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Diversity of microbial communities in open mixed culture fermentations: impact of the pH and carbon source

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Abstract Anaerobic fermentation by an open mixed culture was investigated at different pH values (4–8.5) and with three substrates (glucose, glycerol and xylose). The populations established in each condition were assessed by denaturing gradient gel electrophoresis analysis of the 16S ribosomal RNA gene fragments. The fermentation pattern and the composition of the microbial population were also evaluated when operational variations were imposed (increase of substrate concentration or introduction of a second substrate). The experimental results demonstrated that at low and high pH values, a clearly different fermentation pattern was associated with the dominance of a specialised group of clostridiae. At intermediate pH values, the product spectrum was rather variable and seemed to be sensitive to variations in the microbial community. Different substrates resulted in the establishment of different microbial communities. When fed with a mixture of two substrates, mixotrophic microorganisms (capable of degrading both substrates) were found to overgrow the originally dominant specialists. Overall, the experiments have shown that some operational variables have a clear impact on the fermentation pattern and on the population established. However, a uniform relationship between the process characteristics (associated to a metabolic response) and the microbial population present is not always possible.

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Introduction

Mixed culture biotechnology may become an attractive addition to traditional pure-culture-based biotechnology for the production of bulk chemicals from waste streams (Dai et al. 2007; 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. Additionally, due to the high microbial diversity, these cultures can deal with mixtures of substrates and of variable composition. The conditions in these processes are chosen such that the metabolic conversion of interest provides an ecological advantage to the microorganisms. 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 performance of ecosystem-based processes depends on the 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).



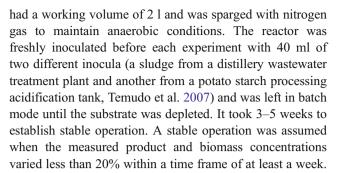
The pH is a powerful parameter that can be used to control both the metabolism and to select the microorganisms that are best able to survive. The pH affects several microbial parameters such as the growth rate, the utilisation of the carbon source, efficiency of the substrate conversion, etc. (Russell 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 cultures fermentation (Temudo et al. 2007; Zoetemeyer et al. 1982). Another important factor that shapes the community structure and product formation is the substrate. The degree of reduction of the substrate and the metabolic pathway used for its degradation determine the product spectrum (Temudo et al. 2008). 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 from the established microbial community.

The aim of this research was to study the link between the functional performance and the microbial community found under each condition. Furthermore, it was of interest to evaluate the response and stability of a certain population towards the introduction of other variables (product concentration or other carbon source). To this end, the microbial population established was assessed in several continuous culture fermentation systems operated under different operational conditions. The variables investigated were the pH and diverse carbon sources (glucose, glycerol and xylose). After achieving a stable performance, the impact of higher inflow substrate concentrations and introduction of a second substrate on the microbial community present was assessed.

Materials and methods

Reactor operation and inoculum source

A continuously stirred tank reactor was operated at 30°C at an influent substrate concentration of 4 g/l. The bioreactor



For the study of the impact of the pH on fermentation by mixed cultures, glucose was used as substrate. 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. 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 influence of different substrates (glucose, glycerol and xylose) on the product spectrum and the composition of the microbial community was studied at pH 8.0, 30°C, and D 0.12 h⁻¹. The influent substrate concentration initially was 4 g/l and later increased to 10 g/l. In the case of the glucose and glycerol cultivated cultures, also 25 g/l was tested (Temudo et al. 2008). The mineral medium composition was described previously (Temudo et al. 2007) and, when needed, was adjusted to keep the C/N/P ratio constant.

Fermentation of a mixture of two substrates was tested on the biomass cultivated on a single substrate. A mixture of glucose and glycerol was added to the glucose grown culture. The same substrates mixture was fed to the glycerol grown culture. A mixture of glucose and xylose was supplied to the reactor cultivated on 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 reactors were opened and cleaned at least once per week to remove wall growth and only allow suspended biomass to remain. The carbon source solutions were sterilised at 110°C for 20 min to avoid microbial growth in the vessel.

Analytical techniques

Reactor broth samples were immediately filtered (Millipore membrane of 0.45 μ m). The substrate and end products were determined and quantified. Xylose, glucose, glycerol, volatile fatty acids (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate 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



online, and the base added to maintain the pH constant was monitored. A detailed description of the analytical methods used can be found elsewhere (Temudo et al. 2007). 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, California, USA) according to the manufacturer's protocol. Extracted DNA was stored at -20°C until further use.

PCR amplification Amplification of 16S ribosomal RNA (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). Polymerase chain reaction (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 for 1 min and extension at 72°C for 20 s, 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 Denaturing gradient gel electrophoresis (DGGE) was performed as described by Schäfer 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 1× TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.5) at constant temperature of 60°C. 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 (Schäfer and Muyzer 2001): 16 h 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). Individual bands were excised, resuspended in 15 μ l of Milli-Q water and stored overnight at 4°C. A volume of 3 to 5 μ l of the supernatant was used for re-amplification with the original primer sets. The re-amplified 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; Schäfer and Muyzer 2001) and aligned using the automatic aligner function. The alignment was further corrected manually, and an optimised tree was calculated using the neighbour-joining algorithm with Felsenstein correction.

Sequence accession numbers The 16S rRNA sequences determined in this study were deposited in GenBank under accession numbers EU435415–EU435434.

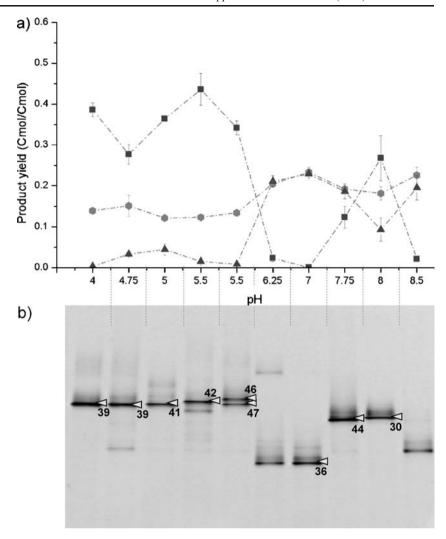
Results

Impact of the pH on glucose fermentation

The pH not only had a significant effect on the product spectrum but also on the dominant type of microorganism as can be seen in Fig. 1a,b. The full product distribution as a function of the pH can be found in Table S1 of the Electronic supplementary material. At low pH values, the product spectrum mainly consisted of butyrate and acetate, while at high pH, it shifted to acetate and ethanol. The microbial population that established at each pH value also differed as can be seen from the DGGE gel in Fig. 1. From the gel, the dominant bands were excised and re-amplified. The successfully sequenced DNA fragments are numbered in Fig. 1 and the respective phylogenetic affiliation is illustrated in the phylogenetic evolutionary tree (Fig. 2). Four microbial communities could be distinguished: at low (4-5.5), medium (6-7) and high pH values, 7.5-8 and 8.5. The dominant microorganisms at low and at high pH were located at two different subclusters of cluster I of the genus Clostridium. At middle pH values, the principal microorganism belonged to genus Klebsiella; facultative anaerobic bacteria that are known for lacking the enzymes involved in butyrate production. This relates to the observation that at pH 6.25 and 7.0, the butyrate yield was lower (Fig. 1).



Fig. 1 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 (square), acetate (circle) and ethanol (triangle). Apart from biomass, the main other products were CO2 and H2 at low pH values and formate at high pH values



Impact of the type of substrate

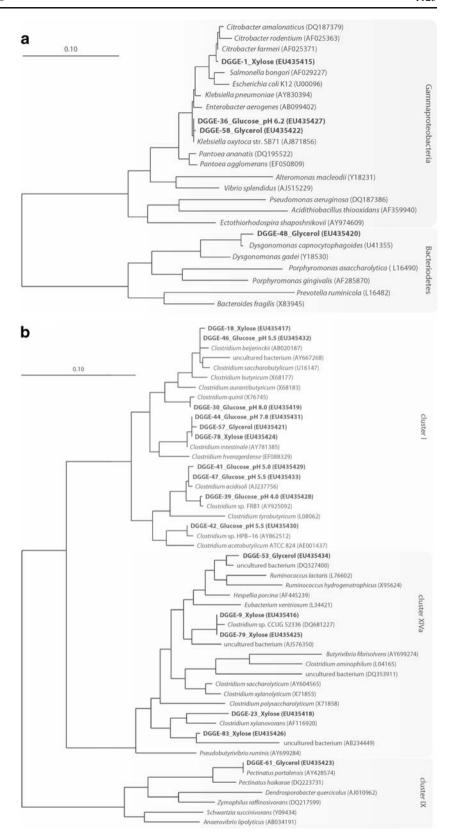
To study and compare the effect of different substrates (glucose, xylose and glycerol), reactors were operated at pH 8, dilution rate 0.12 h⁻¹ and substrate concentration 4 g/l (0.14-0.15 Cmol/l). The system was characterised after 80 volume changes in the case of glucose and glycerol and after 240 volume changes for xylose. The main catabolic products of glucose and xylose fermentation were acetate, butyrate and ethanol (Fig. 3). The complete product spectrum can be found in Table S2 of the Electronic supplementary material. 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, while minor amounts of 1,3-propanediol and acetate were produced (Fig. 3).

The microbial populations that established on the three substrates were different (Fig. 2 and 4). The glycerol and

xylose cultures had a bigger diversity compared to the glucose grown culture. The glycerol community was dominated by an enterobacteria closely related to Klebsiella oxytoca (band number 58), a facultative microorganism which has been 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 Clostridium intestinale, clostridium cluster I (band 57), and the sequence from band 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 Clostridia family, cluster IX, and are strictly anaerobic (Haikara and Helander 2006). In the bioreactor fed with xylose, there were three dominant bands present, represented by numbers 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; however, the result was always the same (two bands in the DGGE gel) and the purified DNA could be



Fig. 2 Phylogenetic tree based on 16S rRNA gene sequences obtained from the DGGE bands. The sequences were mainly affiliated to a two distinct classes: gammaproteobacteria and bacteroidetes and to b three different genera of Clostridia familiae (Collins et al. 1994)





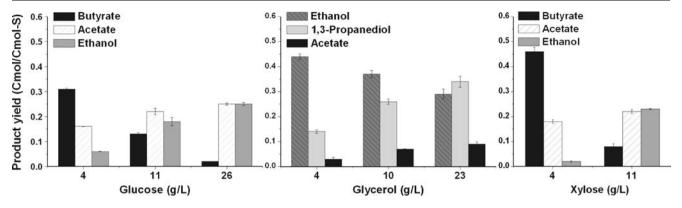


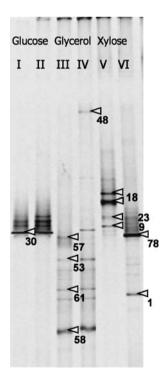
Fig. 3 Main catabolic products of glucose, glycerol and xylose fermentation by open mixed culture at increasing substrate concentration in a chemostat at pH 8, dilution rate $0.12~h^{-1}$ and 30° C. The main other products were biomass, CO₂, H₂ and formate

successfully sequenced. The sequence 18 was affiliated to cluster I of the clostridium genus. The sequences 9 and 23 were affiliated to cluster XIVa of the clostridia family. Many of the described species of this cluster live in ruminant environments, which may explain a higher affinity for xylose.

Impact of the increase of the substrate concentration

After characterising the system at low substrate concentrations in the influent, the substrate concentration of the feeding solution was increased. The system was characterised after reaching stable operation as obtained after approximately 40 volume changes. The main product yields are shown in Fig. 3; full product distribution can be found

Fig. 4 DGGE 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 4 g/l, *III* 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



in Table S3 of the Electronic supplementary material. The substrate concentration inside the reactor was below the detection limit. However, under the new conditions, higher substrate conversion rate and higher product 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. 3).

Figure 4 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 microorganism remained. 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: the glucose and glycerol grown cultures were fed with a glucose–glycerol mixture, and the xylose grown culture was fed with a xylose–glucose mixture. After 1 week feeding with the mixture of substrates, the product spectrum in all reactors was very similar to the average of the product spectra observed when the two substrates were fed separately (Fig. 5). The full product distribution can be found in Table S4 of the Electronic supplementary material. Immediately after switching from a single substrate to a mixture of substrates, the populations present in the reactor were capable of degrading the additional substrate.

The DGGE pattern of the microbial communities at different stages is shown in Fig. 6. Samples were taken before feeding with a mixture of substrates (lane I), 1 day



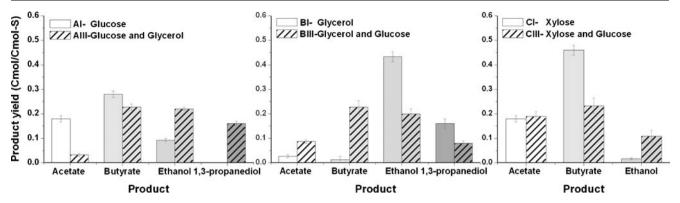
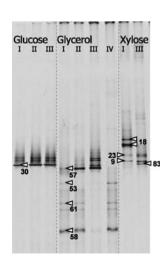


Fig. 5 Main catabolic products on one (I) and two substrates (III) in a chemostat at pH 8, dilution rate $0.12 \, h^{-1}$ and 30° C. A Culture cultivated on glucose before (I) and 1 week after (III) being fed with a mixture of glucose and glycerol; B culture cultivated on glycerol

before and 1 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 1 week

after (lane II) and 1 week after (lane III). The glucosegrown culture could instantaneously convert glycerol. The composition of the microbial population was not affected after 1 week of being fed with the glucose-glycerol mixture. In the glycerol-fed reactor where a more diverse population was present, there was a microorganism (band number 57, Fig. 6) that after only three volume changes took advantage of the presence of glucose besides glycerol. After 1 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 numbers 9 and 23 remained in the system, band 18 disappeared and band 83 emerged.

Fig. 6 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 mixtures of two substrates: glucose, glycerol and xylose. Samples taken one day before (lane I) 1 day after changing the feeding solution (three volume changes, lane II), 1 week after (20 volume changes, lane III) and 2 weeks after being changed back to the initial conditions (lane IV)



Discussion

In this study, a mixture of two inocula was used in order to initially provide a high microbial diversity. The high diversity is reflected in the different microbial populations established with different substrates and under the different conditions studied as indicated in the phylogenetic tree (Fig. 2a,b). A low number of significant bands was found upon feeding with a single substrate: between one and three when glucose was the substrate and less than six for glycerol and xylose. At the relatively high dilution rates applied (0.04–0.13 h⁻¹), it can be safely assumed that the microorganisms identified are metabolically active and directly involved in the substrate conversion. This also means that at each operational condition, the main metabolic functions are catalysed 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 characterised by the absence of dynamics, gradients, not allowing for 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 cultivating in a chemostat, selection occurs for those microorganisms that show a higher specific substrate affinity ($\mu^{\text{max}}/\text{Ks}$). 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 value. Nevertheless, when the conditions were changed as by the increase of influent substrate



Table 1 Free energy of the main reactions observed at different pH values

Reactions	$\Delta G^{0,}$	$Y_{\rm ATPS}$
Glucose+0.53 $H_2O \rightarrow 0.6Butyrate+0.53$ Acetate+0.27glycerol+0.27 H_2 +1.73formate+2.87 H_2 +1.73formate+2.87 H_2 +1.73formate+3	-259.3 -258.3	2.4 2.0

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.

concentration or by the introduction of a different substrate, previously absent bands emerged, suggesting that non-dominant 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 the glucose and glycerol cultures. Nonetheless, the number of significant microorganisms is higher in the xylose-cultivated community. Coexistence of more than one microorganism in chemostat has been reported when more than one nutrient limitation or different environmental factors exert the control on the growth of the different microbial groups (Gottschal 1993; Harder et al. 1977; Powell 1958; Taylor and Williams 1975; Yoon et al. 1977). Another aspect could be the effect that a certain species can have on the growth of other species, creating a symbiotic relation.

Coupling between metabolic function and community composition

The pH was shown to have a clear impact on both the product spectrum and the microbial community structure in a glucose fermenting system. Such an impact of the environmental pH on the process that results in a shift in product spectrum has been reported before (Fang and Liu 2002; Horiuchi et al. 2002; Zoetemeyer et al. 1982). 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. 1982). 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). This suggests that at around neutral pH values, the pH does not impose a strong selective pressure on the system. In these cases, other factors might become important, like an increased bicarbonate concentration that may favour propionate production (via succinate; Koussemon et al. 2003; van der Werf et al. 1997).

Such product shift could not be explained using only bioenergetic considerations at different pH values because both conversions are equally favourable in terms of Gibbs free energy change, and the estimated ATP yields on substrate ($Y_{\rm ATPS}$) are also comparable (Table 1). A higher ATP efficiency, as found at lower pH values, is justifiable due to the higher maintenance requirements:

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

Therefore, at least two metabolic properties can be distinguished with respect to acid toxicity tolerance at low and high pH values (outside the range of pH values 6–7).

The presence of clostridiaceae has been widely reported in similar operational conditions: fermentation of carbohydrates, mesophilic 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; Kim et al. 2006; Ren et al. 2007; Ueno et al. 2001). The cluster I of the genus clostridium are strictly anaerobic "Gram-positive" microorganisms (Collins et al. 1994). Little is known about the difference within this cluster in terms of acid tolerance. 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. The microorganisms found at low pH were closely related to C. acidisoli and C. pasteuranium, species that have been described as acid-tolerant and grow at pH 3.6-7.0 (Wiegel et al. 2006).

When different substrates were tested at the same pH, different populations established. Part of the metabolic pathways utilised for degradation of the different substrates is comparable, but the substrate uptake mechanisms or the substrate oxidation state may induce different pathways. As 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.

Fermentation of a glucose-glycerol mixture resulted in a comparable product spectrum, independent of the fact that it has been previously cultivated on glucose or on glycerol. The response of the microbial community, however, was strongly dependent on the cultivation history. Once a second substrate is added, the present population can either be able to use it or not. Examples of both populations were observed during these experiments. Specialised microorganisms as represented by bands with the numbers 18, 53, 58 and 61 lost some advantage by the presence of a second substrate, whereas mixotrophic microorganisms as 9, 23, 45 and 57 that could convert a second substrate had a competitive advantage under these conditions and started to dominate the microbial community. After reversing the substrate to only xylose or glycerol, the bands that emerged on the substrate mixture disappeared, and the original microbial composition was reestablished.

Mixotrophs have a competitive advantage over the specialists when fed with a mixture of substrates. In a chemostat, mixotrophs convert two substrates while maintaining the same growth rate equal to the dilution rate. This means that the individual growth rates on each substrate can be lower than when growing on a single substrate. As all the kinetic parameters remain the same, a lower rate is only associated to a lower substrate concentration inside the reactor. Under these conditions, the substrates concentrations maintained by the mixotrophs become lower than the concentrations established by the specialist population that will be washed out from the chemostat. This phenomenon has been extensively studied and described with similar results for pure cultures with different substrates (Kovarova-Kovar and Egli 1998; Kuenen 1983; Yoon et al. 1977).

At this stage, the study of the microbial composition is an interesting tool to investigate mixed culture processes. It allows for microbial diversity analysis, identification of dominant metabolic groups in an ecosystem and how these react towards changes in the operation. However, given the largely unpredictable relationship with the metabolic functioning of the ecosystem, it does not provide straightforward insight of the process characteristics.

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References

- Claassen PAM, van Lier JB, Lopez Contreras AM, van Niel EWJ, Sijtsma L, Stams AJM, de Vries SS, Weusthuis RA (1999) Utilisation of Biomass for the supply of energy carriers. Appl Microbiol Biotechnol 52:741–755
- Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandezgarayzabal J, Garcia P, Cai J, Hippe H, Farrow JAE (1994) The phylogeny of the genus *Clostridium*—proposal of 5 new genera and 11 new species combinations. Int J Syst Bacteriol 44(4):812–826
- Dai Y, Yuan ZG, Jack K, Keller J (2007) Production of targeted poly (3-hydroxyalkanoates) copolymers by glycogen accumulating organisms using acetate as sole carbon source. J Biotechnol 129 (3):489–497
- Dejonghe W, Boon N, Seghers D, Top EM, Verstraete W (2001) Bioaugmentation of soils by increasing microbial richness: missing links. Environ Microbiol 3(10):649–657
- Fang HHP, Liu H (2002) Effect of pH on hydrogen production from glucose by a mixed culture. Bioresour Technol 82(1):87–93
- Fang HHP, Liu H, Zhang T (2002) Characterization of a hydrogenproducing granular sludge. Biotechnol Bioeng 78(1):44–52
- Fernandez AS, Hashsham SA, Dollhopf SL, Raskin L, Glagoleva O, Dazzo FB, Hickey RF, Criddle CS, Tiedje JM (2000) Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. Appl Environ Microbiol 66(9):4058–4067
- Findlay SEG, Sinsabaugh RL (2003) Response of hyporheic biofilm metabolism and community structure to nitrogen amendments. Aquat Microb Ecol 33(2):127–136
- Gottschal JC (1993) Growth-kinetics and competition—some contemporary comments. Antonie Van Leeuwenhoek Int J Gen Mol Microbiol 63(3-4):299-313
- Greenberg AE, Eaton AD, Clesceri LS (1992) Standard methods for the examination of water and wastewater, 18th edn. Chapman Hall London
- Haikara A, Helander I (2006) Pectinatus, Megasphera and Zymophilus.
 In: Dworkin M (ed) The prokaryotes. Springer, New York, pp 965–981
- Hanselmann KW (1991) Microbial energetics applied to waste repositories. Experientia 47(7):645–587
- Harder W, Kuenen JG, Matin A (1977) Microbial selection in continuous culture. J Appl Bacteriol 43(1):1–24
- Hawkes FR, Hussy I, Kyazze G, Dinsdale R, Hawkes DL (2007) Continuous dark fermentative hydrogen production by mesophilic microflora: principles and progress. Int J Hydrogen Energy 32(2):172–184
- Herrero AA, Gomez RF, Snedecor B, Tolman CJ, Roberts MF (1985) Growth-inhibition of *Clostridium-thermocellum* by carboxylic-acids—a mechanism based on uncoupling by weak acids. Appl Microbiol Biotechnol 22(1):53–62
- Homann T, Tag C, Biebl H, Deckwer WD, Schink B (1990) Fermentation of glycerol to 1,3-propanediol by *Klebsiella* and *Citrobacter* strains. Appl Microbiol Biotechnol 33(2):121–126
- Horiuchi J, Shimizu T, Kanno T, Kobayashi M (1999) Dynamic behavior in response to pH shift during anaerobic acidogenesis with a chemostat culture. Biotechnol Tech 13:155–157
- Horiuchi JI, Shimizu T, Tada K, Kanno T, Kobayashi M (2002) Selective production of organic acids in anaerobic acid reactor by pH control. Bioresource Technol 82(3):209–213
- Iyer P, Bruns MA, Zhang HS, van Ginkel S, Logan BE (2004) H-2-Producing bacterial communities from a heat-treated soil inoculum. Appl Microbiol Biotechnol 66(2):166–173
- Kim SH, Han SK, Shin HS (2006) Effect of substrate concentration on hydrogen production and 16S rDNA-based analysis of the



- microbial community in a continuous fermenter. Process Biochem 41(1):199–207
- Kleerebezem R, van Loosdrecht MCM (2007) Mixed culture biotechnology for bioenergy production. Curr Opin Biotechnol 18:207–212
- Koussemon M, Combet-Blanc Y, Ollivier B (2003) Glucose fermentation by *Propionibacterium microaerophilum*: effect of pH on metabolism and bioenergetic. Curr Microbiol 46(2):141–145
- Kovarova-Kovar K, Egli T (1998) Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. Microbiol Mol Biol Rev 62(3):646– 666
- Kuenen JG (1983) The role of specialists and generalists in microbialpopulation interactions. ACS Symp Ser 207:230–251
- Langenheder S, Lindstrom ES, Tranvik LJ (2005) Weak coupling between community composition and functioning of aquatic bacteria. Limnol Oceanogr 50(3):957–967
- Lawton JH, Brown VK (1994) Redundancy in ecosystems. In: Schulze E-D, Mooney HA (eds) Biodiversity and ecosystems function. Springer, New York, pp 255–273
- Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G (2004) ARB: a software environment for sequence data. Nucleic Acids Res 32(4):1363–1371
- Muyzer G, Teske A, Wirsen CO, Jannasch HW (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch Microbiol 164 (3):165–172
- Powell EO (1958) Criteria for the growth of contaminants and mutants in continuous culture. J Gen Microbiol 18(1):259–268
- Reis MAM, Serafim LS, Lemos PC, Ramos AM, Aguiar FR, Van Loosdrecht MCM (2003) Production of polyhydroxyalkanoates by mixed microbial cultures. Bioprocess Biosyst Eng 25(6):377–385
- Ren NQ, Xing DF, Rittmann BE, Zhao LH, Xie TH, Zhao X (2007) Microbial community structure of ethanol type fermentation in bio-hydrogen production. Environ Microbiol 9:1112–1125
- Rodriguez J, Kleerebezem R, Lema JM, van Loosdrecht MCM (2006) Modeling product formation in anaerobic mixed culture fermentations. Biotechnol Bioeng 93(3):592–606
- Russell JB (1992) Another explanation for the toxicity of fermentation acids at low ph—anion accumulation versus uncoupling. J Appl Bacteriol 73(5):363–370

- Schäfer H, Muyzer G (2001) Denaturing gradient gel electrophoresis in marine microbial ecology. Methods in microbiology, vol 30. Academic, San Diego, pp 425–468
- Svensson BH (1992) Clostridium-quinii sp-nov, a new saccharolytic anaerobic bacterium isolated from granular sludge. Arch Microbiol 157(2):97–103
- Taylor PA, Williams PJ (1975) Theoretical studies on coexistence of competing species under continuous-flow conditions. Can J Microbiol 21(1):90–98
- Temudo MF, Kleerebezem R, van Loosdrecht M (2007) Influence of the pH on (open) mixed culture fermentation of glucose: a chemostat study. Biotechnol Bioeng 98(1):69–79
- Temudo MF, Poldermans R, Kleerebezem R, van Loosdrecht MCM (2008) Glycerol fermentation by mixed cultures, a chemostat study. Biotechnol Bioeng 100(6):1088–1098
- Ueno Y, Haruta S, Ishii M, Igarashi Y (2001) Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. Appl Microbiol Biotechnol 57(4):555–562
- van der Werf MJ, Guettler MV, Jain MK, Zeikus JG (1997) Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by *Actinobacillus* sp. 130Z. Arch Microbiol 167(6):332–342
- Verstraete W, Wittelbolle L, Heylen K, Vanparys B, de Vos P, van de Wiele T, Boon N (2007) Microbial resource management: the road to go for environmental biotechnology. Eng Life Sci 7(2):117–126
- Walker AW, Duncan SH, Leitch ECM, Child MW, Flint HJ (2005) pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. Appl Environ Microbiol 71(7):3692–3700
- Wiegel J, Tanner R, Rainey F (2006) An introduction to the family clostridiaceae. In: Dworkin M (ed) The prokaryotes. Springer, New York, pp 654–678
- Wittebolle L, Boon N, Vanparys B, Heylen K, De Vos P, Verstraete W (2005) Failure of the ammonia oxidation process in two pharmaceutical wastewater treatment plants is linked to shifts in the bacterial communities. J Appl Microbiol 99(5):997–1006
- Yoon H, Klinzing G, Blanch HW (1977) Competition for mixed substrates by microbial-populations. Biotechnol Bioeng 19 (8):1193–1210
- Zoetemeyer RJ, van den Heuvel JC, Cohen A (1982) pH Influence on acidogenic dissimilation of glucose in an anaerobic digester. Water Res 16(3):303–311

