism belonged to genus Klebsiella; facultative anaerobic bacteria that are not known to be capable of butyrate production from glucose. This explains why at pH 6.25 and 7.0 the butyrate yield was lower (Figure 5.2).



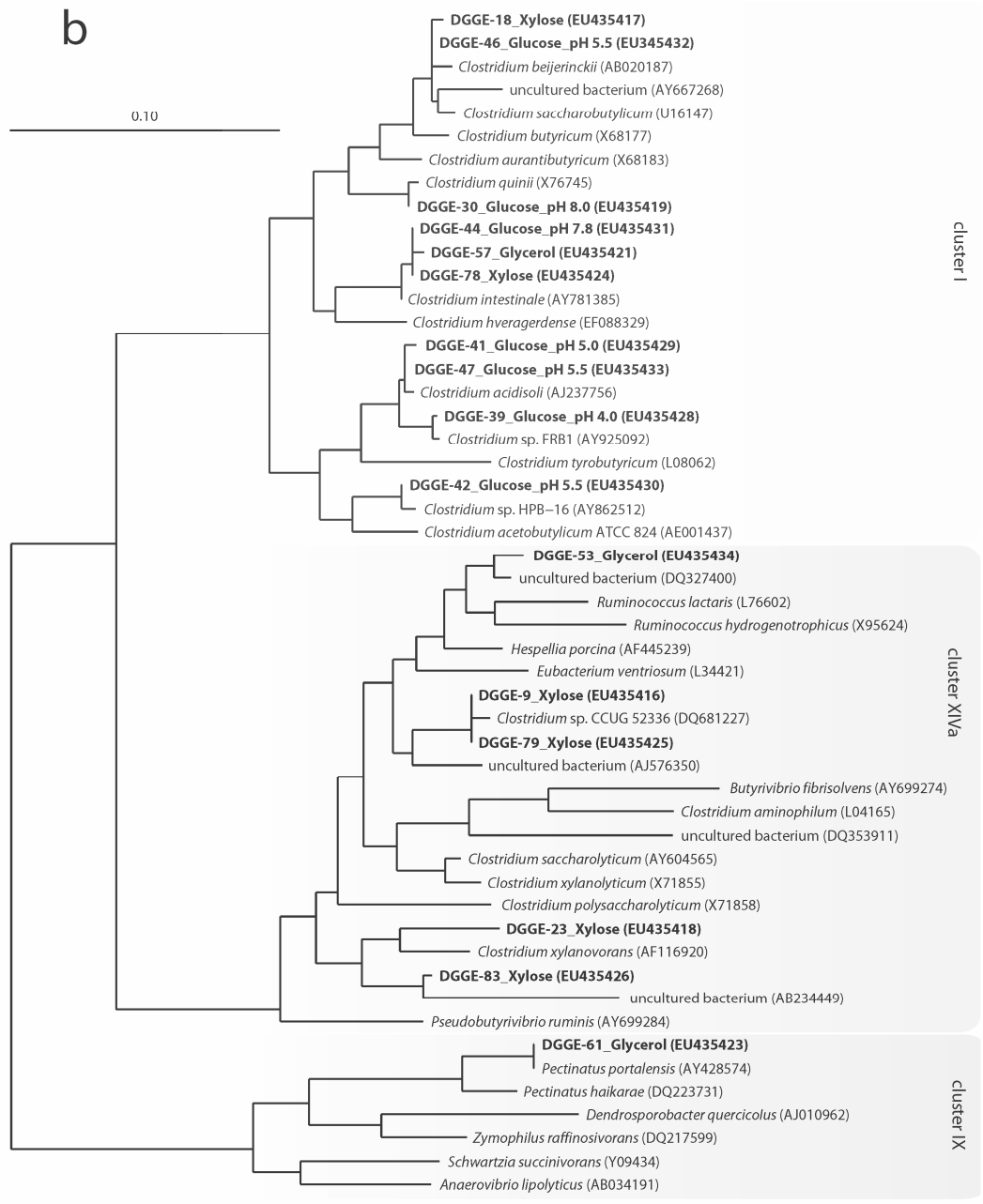


Figure 5.3- Phylogenetic tree based on 16S rRNA gene sequences obtained from the DGGE bands. The sequences were mainly affiliated to three classes: a) gammaproteobacteria and bacteroides and b) clostridia.

Impact of the carbon source

To study and compare the effect of different substrates (glucose, xylose and glycerol), a CSTR was operated at pH 8, dilution rate 0.12 h^{-1} and substrate concentration 4 g/l (0.14-0.15 Cmol/l). The system was characterized after 80 volume changes in the case of glucose and glycerol and after 240 volume changes for xylose. The main catabolic products of glucose and xylose fermentation were acetate, butyrate and ethanol (Figure 5.4). Glycerol has a higher degree of reduction compared to glucose and xylose and therefore a higher fraction is converted to more reduced compounds e.g. ethanol and 1,3-propanediol. In a chemostat at low substrate concentrations, the main product was ethanol, and minor amounts of 1,3-propanediol and acetate were produced as well (Figure 5.4).

The microbial populations that established on the different substrates were also different (Figure 5.5). The glycerol and xylose cultures were more diverse. The glycerol community was dominated by an enterobacteria closely related to *Klebsiella oxytoca* (band 58); a facultative microorganism, which was reported to be able to ferment glycerol (Homann et al. 1990). Other microorganisms in the glycerol grown culture were related to *Pectinatus frisingensis* (band 61), to *C. intestinale*, clostridium cluster I (band 57), and sequence 53 was related to uncultured bacteria of the cluster XIVa of the clostridia family. Species of the genus *Pectinatus* have been reported in spoiled beer and are ethanol tolerant up to 4.8%. These species belong to the Clostridium group, cluster IX, strictly anaerobic (Haikara and Helander 2006). In the bioreactor fed with xylose there were three dominant bands present (9, 18 and 23). The two bands indicated with the number 18 seem to be an artefact of this DNA sequence, several attempts were made to purify, but the result was always unaffected and the purified DNA could be successfully sequenced. The sequence 18 was also affiliated to cluster I of the clostridium group. The sequences 9 and 23 were affiliated to cluster XIVa of the clostridium group. Many of the described species of this cluster live in ruminant environments, which may explain a higher affinity for xylose.

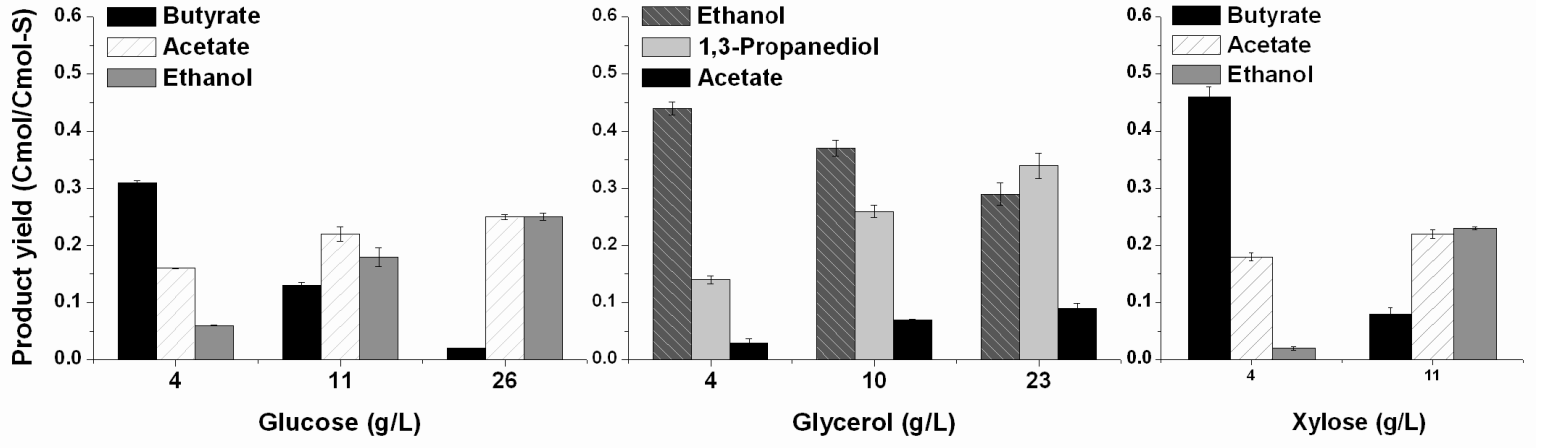


Figure 5.4- Main catabolic products of glucose, glycerol and xylose fermentation by open mixed culture at increasing substrate concentration in a chemostat at pH 8, dilution rate 0.12 h-1 and 30 ^{0}C. The main other products were CO_2, H_2 and formate.

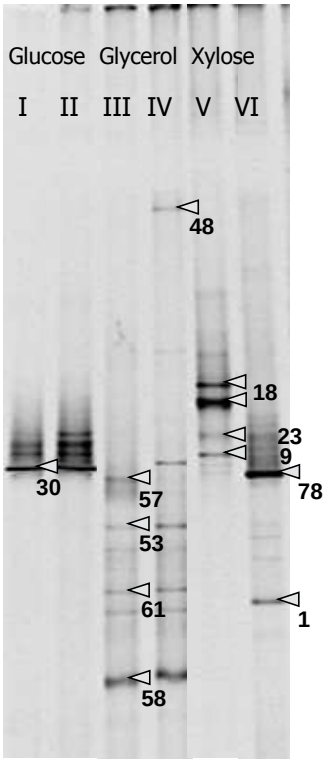


Figure 5.5- DGGE (30-60%) pattern of the 16S rRNA gene fragments of the DNA extracted from the bioreactors cultivated on different substrates: glucose, glycerol and xylose. Impact of the substrate concentration on the microbial community: I-glucose 4g/l, II-glucose 26 g/l, III- glycerol 4 g/l, IV-glycerol 23 g/l, V-xylose 4 g/l and VI-xylose 11 g/l.

Impact of the increase of the substrate concentration

After characterizing the system at low substrate concentrations in the influent, the substrate concentration of the feeding solution was increased. The system was characterized after reaching stable operation as obtained after approximately 40 volume changes. The main product yields are shown in Fig. 5.4. The substrate concentration inside the reactor was below the detection limit. However, under the new conditions, higher substrate conversion rate and higher products concentration, the product spectrum shifted in the three reactors. In the reactor fed with glucose and xylose, butyrate decreased to very low values and ethanol and acetate became the major products (1:1). In the glycerol fed reactor, the ethanol yield decreased, while 1,3-propanediol and acetate yields increased (Fig. 5.4).

Figure 5.5 shows the DGGE pattern of the microbial community at elevated substrate concentrations. Even though a similar shift in the catabolic

product spectrum was observed in the glucose and xylose fed reactors, it only resulted in a clear change in the microbial population in the xylose fed reactor. In the glucose system the main microorganisms remained. The population grown on the lower concentration xylose was probably sensitive to the elevated product concentrations and washed out from the system. In the chemostat fed with glycerol, only small changes in the microbial community occurred, band 57 disappeared and 48 emerged.

Mixed Substrates fermentation

The cultures cultivated on different substrates (glucose, glycerol and xylose) were submitted to a mixture of two substrates (glucose and glycerol for the first two; glucose and xylose for the xylose fed reactor). After one week, the product spectrum was very similar to the average of the product spectra observed when the two substrates were fed in separate (Figure 5.6), see chapter III and IV. Immediately after switching from a single substrate to a mixture of substrates the populations present in the reactor were capable of degrading the substrate added.

In figure 5.7, the DGGE pattern of the microbial communities at different stages is shown. Samples were taken immediately before feeding with a mixture of substrates (I), one day after (II) and one week after (III). The glucose cultivated culture could immediately convert glycerol and the composition of the microbial population seemed not to be affected. In the glycerol fed reactor, where a more diverse population was present, there was a microorganism (band 57, Figure 5.7) that after only three volume changes took advantage of the presence of glucose besides glycerol. After one week, the bands 53, 61 and 58 almost disappeared, and another population emerged represented by the three bands that are positioned above band 57. These three bands seem to be an artefact of band 57, since only one could be successfully purified and gave the same sequence. In the bioreactor initially fed with xylose, an analogous effect was observed to the reactor fed with glycerol. One week after additional glucose was supplied, bands 9 and 23 remained in the system, band 18 disappeared and band 83 emerged.

These results suggest that specialized microorganisms as represented by band 18, 53, 58 and 61 lost some advantage by the presence of a second substrate. Whereas, flexible microorganisms as 9, 23, 45 and 57, could adapt and use another substrate. Bands that emerged after the addition of glucose seem not to be very efficient on the initial substrate, since they disappear when glucose addition is stopped.

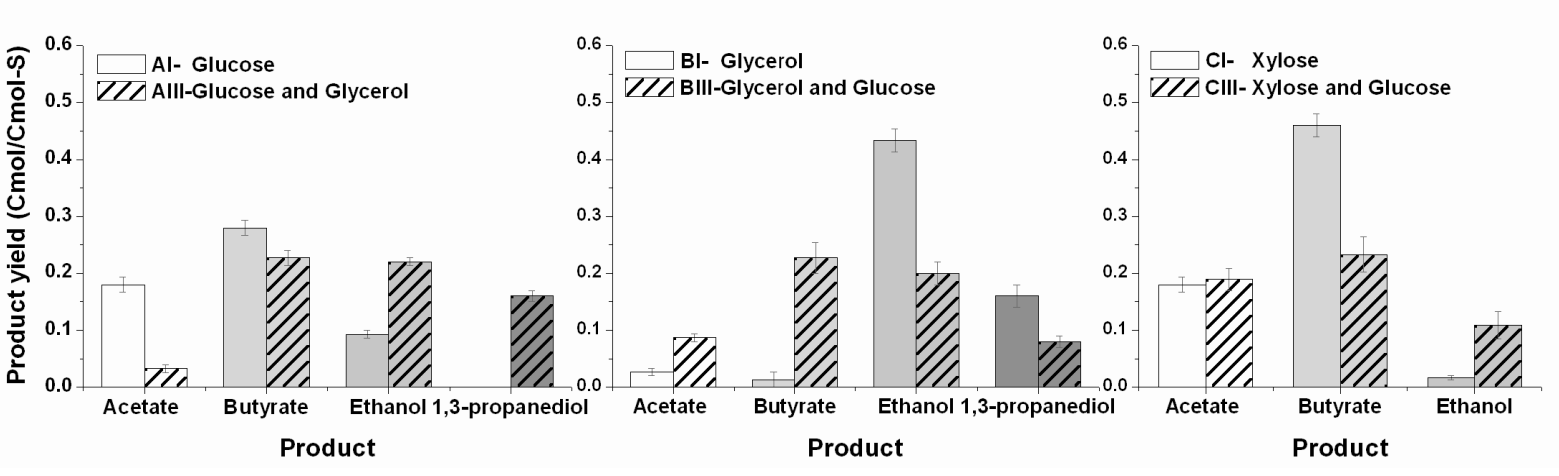


Figure 5.6- Main catabolic products on one (I) and two substrates (III) in a chemostat at pH 8, dilution rate 0.12 h-1 and 30 ^{0}C. A-Culture cultivated on glucose before (I) and one week after (III) being fed with a mixture of glucose and glycerol ; B- culture cultivated on glycerol, before and one week after being submitted to a mixture of glucose and glycerol; C-culture cultivated on xylose before and being submitted to a mixture of glucose and xylose for one week.

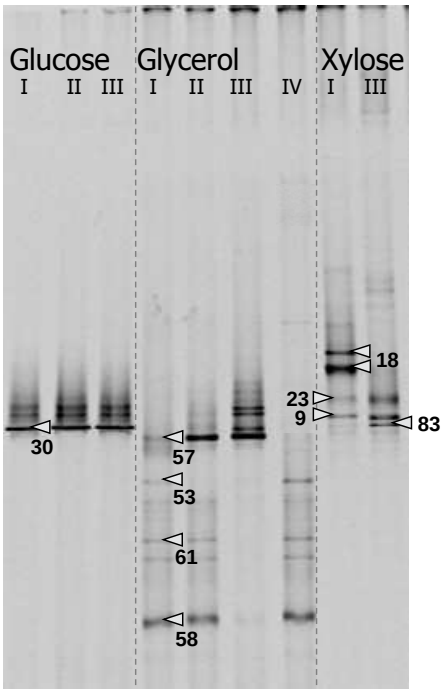


Figure 5.7- DGGE (30-60%) pattern of the 16S rRNA gene fragments of the DNA extracted from the bioreactors cultivated on different substrates before and after being submitted to a mixtures of two substrates: glucose, glycerol and xylose. Samples taken one day before (I), one day after changing the feeding solution (3 volume changes,II), one week after (20 volume changes, III) and two weeks after being changed back to the initial conditions (IV).

Discussion

During this study a mixture of two inocula was used. Both are characterized by a high microbial diversity as can be seen not only by the DGGE pattern of the DNA extracted directly from the inoculum (figure 5.1), but also by the different populations that established at the conditions studied and are indicated in the phylogenetic tree (Figure 5.3). Upon feeding with a single substrate the number of dominant bands found in the systems decreased dramatically: between one and three when glucose was the substrate, and less than six for glycerol and xylose. This means that at each operated condition, the main metabolic functions are catalyzed by a limited number of species. Such a result was expected based on the constant operational conditions for a long period of time (at least 30 volume changes). The chemostat-type reactor is furthermore characterized by the absence of dynamics, gradients, not allowing niche differentiation and establishment of a mixed culture with a high metabolic diversity.

Several theories have been formulated to elucidate the relationship between species diversity and ecosystem functioning (Lawton and Brown 1994; Verstraete et al. 2007). By cultivation in a chemostat selection occurs for those microorganisms that show a higher specific substrate affinity (μmax/Ks). On the other hand, there is significant redundancy in microbial species functions. Fermentation of carbohydrates is a widespread function over many different genera, and as long as all the functional groups are represented, the functioning of an ecosystem does not depend on species diversity (Lawton and Brown 1994). This explains the finding of similar, but different, microorganisms at comparable operational conditions such as a low or high pH values. Nevertheless, when the conditions were changed as by the increase of influent substrate concentration or by the introduction of a different substrate, other bands emerging could be seen, suggesting that other microorganisms were still present in low numbers and took over the main metabolic processes in the bioreactor.

It is noteworthy that the operation time of the xylose grown culture was at least three times longer than of the glucose and glycerol cultures. Nonetheless, the number of dominant microorganisms is higher in the xylose cultivated population. The low number of dominant microorganisms found in the glucose grown culture is in agreement with the theory of microbial selection in continuous culture (Harder et al. 1977), which suggests that only one culture with the highest specific substrate affinity (μ/Ks) will remain in the system. However, coexistence of more than one microorganism is chemostat has also been reported (Boenigk et al. 1993; Harder et al. 1977; Powell 1958; Taylor and Williams 1975; Yoon et al. 1977). In these studies, more than one nutrient limitation or different environmental factors are suggested to exert the control on the different microbial growth. In this way, microorganisms with different kinetic parameters may have comparable apparent parameters in specific conditions and coexist in the process.

Coupling between functioning and community composition

The pH determines the fraction of undissociated weak organic acids in the fermentation broth that are known to be able to permeate cell membranes. The fraction of undissociated acids becomes critical at pH values lower than 6. Inside the cell, the acid dissociates releasing a proton thereby acidifying the cytoplasm. For cellular homeostasis the intracellular pH must be maintained at a near neutral value, and consequently the protons generated have to be extruded. This process requires energy (ATP). The microorganisms that will do this process more efficiently or have highly impermeable cell membrane will have a competitive advantage over the others.

So, at least two metabolic properties can be distinguished in respect to acid toxicity tolerance, at low and high pH values (outside the range of pH values 6-7), but three different communities were detected. One seemingly associated to a higher acid tolerance; another found at mid pH that does not produce butyrate at all and a different group that had an optimum pH at higher values and produced little butyrate. Such an impact of the environmental pH on the process that results in a shift in product spectrum has been reported (Fang and Liu 2002; Horiuchi et al. 2002; Zoetemeyer et al. 1982c). Product spectra similar to those obtained in this study at low and high pH have been found in other studies as well (Zoetemeyer et al. 1982c). At middle pH, however, many other product combinations have been reported for similar operational conditions. These product combinations mainly vary from butyrate, to propionate or ethanol and acetate (Fang and Liu 2002; Horiuchi et al. 1999; Horiuchi et al. 2002; Walker et al. 2005; Zoetemeyer et al. 1982c). This suggests that at the mid-pH range, there is not such a strong selective factor. Other factor might become important, like the increase in the bicarbonate concentration, which may favor the development of propionate producers (Koussemon et al. 2003).

When the influent substrate concentration was increased a shift in the metabolism was observed with all the carbon sources tested. However, this functional shift was not always followed by a shift in the population (glucose and glycerol), where the same communities remained with a different metabolism. This suggests that the product shift was rather a metabolic response to the operational conditions than a different community selection.

Different substrates were tested, at the same pH, enabling different populations to establish. Part of the metabolic pathways utilized for degradation of the different substrates is comparable, but the substrate uptake

mechanisms can be different or the different substrate oxidation states may induce different pathways. This is for example the case with glycerol that is more reduced than xylose or glucose, inducing a different product spectrum (Chapter-IV). Metabolizing each substrate can be seen as a different functional property. Once a second substrate is added, the present population can either be able to use it or not. In the latter case, microorganisms that are able to use the new carbon source may now become more dominant. The three communities established with the different substrates are suggested to be comprised of populations of mixotrophs (able to grow on a mixture of substrates) and specialists (only capable of degrading one substrate).

The glucose-glycerol fermentation resulted in a comparable product spectrum, independent of the fact whether it has been previously cultivated on glucose or on glycerol. The response of the microbial community, however, was strongly dependent on the cultivation history. The glucose grown population remained the same upon addition of glycerol to the influent, but in the reactor cultivated on glycerol a change in the microbial community was observed. It seems that in the glycerol cultivated reactor the population established mainly consisted of glycerol specialists (except for the microorganism represented by band 57). After the addition of glucose to the influent the specialist culture was rapidly overgrown by a mixotrophic microorganism, capable of degrading both glucose and glycerol.

To illustrate the kinetic advantage of a mixotroph degrading multiple substrates instead of multiple cultures degrading a single substrate, a simple model was developed (Fig. 5.8). In this model two populations are defined: a glycerol specialist (X_gly_sp) and a glycerol mixotroph (X_gly_mix, can also grow on glucose). Table 5.2 summarizes the kinetic properties of the cultures. It is assumed that the mixotrophic population grows on each substrate independently, using the Monod model (eq. 5.1) (Yoon et al. 1977). The initial biomass concentrations of X_gly_sp and of X_gly_mix before introducing a second substrate were assumed to be 70 and 30 % respectively of the total biomass present.

$$\mu = \frac{\mu_{\max glucose} \times S_{glucose}}{K_{glucose} + S_{glucose}} + \frac{\mu_{\max glycerol} \times S_{glycerol}}{K_{glycerol} + S_{glycerol}} \quad \text{(eq. 5.1)}$$

This model shows the succession of dominant populations that occurs when two populations cultivated on a single substrate are subjected to a mixture of two substrates. The model demonstrates the fact that the ability to instantaneously convert the other substrate gives an immediate advantage: as

the initial substrate concentration was decreased to half it could directly grow on the new substrate (Figure 5.8b).

TABLE 5.2- Kinetic parameters assumed for the two populations (specialists and mixotrophs).

Population	Growth on glycerol Ks/Ks_sp	Growth on glycerol μmax	Growth on glucose Ks	Growth on glucose μmax
X_gly_sp	1	1	-	-
X_gly_mix	5	1	1	1

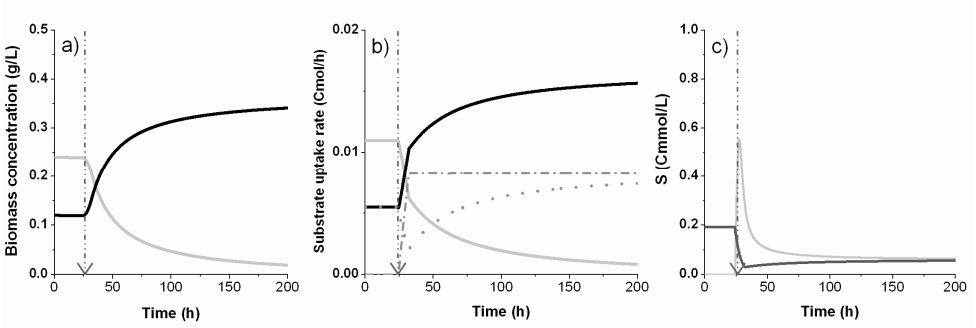


Figure 5.8-Microbial population dynamics when supplied with different carbon sources: a) population concentrations: grey X_gly_sp and black X_gly_mix, b) substrate consumption rate by the different populations: light grey glycerol uptake rate by the specialist population; solid black for the overall substrate uptake rate by the mixotrophic population; grey dash-dot for glucose conversion rate and grey dots for glycerol conversion rate; c) substrate concentration inside the reactor dark grey-glycerol and light grey-glucose. The arrow indicates the moment when fermentation of the two substrates was initiated.

Mixotrophs have a competitive advantage over the specialists when fed with a mixture of substrates. The generalists not only have the ability to adapt to a new situation, but, and more important, the fact they can grow on a different substrate results in a higher biomass concentration. Accordingly, the substrate uptake rate is higher and, consequently, decreases the substrate concentration inside the reactor (eq. 5.1) (Figure 5.8c). This phenomenon has been extensively studied and described for pure culture with several different substrates (Kovarova-Kovar and Egli 1998; Kuenen 1983; Yoon et al. 1977).

Conclusions

The results of this work have demonstrated that there is no generalized conclusion possible concerning a potential relation between ecosystem function and microbial composition. In some cases a clear shift in the metabolic properties, as characterized by the product spectrum, was not associated with a shift in the microbial population. This was for example the case when the substrate concentration was increased in the glucose and glycerol fed chemostat type reactors. In other cases a shift in metabolic properties was directly related to a shift in the microbial population composition. This was the case when glucose grown cultures were established at different pH-values or when the substrate concentration was increased in the xylose grown culture.

To complicate things further, a predictable metabolic response can or cannot be associated with a change in microbial population. This was what we observed when the influent composition of a bioreactor was changed from a single substrate to a mixture of substrates. The product spectra of the mixtures of substrates fermentation were found to be in line with the product spectra of the individual substrates. This anticipated metabolic response, was in some cases associated with a complete shift in population, but in other cases no change in the population composition was observed.

In summary, we suggest that at this stage the microbial composition analysis is an interesting tool to investigate mixed culture processes. It allows for microbial diversity analysis and identification of dominant metabolic groups in an ecosystem. However, given the largely unpredictable relationship with the metabolic functioning of the ecosystem, it does not provide straightforward insight in the process characteristics.

Chapter 6

General conclusions
and
Directions for future research

General conclusions

Mixed culture biotechnology may become an attractive option to convert waste streams into valuable products. Open mixed cultures rely on a mixture of microorganisms with a high diversity as found in nature. In these processes, bacteria are selected based on ecophysiological principles that are best capable to grow under the environmental conditions imposed. In wastewater treatment systems this principle has been successfully applied for removal of sulphur, phosphorous and nitrogen compounds. The work described in this thesis aimed for the application of the same principle to select for the formation of a certain product (VFAs, PHAs or alcohols) by mixed cultures.

Mixed culture biotechnology has specific advantages over pure culture biotechnology. Mixed culture processes do not require sterile conditions and d to the high microbial diversity, mixed cultures are suitable to convert mixtures of substrates. Potential disadvantages, are the limited range of products (catabolic products) and lower product concentrations that can be established, considerably increasing the downstream processing costs. Prosperous applications of mixed culture biotechnology are the fermentation of complex organic carbon sources, such as agricultural wastes, into chemicals that can be used as building blocks in chemical industry. The big challenge of mixed culture biotechnology is to design the operational conditions that will select for the desired metabolic conversion.

In this thesis, fermentation by open mixed cultures was investigated with the aim to control the conversion of organic compounds in a waste stream into a known and stable mixture of fatty acids and/or solvents. To achieve these objectives, open mixed microbial cultures were grown in continuously operated lab scale bioreactors. The impact of the following operational variables on the product spectrum in a fermentation process was investigated:

- the impact of the pH on glucose fermentation;
- the dependence of the fermentation process on the substrate (glucose, xylose, glycerol)
- mixtures of these substrates
- the substrate concentration in the bioreactor influent

The main conclusions derived from these experiments are the following:

1. The pH has a strong impact on the product spectrum of glucose fermentation (chapter 2):

- at low pH values (< 6) the main products were: butyrate, acetate and CO_2 and H_2
- at high pH values (>7) the products were mainly: acetate, ethanol and formate
- A pH-dependent thermodynamic equilibrium is established between H_2 and CO_2 and formate.
- No clear reason was found for the shift in the catabolic products from butyrate and acetate to acetate and ethanol at higher pH values.
- The composition of the microbial population established was depending on the pH range (chapter 5). At high and low pH values, most of the microorganisms found belonged to the clostridium genus (cluster I). At intermediate pH-values, other bacteria dominated the culture.

2. Xylose, the second main sugar in agricultural wastes, was found to be converted into a comparable product spectrum as glucose (chapter 3). The biomass yield on this substrate was shown to be significantly lower (80%) compared to glucose. This result is likely related to the xylose uptake system as was found in studies with pure cultures.

3. Glycerol has a higher degree of reduction compared to glucose, and this was reflected in product spectrum consisting of more reduced compounds. At pH 8, the main catabolic product was ethanol (chapter 4).
   - On the different substrates a completely different microbial population was established (chapter 5).

4. The main consequence of increasing the influent substrate concentration was the increase in the product concentrations (chapter 3 and 4). It was found that, maintenance energy requirements increased at increasing product concentrations, resulting in a decrease in the biomass yield and in an increase in the specific substrate conversion rate. Additionally, a product shift was observed. In the glucose and xylose fed reactors, the butyrate production almost ceased, and instead ethanol and acetate were produced in equal molar amounts. In the glycerol fed reactor, the ethanol yield considerably decreased and, 1,3-propanediol and acetate production increased.

- This product shift observed upon an increase in the substrate concentration sometimes was, and sometimes was not associated to a shift in the microbial population (chapter 5)

5. When a second substrate was introduced into the bioreactor influent (e.g. glycerol besides glucose), it was readily converted by the culture present, showing the flexibility of an open mixed culture. The product spectrum was comparable to the average of what was obtained for the single substrates fermentations (chapter 3 and 4).
   - The microbial populations initially selected consisted of specialists and mixotrophs. It was observed that the introduction of a second substrate favours mixotrophic microorganisms (chapter 5).

Directions for future research

Predicting product formation

The pH value of the system showed to have a large impact on the product spectrum and also on the composition of the microbial population established. It is known that different bacteria have developed different strategies to overcome stressful conditions or even adapt to those conditions. This explains the occurrence of different microbial populations at different pH values. However, the exact effect of the pH on the catabolism remained unclear and therefore difficult to explain in a general metabolic model (Rodriguez et al. 2006). Especially the mechanism responsible for the switch from production of an ethanol/acetate mixture to butyrate as a function of the pH is unclear.

The intracellular pH is generally assumed to be close to neutral values and independent of the medium pH-value. However, several studies have shown that the intracellular pH may vary from 6 to 8.5, when the external pH ranges between 4 and 9 (Breeuwer et al. 1996; Huang et al. 1986; Padan et al. 1981; Siegumfeldt et al. 1999; Thomassin et al. 2006). None of these studies, however, has reported the catabolic products that were formed at each condition. It would be interesting to know if there is any relation between the intracellular pH and the shift from butyrate to ethanol production, as it has been shown in lactic acid bacteria (LAB). The increase of the proton concentration was shown to exert an allosteric effect on lactate dehydrogenase and, in a similar way, deactivate the pyruvate formate lyase (Mayr et al. 1982; Russell et al. 1996). In the butyrate pathway, there is a reduction reaction (crotonyl-CoA to butyryl-CoA) catalyzed by Butyryl-CoA dehydrogenase, which was shown to be very sensitive to pH values higher 7 (Fink et al. 1986). This reaction is rather important, since it involves the regeneration of NAD, and if it becomes inhibited, the cell must find other pathways to oxidize the NADH, for instance by producing ethanol.

The determination of the intracellular concentrations of NADH/NAD and Co-A/Acetyl-CoA at different pH values could provide further information about the impact of the cell internal conditions on the product spectrum. Both NAD and free CoA are conserved moieties, exist in limited amounts inside the cell.

The fate of the acetyl-CoA mainly depends on the activity of three enzymes: phosphotransacetylase (acetate pathway, eq.1), acetaldehyde dehydrogenase (ethanol pathway, eq.2) and β-ketothiolase (butyrate pathway,

eq.3). How the cell regulates these enzymes activities, is not very clear yet. Acetyl-CoA was shown to be an allosteric activator, while free CoA inhibited the activity of the pyruvate ferredoxin oxido-reductase (PFOR) (Thauer et al. 1977). Furthermore, thiolase has also been reported to be inhibited by small amounts of free CoA (Boynton et al. 1994; Wiesenborn et al. 1988). So, the ratio free CoA/acetyl-CoA plays a determinant role in the control of the flow through acetoacetyl-CoA. The determination of the ratio free Co-A/Acetyl-CoA at different pH values could provide information about this regulation mechanism.

$$Acetyl-CoA+Pi \longrightarrow Acetyl-Pi+HSCoA--> Acetate.....eq.1$$

$$Acetyl-CoA+NADH \longrightarrow Acetaldehyde+HSCoA--> Ethanol.....eq.2$$

$$2Acetyl-CoA \longrightarrow Acetoacetyl-CoA+HSCoA--> Butyrate.....eq.3$$

The NADH/NAD ratios have been reported to increase with increasing pH values and were shown to be associated to the flux through the pyruvate formate lyase in Enterococcus faecalis cells (Snoep et al. 1991). These results showed that the increased redox potential of the NADH/NAD couple had an impact on the activity of the enzymes that convert pyruvate. Such an increase of the redox potential has furthermore been reported to be associated to the shift from acetogenesis to solventogenesis in butanol producing clostridia (Girbal and Soucaille 1994; Grupe and Gottschalk 1992; Kim and Kim 1988). By confirming that in mixed cultures the ratio NADH/NAD increases with the pH value, would allow to describe mathematically the shift from butyrate to ethanol as a function of the pH.

One important aspect to take into consideration is that different bacteria may be adapted to specific intracellular ratios, which would make more difficult to draw conclusions from these analyses when different populations are selected at each pH value. Therefore, these kind of analyses will always need to be accompanied by methods for characterization of the microbial population.

Directing product formation

The results presented in this thesis were shown to be stable and reproducible, however, the product concentrations were generally low compared to industrial pure culture fermentations and, in general, a mixture of at least two catabolic products was always obtained. These effluents could be used to feed other bioprocesses, e.g. the production of biopolymers (PHA). But it would also be interesting to develop strategies that could narrow the product spectrum to a single product. It has been shown that specific kinetics can contribute to a change in product spectrum (chapter 3 and 4). A clear shift was observed in the glycerol grown culture (chapter 4): at low biomass specific substrate conversion rates, ethanol and formate are the main products, while at higher uptake rates the ethanol yield decreased. This was observed when the inflow substrate concentration increased and during batch experiments. It seems to be a consequence of the increase of the intracellular NADH turnover: when the glycolysis derivatives cannot respond to the cell demand of NADH oxidation, 1,3-propanediol is produced as an electron sink. It would be interesting to try to control this product shift by changing the feeding pattern. In order to obtain a high ethanol concentration, a sequencing fed-batch reactor could be designed (Bravo et al. 2000; Jobe et al. 2003; Xu et al. 1996). In this process, the substrate would be added at the rate it is converted to ethanol and would avoid overflow metabolism. In this way, higher amounts of glycerol could be converted into mainly ethanol. An opposite strategy could be applied to enhance 1,3-propanediol production instead of ethanol.

Higher temperatures have been suggested to improve ethanol production by bacteria, not only the productivity but also the separation process are enhanced under these conditions (Benbassat et al. 1981; Herrero and Gomez 1980; Lamed and Zeikus 1980; Lovitt et al. 1984; Lovitt et al. 1988). Furthermore, in some studies, the decrease of the stirring speed was studied and seemed to enhance ethanol productivity, because of the increase of the dissolved hydrogen concentration (Lamed et al. 1988). During our studies, however, the stirring speed and sparging (or not) the reactor (associated with a change in the hydrogen partial pressure) did not show to have a significant effect on the product spectrum (data not reported).

To produce lactic acid, the operational conditions could be modified to resemble the conditions used for enriching lactic acid bacteria (LAB). Nutrient-rich environment, anaerobic or microaerophilic, low pH (6.0±1) and batch mode are consistently reported in industrial processes (Hofvendahl and Hahn-Hagerdal 2000). In the absence of LAB, other strategies could be applied to enhance lactate production by other species as certain nutrients limitation.

Iron and/or sulphate limitation have shown to trigger lactate production in Clostridium butyricum or Cl. acetobutylicum (Dabrock et al. 1992; Dubourguier personal communication; Freier and Gottschalk 1987; Hanson and Rodgers 1946; Katagiri et al. 1960). Iron and sulphate are key elements in the clostridium metabolism; both are constituents of the active centre of pyruvate ferredoxin oxido-reductase (PFOR). If they become limiting, pyruvate conversion into acetyl-CoA decreases, and production of lactate is an alternative for the cells. When glycerol is the substrate, iron limitation strongly enhances 1,3-propanediol production (Dabrock et al. 1992; Reimann et al. 1996).

Nutrients limitation as: phosphate, ammonia, sulphate have also been reported to improve butanol production (Bahl et al. 1982; Bahl et al. 1986). By limiting the cell growth, the carbon is directed into other products. Despite the improvements, there is still no strain (natural or modified) that is sufficiently butanol tolerant that could make this process viable. Furthermore, producing butanol seems to be a transitory advantage for the bacteria to protect from the acids present, but not to out compete other species present. In situ specific product removal while keeping the acids at high concentrations could be a way to direct into more butanol production.

Use of real waste streams

During this study, all experiments were conducted with synthetic medium. The aim was to study a stable conversion of certain substrates and the microbial populations that established at each condition. For practical implementation of the process proposed here, experiments will need to be conducted with mixtures of substrates and real waste streams to verify the pure substrate results. In this respect, it is important to note that difficulties have been reported with the use of real waste streams; e.g. due to the presence of toxic compounds. These issues would have to be addressed before the application of a certain organic waste.

Another aspect of relevance is whether the waste streams are rich in readily biodegradable substrates, as sugars and glycerol, or in polymers that still have to be hydrolyzed. In this situation, fermentation is not the limiting step and retention times may need to be extended, which may allow other bacteria to convert further the fermentation products, by e.g. methanogenic bacteria. This suggests the requirement to run the mixed culture fermentation process in conditions that are unfavourable for methanogens, e.g. by (periodically) lowering of the operational pH.

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Curriculum vitae

Margarida Freire Temudo Ribeiro Ferreira was born on the 24th June 1978 in Lisbon, Portugal. In 2002 she graduated in applied chemistry, specialization in biotechnology at the Faculty of Science and Technology, New University of Lisbon.

In 2000, she went to the University of Bayreuth, Germany, for a training. The aim was to determine the toxicity effect of fog and rain waters on bacterial activities. In 2002, she did her thesis with the group of Biochemical and bioseparation engineering, at the New University of Lisbon, under the supervision of professor Maria Ascensão Reis. Her thesis was on biological nutrients removal from domestic effluents: Characterization and optimization of a biological process for removing phosphorus, ammonia and organic load, in a SBR operated with short cycles.

From January 2004 to January 2008, she worked as a PhD student at the Environmental Biotechnology group, Delft University of Technology. Her supervisors were professor Mark van Loosdrecht and Robbert Kleerebezem. The results of this research are described in this thesis.

Publications

Publications List:

-Temudo, MF, R. Kleerebezem, & MCM van Loosdrecht (2007). Influence of the pH on (open) mixed culture fermentation of glucose: a chemostat study. Biotechnology and bioengineering, 98(1), 69-79.

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- Freitas F., M. Temudo, J.S. Almeida, M.A.M. Reis. Optimization of Nutrient Removal in a Sequencing Batch Reactor Operated with High Frequency Oxygen Oscillations. 17th Forum for Applied Biotechnology-FAB, Gent, Belgium, September 2003.

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After four years, many people have contributed to gladly achieve this moment.

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