EFFECT OF AERATION STRATEGY ON THE PERFORMANCE OF A VERY HIGH GRAVITY CONTINUOUS FUEL ETHANOL FERMENTATION PROCESS

by

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ABSTRACT

Normand Cyr M.Sc. (Bioresource Eng.)

Effect of aeration strategy on the performance of a very high gravity continuous fuel ethanol fermentation process

The fuel ethanol industry is now making use of a very efficient process where virtually all sugar substrates are converted to ethanol. Nevertheless, some metabolic by-products excreted from *Saccharomyces cerevisiae* tend to reduce the ethanol yield. Of such, glycerol is the major one, accounting for about 5-10% relative to the amount of ethanol produced.

Glycerol plays an important role in maintaining the redox balance within the cells by oxidizing the cytosolic NADH under anaerobic conditions. It is also believed that it acts as an osmoprotectant and would be favourably produced in high osmotic pressure conditions.

In order to mitigate the production of glycerol, various aeration strategies were investigated in a single-stage continuous fermentation system. Oxygen dissolved in the fermentation medium put the yeast in aerobiosis, acted as an oxidizing agent and hence minimised the specific glycerol production by 36 % as compared to a completely anaerobic fermentation.

This has hardly been reproduced in a more industrially relevant system using a multi-stage continuous fermentation process. Indeed, oscillations in the concentrations of the various metabolites over time made difficult the assessment of significant changes. Nevertheless, these findings open the door to further investigations in order to understand the effect of oxygen in continuous fermentations using very high gravity feeds, such as in the fuel ethanol industry.

RÉSUMÉ

Normand Cyr M.Sc. (Bioresource Eng.)

Effet de la stratégie d'aération sur la performance d'un système de fermentation à haute gravité en continu pour la production d'éthanol de carburant

L'industrie de l'éthanol de carburant utilise de nos jours un procédé très efficace où presque tous les sucres fermentescibles sont transformés en éthanol. Néanmoins, certains métabolites secondaires produits par *Saccharomyces cerevisiae* occasionnent une perte de rendement en éthanol. Parmi ces métabolites secondaires, le glycérol est le principal et compte pour 5 % à 10 % de la concentration d'éthanol retrouvée durant une fermentation alcoolique.

Le glycérol joue un rôle important dans le maintien de l'équilibre oxydo-réductif intracellulaire, en oxydant le NADH cytosolique lorsque des conditions d'anaérobiose sont présentes. Il agit aussi en tant qu'osmorégulateur et sa production est favorisée lorsque la cellule est soumise à des conditions de pression osmotique élevée.

Dans le but de réduire la production de glycérol, plusieurs stratégies d'aération ont été élaborées et testées dans un système de fermentation en continu à simple pallier. L'oxygène dissout dans le moût a

permis de maintenir la levure en aérobiose, tout en agissant à titre d'agent oxydant. Par conséquent, la production spécifique de glycérol fut réduite de 36 % lorsque comparée à celle obtenue en fermentation anaérobique absolue.

Les résultats se sont révélés peu reproductibles dans un système industriel modèle à l'échelle du laboratoire, pour lequel un système de fermentation en continu à paliers multiples a été utilisé. En effet, des oscillations dans la concentration des composés en solution ont rendu délicat l'évaluation de changements significatifs. Cependant, ces résultats ouvrent la porte à de futures recherches ayant pour but de comprendre l'effet de l'oxygène dans les fermentations en continu utilisant un substrat à concentration élevée, tel qu'employé dans l'industrie de l'éthanol de carburant.

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1. INTRODUCTION

1.1. Background

1.1.1. Fuel industry situation and link with environment issues

The petroleum industry presently faces important challenges: geological reserves are rapidly declining; energy demand is projected to increase by over 50 % by 2050 and many environmental issues are being raised with regard to fossil fuel usage (38,71). Moreover, the tragedy of the Hurricane Katrina (August 2005) and the invasion of Iraq by the United States (March 2003 to date) have caused important price volatility for gasoline and demonstrated once again a requirement for energy security. Therefore, the industry is constrained to turn into more locally produced energy sources that will not favour the release of considerable amounts of greenhouse gases (GHG) which leads to global warming (30). Examples of avenues investigated are solar power, fuel cells, hydrogen, wind power, nuclear energy, biodiesel and bioethanol.

1.1.2. Place of ethanol in the fuel industry

Ethanol is being used extensively in the fuel industry since more than 30 years (94). Initially implemented as a response to the oil crisis faced in the 1970's, its current utilisation relies mainly on environmental concerns. Moreover, the actual crude oil price being close to

75 $US/barrel (August 2006 data), introduction of ethanol becomes even more economically attractive.

As a result, the fuel ethanol industry is growing at a tremendous rate: according to the 2006 Ethanol Industry Outlook Report from the Renewable Fuels Association, the United States have produced over 16.1×10^9 L of anhydrous ethanol in 2005, and a growth in the range of 20 % is expected annually.

1.1.3. Challenges faced by the fuel ethanol industry presently

Nevertheless, the fuel ethanol industry has to remain competitive and many hurdles are faced with respect to production. Apart from raw material prices that are artificially kept low by subsidies given to farmers, fuel ethanol production costs should remain as low as possible and this relies on an efficient conversion of the substrates to ethanol. A few challenges are described below.

The current process involves a yeast-catalyzed transformation of fermentable sugars in ethanol and carbon dioxide. However, at the industrial scale, maintaining a pure culture of yeast is truly a challenge. Ethanol producers are faced with bacterial contaminants that will compete with the yeast for sugars, thus reducing the yield of ethanol (8,10,63,80,87). Moreover, bacteria present in a fermentation broth will liberate acids that are inhibitory to the performance of the yeast (64).

Another challenge resides in the ethanol tolerance of yeast strains. *Saccharomyces cerevisiae*, which is the yeast species used in fuel ethanol

production, is growth-inhibited above certain ethanol concentration. On the other hand, being able to supply the distillation procedure with high ethanol concentration reduces energy requirements and production costs. Improvements in ethanol tolerance are thus still required.

Thirdly, there are some fractions of the carbon that is redirected in the cell towards production of metabolic by-products for proper cell maintenance, which in return reduces the yield of product (67). This thesis will demonstrate a new approach that can be implemented to mitigate such by-products.

1.2. Objectives

The following three objectives were identified:

- Study the effect of aeration on metabolic by-products formation during fermentation of glucose by *Saccharomyces cerevisiae* in conditions of very high substrate concentrations.
- Quantify the production yields of such metabolic by-products in the various aeration strategies studied.
- Evaluate the adjustments in production cost at the industrial scale of such changes using a process simulation model.

2. LITERATURE REVIEW

2.1. Ethanol production processes

There are two ways of producing ethanol, one which involves a chemical synthesis and a second which employs microorganisms.

2.1.1. Chemical synthesis

The chemical synthesis of ethanol involves the hydration of ethylene derived from petroleum refining activities (Figure 2.1). Phosphoric acid attached to an inert support, such as zeolite or silica gel, acts as catalyst which favours the reaction. The acid can later be recycled and reused.

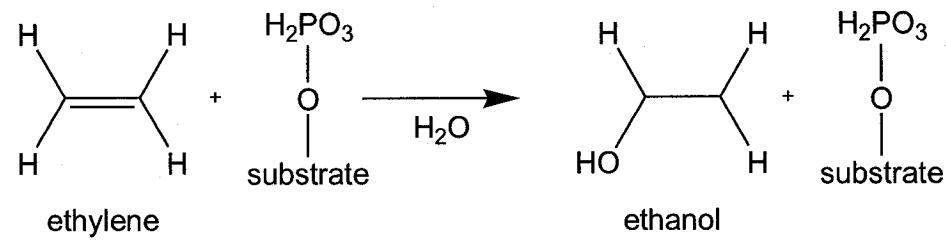

Figure 2.1. Hydration reaction of ethylene to ethanol

2.1.2. Microbial fermentation

2.1.2.1. Bacterial

A multitude of bacterial species are capable of producing ethanol, mainly from sugar substrates. However, several species generate other end products (higher alcohols, organic acids, ketones, gases etc.) thus

negatively affecting the yield of ethanol. According to Wiegel, (95), a molar conversion ratio, from glucose to ethanol, of 1.0 is minimal to make the fermentation economically appealing. From the variety of bacterial species investigated, *Clostridium sporogenes, C. indolis, Leuconostoc mesenterioids, Streptococcus lactis and Zymomonas mobilis* are amongst the most promising ones.

Z. mobilis has attracted the most attention since it can be considered a strict ethanol producer. Moreover, it gives a very high product concentration with no noticeable inhibition (60,73). Although glucose is the substrate of choice for most strains, other carbon sources, such as sucrose and fructose can be utilized by the bacteria, but will favor the production of organic acids over ethanol. This issue raises an important industrial limitation where mixed sugar substrates are used, such as with molasses, cereal mashes and cellulosic material.

Other more recent attempts have been made in metabolic engineering to introduce bacterial strains that would be capable of performing effective fermentation of cellulosic material, in which xylose and other pentoses are present in considerable amounts (21). Among them, the Gram-negative bacterium *Escherichia coli* exhibits interesting characteristics: it is able to ferment a wide variety of sugar, does not require complex growth factors and has been extensively used in other industrial processes.

The metabolic hurdle present in *E. coli* is that it is heterofermentative: it produces both ethanol and organic acids during anaerobic fermentation. Effectively, the bacteria produce ethanol via pyruvate using pyruvate formate lyase which is an unbalanced pathway: only one NADH is produced from the production of pyruvate from sugars whereas two nicotinamide adenine dinucleotide (NADH) molecules are regenerated back to NAD^+ when pyruvate is converted into ethanol. Consequently, the lack of NADH is counterbalanced by the production of organic acids. On the other hand, homofermentative bacteria such as *Z. mobilis* transform pyruvate to ethanol via a pyruvate decarboxylase (PDC), which only consumes one NADH molecule. The second obstacle resides in the strong alcohol dehydrogenase (ADH) activity in *E. coli*, which oxidises ethanol to acetaldehyde. Ingram *et al.* (42) developed a strain co-expressing *pdc* and *adh II* (favouring the reduction of acetaldehyde to ethanol) from a plasmid, and achieved the production of almost solely ethanol, but noticed the poor resistance of the transformant to harsh fermenting conditions. However, other authors (23,27) have notice a weak genetic stability of such plasmids when the strain was used in continuous cultures.

A more recent attempt successfully engineered the bacteria of interest to yield ethanol at around 90 % of the theoretical maximum within 60 h. The authors used an *E. coli* strain (FMJ39x) lacking lactate dehydrogenase (*ldhA*) and pyruvate formate lyase (*pfl*) activity, thus

making it incapable of growing anaerobically. Consequently, it is unable to fermentatively reduce pyruvate and regenerate NAD^+ reduced during glycolysis. By transforming a plasmid, pLOI297 (37,42), containing *Z. mobilis* genes responsible for metabolism of pyruvate to ethanol (*pdc*, pyruvate decarboxylase and *ldh*, lactate dehydrogenase) in FMJ39x, they were able to restore the fermentative metabolism since NAD^+ can be recycled back to NADH when pyruvate is converted to ethanol (24).

2.1.2.2. Yeast-based

For many centuries, humankind has been using yeast to make various fermented products such as bread, wine and beer. Amongst the yeast species, only a few are now of primary industrial relevance: *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*, *Schizosaccharomyces pombe* and *Kluyveromyces lactis.*

Nevertheless, *S. cerevisiae* is the organism of choice in ethanol production. It is able to grow and ferment ethanol at pH values of 3.5-6.0 and temperatures of 28-35 °C It exhibits rapid fermentation and tolerates high ethanol and high initial sugar concentrations (49).

Biochemically, 1 mole of glucose is converted to 2 moles of ethanol, 2 moles of carbon dioxide and 2 moles of adenosine triphosphate (ATP), anaerobically, via the Embden-Meyerhof Parnas (EMP) pathway (Figure 2.2). Therefore, for each gram of glucose, theoretically 0.51 g of ethanol can be produced.

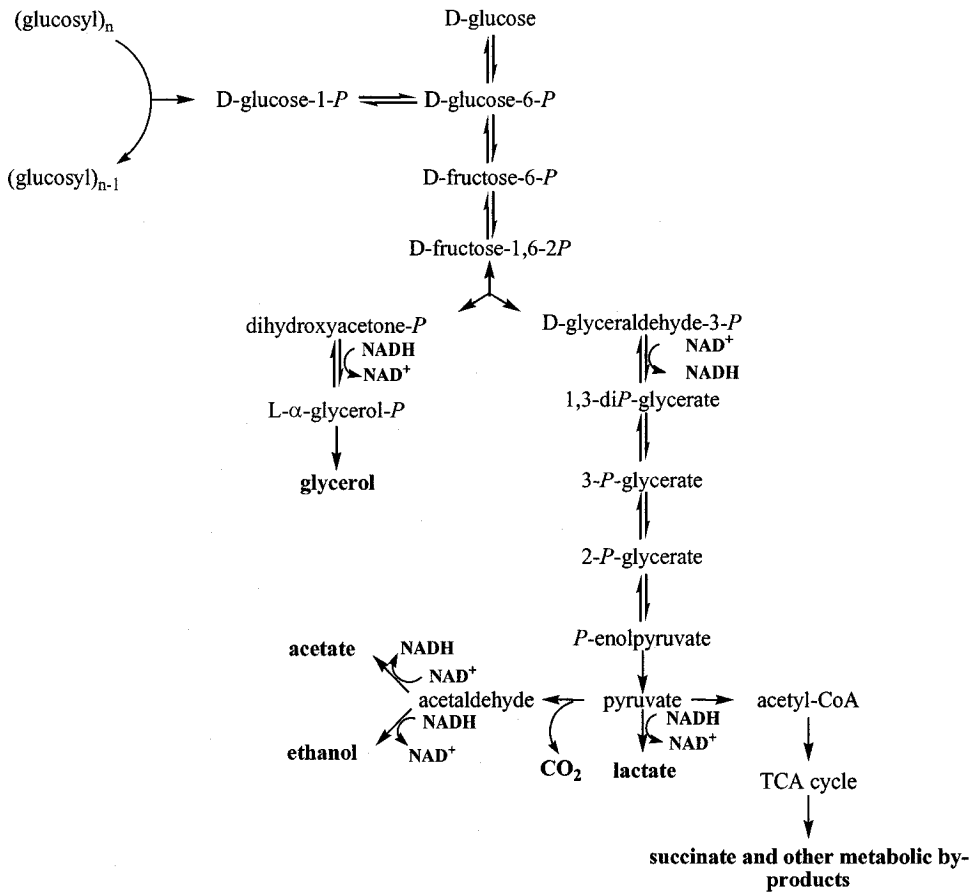

Figure 2.2. EMP pathway. (NAD^+/NADH: nicotinamide adenine dinucleotide; *P*: phosphate; CO_2: carbon dioxide)

Practically, however, yield is about 75 % of the theoretical attainable concentration. This can be explained by side reactions, in which metabolic by-products (glycerol, succinate, acetate) are generated, mainly for cell maintenance purposes. In fact, Oura (67) theorised that if no glycerol or succinate were produced, a yield of ethanol on sugar would increase by 2.7 %, which is significant to any producer of fuel ethanol or distilled beverage.

Current industrial fuel ethanol processes involve the fermentation of starch-based materials, such as corn, wheat and barley and sugar crops,

such as sugar beet and sugarcane. Some ethanol plants also deal with defective syrups, waste beer and whey. All of these substrates constitute inexpensive sources of carbon. Nevertheless degradation of starch, especially with cereals, is essential for proper fermentation since *S. cerevisiae* is not able to hydrolyze it to glucose. Many recent efforts have been made to construct recombinant yeast strains capable of producing starch-degrading enzymes (α-amylase and glucoamylase), that would facilitate the metabolism of starch, and promising results have been demonstrated (28,54,78,88).

Another limitation with *S. cerevisiae* resides in the fact that it does not naturally ferment pentoses, which are greatly prevalent in cellulose-based material. Hence, baker's yeast is not well adapted to ferment this type of substrate to ethanol. Four excellent reviews on microbial cellulose utilisation (43,56,57) and consolidated bioprocessing of cellulosic biomass (58) describe well the challenges face in that area.

Much effort has been made to engineer strains that can excrete heterologous cellulase enzymes (32,33) and ferment the pentoses D-xylose and D-arabinose (15,44,50,92).

2.2. Fermentation process designs

Three types of processes are industrially-relevant for the production of ethanol: batch, fed-batch and continuous processes, and each has its own advantages and disadvantages.

2.2.1. Batch fermentation

Currently, the majority of the fuel ethanol produced worldwide is derived from batch processes. In this method, which was developed by the alcoholic beverage industry (beer, wine, and spirits), the substrate is inoculated with yeast and allowed to ferment in a bioreactor until exhaustion of fermentable sugars occurs. In this way, the process is easily controlled, the investment costs are low and the risks of contamination by spoilage organisms are minimised.

This process, however, requires downtimes (10-20 % of the utilisation time) of the bioreactor for cleaning and sterilisation, decreasing productivity. Moreover, yeast cells undergo a lag phase at the beginning of the fermentation, and their action is further inhibited by high ethanol concentrations at the end of the process. A plant using a batch process will require a propagation facility in order to prepare the inoculum for each batch, which necessitates skilled labour to maintain pure cultures and avoid possible entrance of contaminants.

A batch process for ethanol fermentation is desired for small plants which engages small capital investments. Staggered operations with multiple fermenters definitely improve the throughput. Also, reutilisation of the biomass by cell recycling will likely reduce the lag phase of the next batch by providing a large quantity of inoculum.

2.2.2. Fed-batch fermentation

Fed-batch fermentation systems are mainly design in order to limit the inhibitory effect of high substrate, or product concentration. It is typically started as a batch with a volume considerably smaller than the maximum working volume of the bioreactor. Once adequate inoculum density is reached, a feeding strategy is initiated.

Many different feeding techniques can be employed. Simplistically, one can feed nutrients at a constant rate throughout the process until full volume is attained. However, an exponential feeding regime that follows the biomass requirements is likely to demonstrate a more effective substrate conversion to product. A third way of feeding the nutrients to the vessel is via a control system. In the case of fuel ethanol production, sugar concentration may be monitored. Whenever the sugar concentration falls below a certain set point, the feeding pump is activated and delivers the required amount of substrate to bring the sugar concentration back up to the set point.

Using a fed-batch fermentation system in the fuel ethanol industry, one can minimise the effect of high substrate inhibition by keeping fermentable sugars concentration below a certain level, which would otherwise causes osmotic stress to the yeast in batch systems.

Nevertheless, two main disadvantages remain: (a) requirement for expensive control equipment in order to obtain optimized feeding strategy

and (b) loss of productivity when fermenters are being emptied, cleaned, sterilised and filled between each batch (49).

Excellent demonstrations of new process development in fuel ethanol production using fed-batch fermentation systems have been described in the following references: (2,3,14,93).

2.2.3. Continuous fermentation

The solution to the downtime disadvantage encountered with batch and fed-batch fermentation systems is to operate the fermentation process continuously (Figure 2.3). This implies that the substrate (S_{in}) is fed continuously at a constant rate (F_{in}) to the fermentation vessel and a second pump, running at the same medium flow rate ($F_{in} = F_{out}$), is removing broth, containing residual substrate (S_{out}), product (P) and biomass (X) in order to keep the internal volume (V) constant (69). In this way, a constant environment, called steady-state, should be theoretically maintained within the bioreactor. Moreover, if appropriate conditions are applied, cells will continuously reproduce and a constant biomass will be sustained.

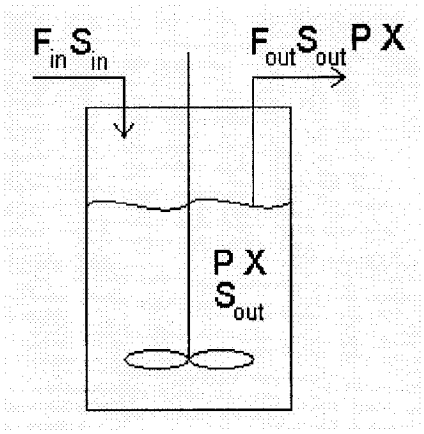

Figure 2.3. Diagram of a single-stage continuous fermentation system. (F: feed flow rate; P: product concentration; S: substrate concentration; X: biomass concentration)

This type of process is largely used in the chemical industry but is not very popular when microbially catalyzed reactions are involved. This can be explained by the fact that most bioprocesses require the utilisation of a pure culture. However, at the industrial level, it is very difficult to keep contaminants out of the fermenters, which will later reduce the volumetric productivity, and possibly cause the failure of the system (see section 2.3.1). Other disadvantages include a requirement for uniform quality of the raw material, a high probability of microorganism mutation over long periods and a need for sterilising the new raw material continuously which is energy consuming.

Despite that, some fuel ethanol plants have been converted to a continuous system for its advantages. Indeed, as clearly illustrated by a study done by Cysewski and Wilke (20), utilisation of a continuous fermentation system over a batch system in a fuel ethanol plant would suggest savings of above 50 % in fixed capital investment. This can be

explained by many advantages gained: (a) long-term continuous productivity, (b) higher volumetric productivity, (c) reduced labour costs and (d) reduced vessel down time for emptying, cleaning, filling and sterilising (9).

2.2.3.1. Multi-stage continuous fermentation

The simplest mode of continuous culture is the single-stage continuous fermentation (SSCF), which involves only one vessel, is operated: product is harvested directly from the outlet of the fermenter (Figure 2.3). Nonetheless, it has been mathematically shown that the residence time required to obtained a certain ethanol concentration using two fermenters of equal volumes was reduced by more than 50 % than when using only one fermenter having the corresponding volume (34,89). Indeed, since product inhibition occurs linearly as ethanol concentration increases above a certain threshold, around 25 gL^{-1}, during the fermentation (39), it is likely that multiple fermenters connected in series, will minimise such effect by presenting different product concentrations in each fermenter (26).

In the specific case of fuel ethanol production, sacrifices have to be made: in order to achieve an adequate yeast cell concentration, large amounts of oxygen must be supplied for aerobic growth. This likely results in a drop in the ethanol yield per substrate since more carbon is directed towards biomass production instead of being utilised for ethanol formation.

Consequently, to overcome such limitation, the use of a multi-stage continuous fermentation (MSCF) system (Figure 2.4) would permit the ethanol producer to dedicate an initial vessel for biomass production where oxygen is supplied. Then, the following tank(s) will be used for the anaerobic conversion of the sugars into ethanol.

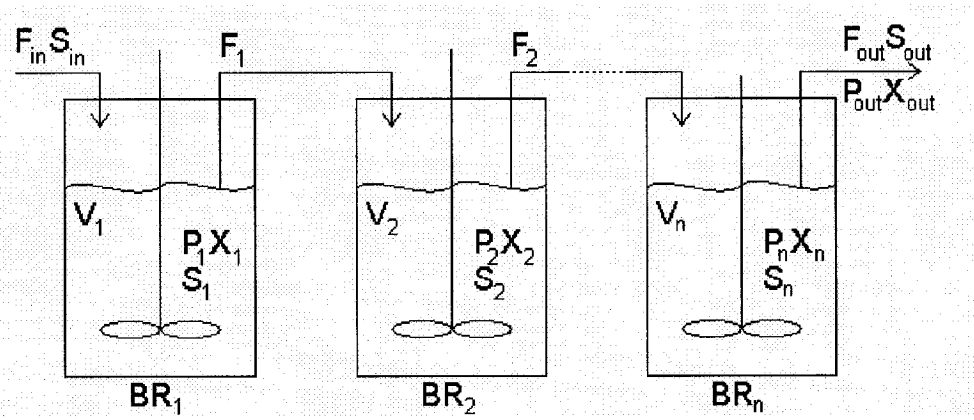

Figure 2.4. Diagram of a multi-stage continuous fermentation system. (BR_n: bioreactor number n; F_n: feed flow rate from vessel; P_n: product concentration; S_n: substrate concentration; V_n: volume in vessel; X_n: biomass concentration)

Bayrock and Ingledew (9) demonstrated the feasibility of such system for efficient production of high concentrations of ethanol (132 $g \cdot L^{-1}$) within a reasonable residence time (116 h) in very high gravity (VHG) conditions (312 $g \cdot L^{-1}$ glucose). Moreover, in a second study, the same group compared the productivity of a SSCF system to a MSCF system (11) for fuel ethanol production in similar VHG conditions. They obtained a considerable increase of over 75 % in the final ethanol concentration using the MSCF system, over the SSCF system, with identical overall residence times.

2.2.3.2. Cell recycling

Cell recycling is being used for ages in the brewing industry. Once fermentation of a first batch is terminated, yeast is recuperated and reused as inoculum for the following batch. This enables the increase in initial biomass concentration in order to limit the consumption of substrate for its formation during the fermentation, consequently improving the yield of ethanol.

To increase the biomass concentration in a fuel ethanol MSCF system and attain very high cell density cultures, one can use a cell separation device, such as a centrifuge or a membrane. The supernatant, or the permeate, is sent to the distillation columns while the precipitate, or the retentate, is pumped back in to the system, normally at the first stage of the train (Figure 2.5).

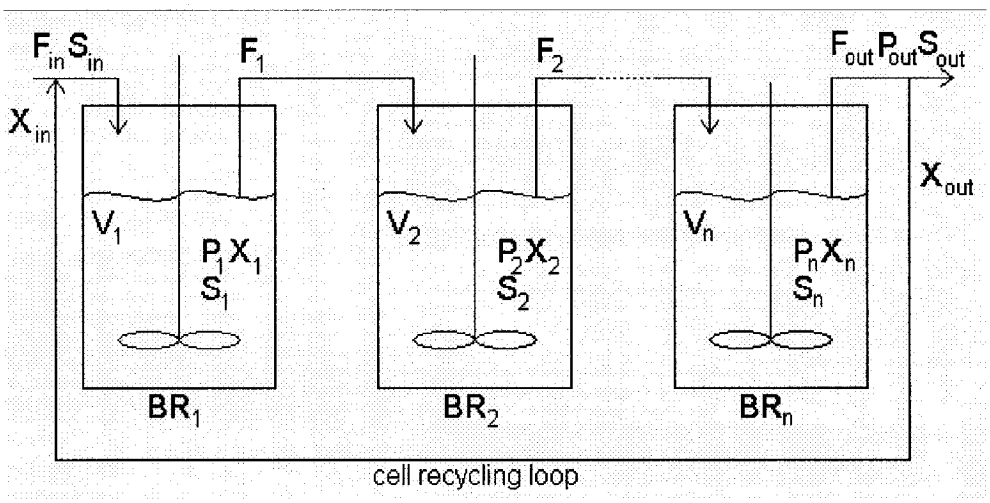

Figure 2.5. Diagram of a cell recycling multi-stage continuous fermentation system. (BR_n: bioreactor number n; F_n: feed flow rate from vessel; P_n: product concentration; S_n: substrate concentration; V_n: volume in vessel; X_n: biomass concentration)

As an example, in a typical two-stage system with cell recycle using an ultrafiltration apparatus, very high volumetric ethanol productivity (40 $g \cdot L^{-1} \cdot h^{-1}$) has been achieved experimentally (13). Recycling the biomass back to the first stage of the MSCF system mitigates the utilisation of the substrate for biomass production. Moreover, cells from the outlet of the system are already naturally conditioned to tolerate high ethanol concentrations and will hence tend to be more robust throughout the process.

2.2.3.3. Pervaporation

Product inhibition is a typical hurdle faced by the fuel ethanol industry: as the ethanol accumulates in the fermentation broth, thus slowing down the volumetric productivity of the yeast at ethanol concentrations reaching above 45 $g \cdot L^{-1}$ (46,86). MSCF system currently allows the reduction of such effect in the early stages of the process but the obstacle remains in the final stages.

Pervaporation is a separation process involving the separation of chemical entities based on their volatility and permeability through a membrane (31,91). This is accomplished by submitting the permeate side of the membrane to a vacuum to evaporate the compounds of interest (Figure 2.6).

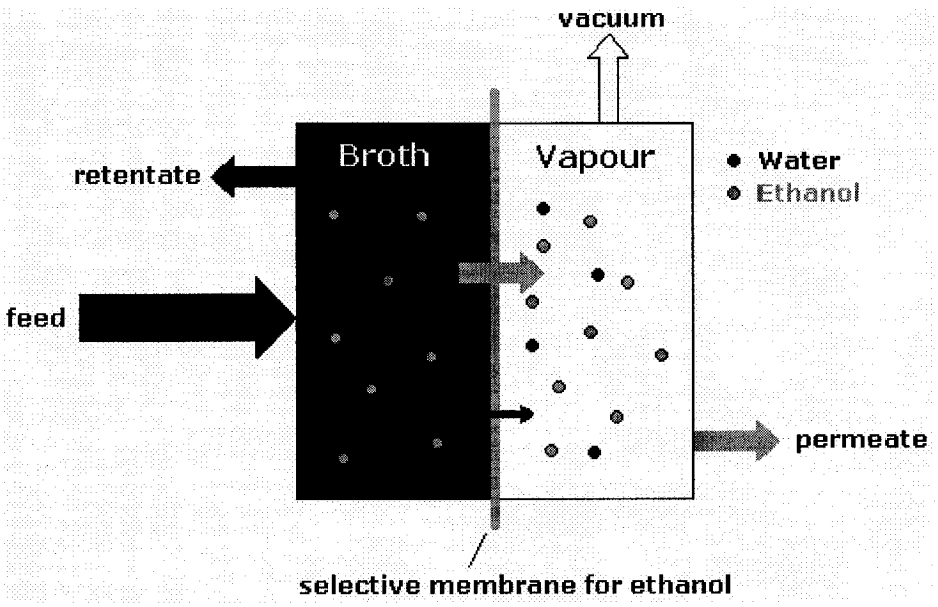

Figure 2.6. Schematic diagram of a pervaporation process (adapted from (91))

In the case of ethanol in a fermenting broth, pervaporation can be used at low-temperature (fermentation temperature), low-pressure system using an organophilic membrane (*e.g.* polydimethylsiloxane), and because of the nature of the vapour-liquid equilibrium of ethanol-water mix, the process is likely to have a built-in selectivity for ethanol at concentration ranges typically found in fuel ethanol plants (5-15 %w/v) (65).

Costs associated with the technology makes it still prohibitive as compared to well-established distillation apparatuses. Nevertheless, the advantages of distillation over pervaporation technology for biofuel recovery fade as the scale of the operation is reduced, thereby opening the door for such tool to be implemented in the future (66,91).

Indeed, smaller working volume fermenters could be used with pervaporation without affecting the productivity. O'Brien and Craig Jr. (65)

have actually demonstrated that such technology could lead to above five-fold increase in ethanol volumetric productivity by maintaining the product concentration in the fermentation broth between 4.5 $g \cdot L^{-1}$ and 6.5 $g \cdot L^{-1}$ as compared to performances observed in a traditional MSCF system.

2.3. Current challenges in the industry

Many challenges are faced by the fuel ethanol industry these days. Indeed, a reduction in the production cost of fuel ethanol will enables ethanol to remain competitive with regular gasoline, and thus creating a viable green fuel that will be locally produced, and that will not be totally dependant on supplies from foreign countries where the political instability of their governments make them a source at risk.

Of the current hurdles, two opportunities are considered to be major and need special attention: the bacterial contamination of fermentation broth and the yield of ethanol over substrate.

2.3.1. Bacterial contamination

One of the major problems in continuous ethanol production is the prevalence of bacterial contamination by lactic acid bacteria (LAB). Typically, 60 % of the contaminants are from the *Lactobacilli* genus, mainly *Lactobacillus delbrueckii* subsp. *delbrueckii*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *L. brevis*. Other LAB found in fermenting broth include *Pediococcus* sp. and *Leuconostoc* sp. (80). Indigenous bacteria contaminating the fermentation process by *Saccharomyces*

cerevisiae compete aggressively for trace nutrients and therefore reduce the ethanol yield by affecting the viability of the main organism (10). Moreover, lactic acid and acetic acid, the two main products from the fermentation of glucose by LAB, are observed to have an inhibitory to the growth of *S. cerevisiae* at levels corresponding to 2.5 %w/v and 0.6 %w/v respectively (59,64).

Currently, expensive antibiotics (virginiamycin, penicillin) are commonly used to control the bacterial invasion in the fermentation broth (41). However, resistance among indigenous species will develop and can create problematic situations which would lead to shutting down the continuous system. Moreover, the utilisation of such antibiotics may cause wastewater problems (issue that is presently ignored) and can lead to liberation of those toxic chemicals into the environment (45,76), or even worst, in the distiller's dry grains, which will later be consumers by cattle and dairy cows.

Consequently, new research efforts are being made to develop natural antimicrobial agents that would mitigate bacterial contaminations in fuel ethanol fermentations. Of such, hop acids, which are well known for their bittering and antibacterial effect in beer (75,79,82), constitute an interesting avenue (61,74).

2.3.2. Yield improvement by reduction of metabolic by-products

The other interesting challenge faced by the fuel ethanol industry is the yield (Y) of product (P – ethanol) over substrate (S – glucose) ($Y_{P/S}$). As stated previously in section 2.1.2.2, for each mole of glucose, theoretically two moles of ethanol can be theoretically produced, which corresponds to 0.51 g of ethanol per gram of glucose ($Y_{P/S}$ = 0.51 $g \cdot g^{-1}$). However, typical yields in industrial fermentations using a MSCF system range from 0.35 $g \cdot g^{-1}$ to 0.42 $g \cdot g^{-1}$ (personal communications with distillers and fuel ethanol producers). This difference is directly linked to a loss of productivity, which can be translated to a higher requirement for substrate to produce the same amount of ethanol. Considering that substrate accounts for about 75 % of the operating cost, a small reduction in its requirement could result in considerable savings (51,62,85).

The avenue chosen in the current work was to mitigate the production of some metabolic by-products via an attempt to modify the redox balance in the yeast cells.

2.3.2.1. Higher alcohols

The production of higher alcohols is directly linked to amino acid and protein synthesis in yeast (77). The biosynthesis of amino acids results in the production of aldehydes that are decarboxylated by pyruvate decarboxylase (PDC) and further reduced to the corresponding alcohols

(Figure 2.7). The reduction is mediated by a NADH dependent alcohol dehydrogenase (ADH) enzyme (70).

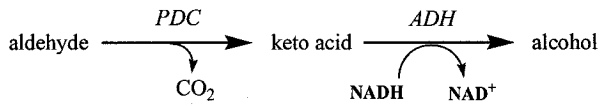

Figure 2.7. Biosynthetic route to alcohols in yeast. (ADH: alcohol dehydrogenase; NAD^+/NADH: nicotinamide adenine dinucleotide; PDC: pyruvate decarboxylase)

Quain and Duffield (70) have demonstrated that production of such higher alcohols was linked to the necessity of the yeast to re-establish its redox balance. Nevertheless, in industrial fermentations involved in fuel ethanol production, concentrations of higher alcohols are negligible and a reduction in their concentration would not be significant enough to display an increase in $Y_{P/S}$ (67).

2.3.2.2. Succinate

Succinate is produced by yeast via two mechanisms, one being oxidative through the TCA cycle and the second being reductive through formation of malate and fumarate as intermediates (Figure 2.9). The latter is proposed as a way to balance excess NADH in the mitochondria to obtain an overall redox neutral state in the cell (18,67). This action should be directly correlated with glycerol formation (see 2.3.2.3). Nonetheless, as stated by Oura (67), it is improbable that succinate is formed through the reductive pathway during fermentation, but will likely occur to a small extent growth under anaerobic conditions is observed.

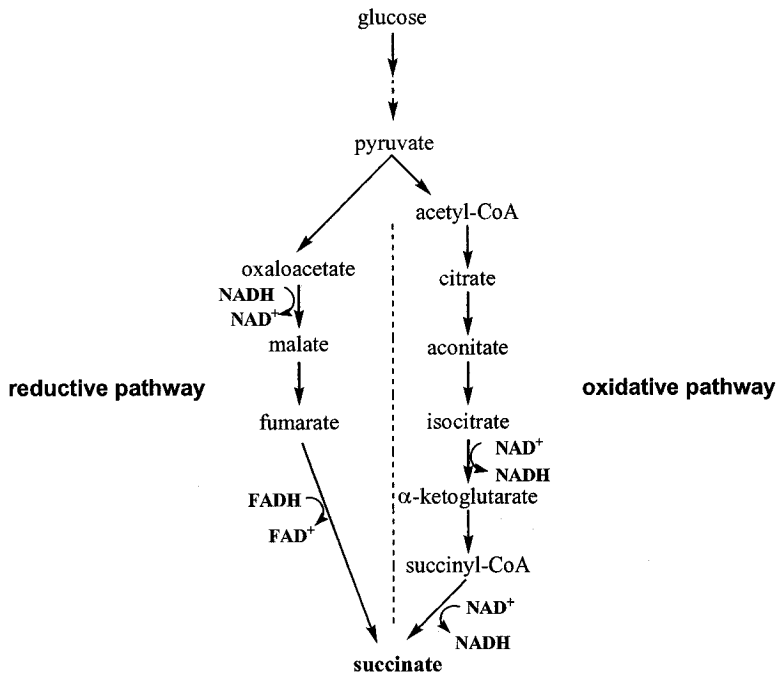

Figure 2.8. Metabolic pathways involved in succinate production. (FAD^+/FADH: flavin adenine dinucleotide; NAD^+/NADH: nicotinamide adenine dinucleotide)

2.3.2.3. Glycerol

Glycerol production in yeast metabolism is involved in two functions: osmoregulation and maintenance of the redox balance of the cell (4,5,14,19,72). It is associated with two cellular processes: growth and ethanol production. During cell growth, a slight excess of NADH is liberated and needs to be reoxidised to NAD^+ to keep vital functions active (52). Formation of glycerol is thus activated via the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate, which is later transformed to glycerol, to replenish the pool of NAD^+ (Figure 2.9).

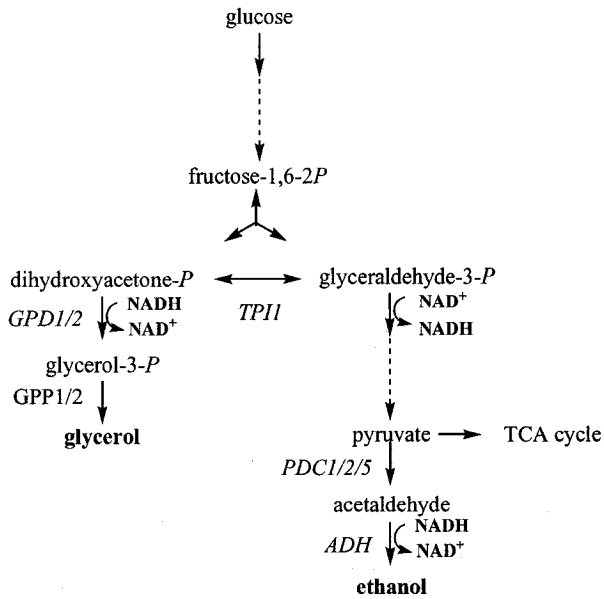

Figure 2.9. Metabolic pathways involved in glycerol production. (ADH: alcohol dehydrogenase; GDP1/2: glycerol-3-phosphate dehydrogenase isoforms 1 and 2; GPP1/2: glycerol-3-phosphatase isoforms 1 and 2; PDC1/2/5: pyruvate decarboxylase isoforms 1,2 and 5; TPI1: triose phosphate isomerase; NAD^+/NADH: nicotinamide adenine dinucleotide)

On the other hand, ethanol production by *S. cerevisiae* is considered redox neutral (equal amounts of NAD^+ and NADH are reduced/oxidised). However, acetaldehyde may be released from the cell prior to its reduction to ethanol. Consequently, the step involving alcohol dehydrogenase, and oxidizing NADH, does not occur and redox imbalance is observed (67). Second, if inappropriate supply of amino acids is provided, *de novo* synthesis will be required (from glucose and ammonia) which will result in a surplus of NADH. In order to counterbalance such problematic in fuel ethanol industrial fermentations the yeast will generate glycerol which will lead to the reoxidation of NADH to NAD^+ (1).

Moreover, under anaerobic conditions, glycerol formation has been proven to be strictly required as a redox balancing for excess NADH (5,53). In fuel ethanol production, such conditions are prevalent and as a result, carbon from substrate is redirected partly away from ethanol formation hence lowering the product yield (1,15,19,90). Aeration strategies have therefore been investigated in order to supply the culture with proper oxygen for both aerobic and anaerobic metabolism (2). Grosz and Stephanopoulos (2,35,36) studied the effect of micro-aeration by varying the concentration of oxygen in the sparging gas. Using a defined feeding medium containing around 100 $g \cdot L^{-1}$ of glucose and changing the oxygen concentration in the sparging gas from 0.0 % to 1.7 % in a chemostat running at a dilution rate of 0.2 h^{-1}, they discovered that only a narrow range of oxygen supply would enable important improvements. They were actually able to stimulate by 50 % the specific ethanol productivity (ν_{etoh}) and specific glucose uptake rate (ν_{glu}) (yield of product/substrate per unit of biomass present per hour), while significantly reducing the specific glycerol productivity (ν_{gly}).

In another study, Alfenore et al. (2) designed a high ethanol performance (147 $g \cdot L^{-1}$ in 47 h) fed-batch fermentation system and evaluated the effect of aeration on glycerol production. They demonstrated that a full aeration strategy (0.2 vvm) led to a 23 % increase in cell viability and three-fold reduction in glycerol (from 12 $g \cdot L^{-1}$ to 4 $g \cdot L^{-1}$) as compared to a micro-aeration system (headspace of fermenter flushed with air).

Nevertheless, such approach in a continuous fermentation system has not been investigated. The following research project examined this avenue in a more industrially relevant process (MSCF system).

3. EXPERIMENTAL SETUP

3.1. Defined fermentation media

For the experiments of this project, the same media was used, and was named the defined fermentation media (DFM). It has been created from controlled quantities of defined ingredients which would avoid any nutrient limitations (40) and would best replicate the composition of a typical corn mash used in the fuel ethanol industry. Unless specified, the composition of the DFM was as described in Table 3.1.

For the continuous systems runs, batches of 9 L of the DFM were prepared: 2700 g of glucose was partly dissolved in 6650 mL of deionised water in a 10 L carboy. The solution was sterilised for 2 h at 121 °C/15 psi. Separately, a 250 mL nitrogen solution containing 77.04 g of $(NH_4)_2SO_4$ and 24.75 g of $(NH_4)_2HPO_4$ was sterilised for 30 min at 121 °C/15 psi.

All the other nutrients were prepared as concentrated solutions and aliquots were dispensed in the medium batches following an overnight cool down following autoclaving. The phosphate salts were dissolved together to 1000 mL at a hundred fold concentrated solution in deionised water (used 10 mL per litre of DFM). 32.0 g of $CaCl_2 \cdot 2H_2O$ was dissolved alone to 1000 mL in deionised water (used 10 mL per litre of DFM).

Table 3.1. Chemical composition of the defined fermentation media

Compound	Concentration
Carbon [1]	
Glucose	300.0
Nitrogen [1]	
$(NH_4)_2SO_4$	8.56
$(NH_4)_2HPO_4$	2.75
Phosphate [1]	
NaH_2PO_4	0.47
Na_2HPO_4	1.64
Minerals [1]	
KCl	0.67
$MgSO_4 \cdot 7H2O$	0.35
$CaCl_2 \cdot 2H_2O$	0.32
Trace minerals [2]	
$FeCl_3 \cdot 6H_2O$	11.18
$MnSO_4 \cdot H_2O$	8.15
$ZnSO_4 \cdot 7H_2O$	7.00
$CuSO_4 \cdot 5H_2O$	1.81
Vitamins [2]	
myo-Inositol	46.60
Calcium pantothenate	23.30
Thiamine hydrochloride	4.66
Pyridoxine hydrochloride	1.16
Biotin	0.023

[1] in $g \cdot L^{-1}$
[2] in $mg \cdot L^{-1}$

The two other main mineral salts were dissolved together at a hundred fold concentration to 1000 mL in deionised water (used 10 mL per litre of DFM). A trace minerals solution was prepared by dissolving all the trace minerals together to 250 mL in deionised water, at a thousand fold concentration (used 1 mL per litre of DFM). All the vitamins, except biotin were prepared the same way, but in 100 mL of deionised water (used 1 mL per litre of DFM). Biotin solution was made up by dissolving 23 mg of it in 100 mL of deionised water (used 100 µL per litre of DFM).

All the solutions were autoclaved for 30 min at 121 °C/15 psi except the two vitamin solutions that were filter sterilised with 0.22 µm syringe filters (Fisher Scientific, Ottawa, ON, Canada).

3.2. Strain selection

It was necessary to select an appropriate *Saccharomyces cerevisiae* strain suitable for VHG fermentation. The National Center for Agricultural Utilization Research (Peoria, IL, USA) was contacted in order to obtain yeast strains from their Agricultural Research Services Culture Collection (United States Department of Agriculture). Six strains were kindly provided: NRRL Y-268, NRRL Y-567, NRRL Y-634, NRRL Y-635, , NRRL Y-637 and NRRL Y-978. They were shipped in lyophilised form in individual glass ampoules.

Upon receipt of the material, dry yeasts were revived according to the provided instructions. First, each strain was suspended in 10mL of sterile YM broth (10 $g \cdot L^{-1}$ glucose, 5 $g \cdot L^{-1}$ peptone, 3 $g \cdot L^{-1}$ malt extract and 3 $g \cdot L^{-1}$ yeast extract). Growth then took place for 48 h at 30 °C in a Forma orbital shaker (Thermo Electron, Marietta, OH, USA), with agitation set at 150 rpm. Following this, 100 µL of culture was aseptically spread on plates of YPD agar (20 $g \cdot L^{-1}$ glucose, 20 $g \cdot L^{-1}$ peptone, 10 $g \cdot L^{-1}$ yeast extract and 20 $g \cdot L^{-1}$ agar). The plates were incubated another 48 h at 30 °C. Then, a single colony from the agar plate was transferred to a flask containing 100 mL of sterile DFM having 120.0 $g \cdot L^{-1}$ glucose. The yeast was allowed to grow for 48 h at 30 °C under slight agitation (150 rpm) provided by an

orbital shaker. An assessment of the viable cells concentration was then performed according to procedure describes in section 3.6.4.

Adequate volume to inoculate 1.0×10^6 viable cells per millilitre was transferred to a new flask containing 90 mL of sterile DFM having 133.3 $g\cdot L^{-1}$. Sterile deionised water was added to complete the volume to 100 mL and adjust the glucose concentration to 120.0 $g\cdot L^{-1}$. Fermentation then took place at 30 °C, with 150 rpm of agitation in an orbital shaker. Ethanol was determined by gas chromatography (GC) according to the procedure described in section 3.6.1. Selection of the strain was based on the fermentation rate (ethanol produced over time). NRRL Y-634 was the candidate that presented the fastest production of high concentrations of ethanol and was chosen for the further experiments.

3.2.1. Conservation of the yeast strains

In order to conserve each strain, a single colony was picked from their respective YPD plate and used to inoculate 10 mL of YM broth. Growth then took place for 24 h at 30 °C in an orbital shaker with agitation set at 150 rpm. A 500 µL aliquot was later transfer to a sterile 1.8 mL centrifuge tube and 500 µL of sterile 40 %v/v glycerol was added as cryoprotectant (55). The tube was then flash frozen in liquid nitrogen prior to storage at –80 °C.

3.3. Description of the bioreactors

In order to perform the fermentations, 3.7 L KLF2000 Bioengineering bioreactors (Wald, Switzerland) were used. Temperature was tightly controlled using the Bioengineering console and its temperature controller module that could heat or cool the fermenters as necessary, based on output from an on-line thermistor. Fluctuations in temperature were never observed above 0.1 °C. Agitation was maintained at 125 rpm to prevent short-circuiting of the continuous inlet and outlet flows and to keep the yeast in suspension.

The pH was measured in the fermenters with an on-line gel-type InPro3030 pH probe (Mettler Toledo GmbH, Urdorf, Switzerland) that was calibrated with two points prior to sterilisation of the bioreactor. The bioreactor console was equipped with a proportional-integral-derivative (PID) controller that enabled the control of pH by activating pumps delivering whether acid or base.

Oxygen was supplied via a compressed air supply line. A gas flowmeter (Aalborg Instruments and Controls, Orangeburg, NY, USA) was connected inline in order to determine precisely the flow of air going in the fermenter. The air was filter sterilised prior to enter the sparging tube using an 25 µm Hepa filter (Fisher Scientific, Ottawa, ON, Canada) . The latter was equipped with a porous stainless steel end that was acting as a diffusing stone. The dissolved oxygen (dO_2) was monitored using an InPro6800 dO_2 probe (Mettler Toledo GmbH, Urdorf, Switzerland) that was

calibrated with two points (0% and 100% saturation) following sterilisation. The probe was connected to the KLF2000 console in order to provide the digital reading, and a PID controller was linked to a solenoid valve in order to set the dissolved oxygen at a certain level, if required.

A water-jacketed condenser was installed at the exhaust of the fermenters in order to limit the loss in volatile compounds, especially ethanol, which would have likely happen due to gas stripping (83,84).

3.4. Single-stage continuous fermentation system setup

The first set of experiments was conducted in a SSCF system which was mimicking the first stage of a MSCF system typically found in the fuel ethanol industry. Figure 3.1 illustrates the process design.

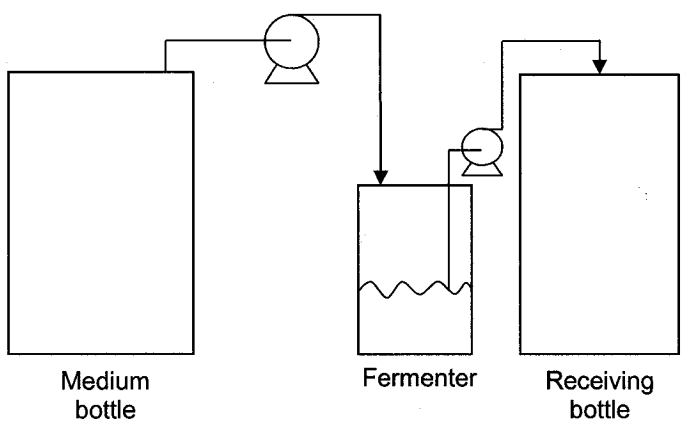

Figure 3.1. Schematic of the single-stage continuous fermentation system

An Ismatech multi-channel variable speed peristaltic pump (Cole-Parmer, Anjou, QC, Canada) and 0.12 in. ID peroxide-cured silicone tubing (Cole-Parmer, Anjou, QC, Canada) were used for obtaining the

appropriate inlet flow of DFM. The outlet pump was a Bioengineering fixed speed pump (Wald, Switzerland) in order to maintain a constant volume in the fermenter based on the placement of the draw-off tube at a specific height.

The working volume in the fermenter was 1.2 L. With a DFM flow rate of 25 $mL \cdot h^{-1}$, a dilution rate of 0.021 h^{-1} was obtained; with a total residence time in the system of 48 h. Temperature was controlled at 30 °C. The pH was controlled at 4.50 (±0.10) by aseptically pumping 1.0 M sodium hydroxide (NaOH) whenever pH was dropping. The bioreactor console acted as the controller. A strict P-band of 0.5 was used. The volume of NaOH solution delivered was too small to make a difference in the dilution rate.

3.4.1. System start-up

Initially, a volume of water was sterilised inside the fermenter for 15 min at 121 °C/15 psi and sterile DFM was then gradually fed in. At the same time, the inoculum was injected into the sterile fermenter and the feed rate of DFM was allowed to increase gradually, up to the operating rate after 48 h. This permitted the yeast to acclimatise with its new environment (12), and avoid additional osmotic stress caused by excessive sugar concentration.

From that time on, measurements for biomass concentration, glucose, glycerol and ethanol, as well as succinate, lactate and acetate were monitored on a daily basis, for at least 21 days.

3.4.2. Aeration strategies

Two different aeration strategies were tested in the SSCF system in order to evaluate the effect of oxygen on the production of metabolic by-products occurring during the growth of the yeast, which is mostly happening in the first stage of a MSCF system.

Anaerobic conditions were initially tried: no oxygen was supplied at all. Dissolved oxygen (dO_2) was maintained at 0.0 % at all time. The second aeration strategy involved supplying 60 $mL \cdot min^{-1}$ of air (0.05 vvm), which corresponds to approximately 15 mg of oxygen delivered per minute. Such aeration was considered micro-aerobic since yeast was subjected to limiting oxygenation, as determined by a dissolved oxygen measurement of zero.

3.5. Multi-stage continuous fermentation system setup

A typical MSCF system found in the fuel ethanol industry was reduced to the bench scale level. The particularity to it was that the feed of substrate was split in two between the first (F1) and the second (F2) fermenter. The following figure illustrates schematically the setup:

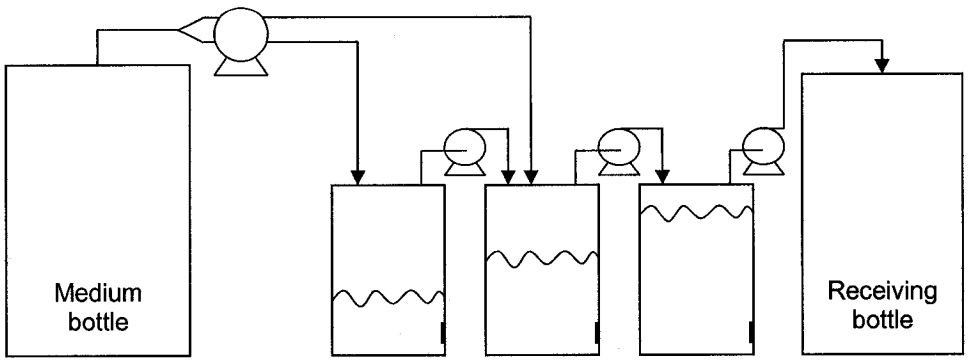

Figure 3.2. Schematic of the multi-stage continuous fermentation system

Peristaltic pumps and 0.12 in. ID peroxide-cured silicone tubing (Cole-Parmer, Anjou, QC, Canada) were used for obtaining the appropriate inlet and outlet flow rates. The main feed to F1 and F2 was provided by a MasterFlex L/S variable speed pump (Cole-Parmer, Anjou, QC, Canada) while the outlet pumps from F1, F2 and F3 were fixed speed pumps (Bioengineering, Wald, Switzerland) and maintained a constant volume in each fermenter based on the placement of the draw-off tube at a specific height.

The working volumes for F1, F2 and F3 were respectively 1.2 L, 2.5 L and 2.5 L. With a DFM flow rate of 50 $mL \cdot h^{-1}$ to F1, and 100 $mL \cdot h^{-1}$ to F2 and F3, a dilution rate of 0.04 h^{-1} was obtained, with a total residence time in the system of 60 h (20 h per fermenter). Temperature was controlled at 30 °C in F1, and 32 °C in F2 and F3. pH was controlled at 4.50 (±0.10) in F1 by aseptically pumping 1.0 M NaOH whenever pH was dropping. The bioreactor console acted as the controller. A strict P-

band of 0.5 was used. The volume of NaOH solution delivered was too small to make a difference in the dilution rate.

3.5.1. System start-up

Prior to inoculating the first fermenter (F1), its working volume was set to 1.2 L. Initially, a volume of water was sterilized inside the fermenter and DFM was then gradually fed in. At the same time, the inoculum was injected into the sterile fermenter and the feed rate of DFM was allowed to increase gradually, up to the operating rate after 48h.

From that time, measurements for biomass concentration, glucose, glycerol and ethanol, as well as succinate, lactate and acetate were monitored on a daily basis, and after having reached steady state and maintained it confidently, a second fermenter (F2) was connected in series/parallel to F1.

Since DFM was fed to both F1 and F2, a strategy had to be formulated to make sure that both fermenters were supplied at the exact same rate. Different setups were tried and the use of a Y to split the feed before the pump head into two lines resulted in the best reproducibility and stability in the rate. Finally, after steady-state was reached in F2, the third fermenter (F3) was connected to the sequence.

Each time a new fermenter was added to the system, it was previously sterilized with a volume of water inside. Then, water was pumped out and fermenting medium (including yeast) was allowed to fill the new fermenter at the system's normal feeding rate.

3.5.2. Aeration strategies used

Three different aeration strategies were experimented on the MSCF system in order to evaluate the effect of oxygen on the production of metabolic by-products in an industrially relevant process. Continuous fermentations were operated until completing of height changes in working volume (480 h, or 20 days) following steady-state establishment, as determined by similar glucose concentrations after three consecutive days. Then, the reactors were shut down, emptied, cleaned up and the system was started-up again with different operating conditions. First, a complete anaerobic fermentation, with no oxygen supply, was performed. This oxygenation state is typically found in the fuel ethanol industry where MSCF systems are used since the oxygen supply is negligible as compared to the fermentation volume (personal communications).

The second experiment conducted involved saturating the fermenting medium with oxygen in the first stage (F1) of the system. This was verified by keeping the dO_2 at 100 % at all time.

In a third attempt to minimize the formation of metabolic by-products, especially glycerol, in a laboratory scale fuel ethanol MSCF system, micro-aerobic conditions in F1 were used. In that case, oxygen was maintained at only 5 % of its saturation (meaning that sufficient oxygen was supplied for yeast metabolism, but without excess), by using the dO_2 PID controller linked to the console of the bioreactor. This device was managing the opening of a solenoid valve which would allow, upon

opening, air to go through. Shutting of the valve resulted in no air supply. Thus, pulse addition of oxygen in order to maintain 5 % saturation was carried out.

3.6. Analytical methods used

In order to assess the performance of the fermentation systems experienced over the course of the current project, several parameters were evaluated. The procedure used to analyse them is described below.

3.6.1. Ethanol and other volatiles by gas chromatography

Ethanol and other higher alcohols of interest were determined by gas chromatographic methods previously developed (16,29,68) and were further adapted to the system available. The samples were first filtered through a 0.45 µm filter and then supplemented with propionic acid as an internal standard prior to injection.

A HP 5980 Series II gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a HP-Innowax 25 m × 0.25 mm (0.25 µm) column (Agilent Technologies, Santa Clara, CA, USA) or a Zebron ZB-WAX 25 m × 0.25 mm (0.25 µm) column (Phenomenex, Torrance, CA, USA) was used.

3.6.2. Glucose, glycerol and ethanol by high performance liquid chromatography

Glucose, glycerol and ethanol were determined by high performance liquid chromatography (HPLC) using an Agilent 1110 HPLC system (Agilent Technologies, Santa Clara, CA, USA). The elution was performed using a 300 mm × 7.8 mm (8 μ) Resex-ROA column (Phenomenex, Torrance, CA, USA). The mobile phase utilised was 5 mM H_2SO_4 that was pumped isocratically at a flowrate of 0.60 $mL \cdot min^{-1}$. The column was heated at 60 °C and the detection of the compounds was made by a refractive index detector. Quantification was carried out using a standard curve built with standards within the range of analysis (0.1 $g \cdot L^{-1}$ to 10 $g \cdot L^{-1}$). Duplicate samples diluted ten-folds were injected and variability was maintained below 5 %.

3.6.3. Succinate, lactate and acetate by high performance liquid chromatography

Simultaneously to the analysis of glucose, glycerol and ethanol (see section 3.6.2), succinate, lactate and acetate were eluted using the same column, but detected and quantified using a variable wavelength detector set at 210 nm. Quantification was carried out using a standard curve built with standards within the range of analysis (0.1 $g \cdot L^{-1}$ to 10 $g \cdot L^{-1}$). Duplicate samples diluted ten-folds were injected and variability was maintained below 5 %.

3.6.4. Cell count and viability determination

The cell concentration was determined by a direct microscopic cell count of a diluted sample using a haemocytometer. A twenty-fold dilution was best for samples in all three fermenters. This permitted the enumeration of at least 200 cells, most of the time. Viability was determined using an aqueous solution containing 10 $mg \cdot L^{-1}$ methylene violet 3 RAX (Sigma-Aldrich, St-Louis, MO, USA) and 2.0 $g \cdot L^{-1}$ sodium citrate. Mixing of one part of cell suspension with one part of citrate-buffered methylene violet enabled the proper staining of the cells. Non-viable cells were identified as being stained purple while viable one stayed colourless (81).

3.6.5. Biomass

Biomass was also quantified by of dry weight. A 40.0 mL sample was withdraw from the fermenter and centrifuged for 10 min to pelletise the yeast. Supernatant was removed and kept for further analysis, and the pellet was washed twice with 35 mL of deionised water. Finally, the pellet was re-suspended in about 5 mL of deionised water, transferred to a pre-weighted aluminium drying dish (Fisher Scientific, Ottawa, ON, Canada) and dried overnight in an oven at 105 °C. The dry weight was calculated as follow:

Equation 1. Determination of the dry mass of a cell culture

$$dry\ weight\ (g \cdot L^{-1}) = \frac{\text{mass of cell suspension} - \text{mass of dried cells}}{\text{volume of sample}}$$

3.6.6. Yield calculations

In order to assess the performances of the continuous fermentation systems, fermentations yields were calculated based on the following equations:

Equation 2. Yield of biomass over glucose

$$Y_{X/S} = \frac{\text{number of viable yeast cells present}}{\text{mass of glucose consumed}}$$

Equation 3. Yield of ethanol over glucose

$$Y_{etoh/S} = \frac{\text{mass of ethanol produced}}{\text{mass of glucose consumed}}$$

Equation 4. Yield of glycerol over glucose

$$Y_{glyc/S} = \frac{\text{mass of glycerol produced}}{\text{mass of glucose consumed}}$$

Equation 5. Volumetric consumption rate of glucose

$$Q_{gluc} = \frac{\text{mass of glucose consumed}}{\text{residence time in fermenter}}$$

Equation 6. Volumetric productivity of ethanol

$$Q_{etoh} = \frac{\text{mass of ethanol consumed}}{\text{residence time in fermenter}}$$

Equation 7. Volumetric productivity of glycerol

$$Q_{glyc} = \frac{\text{mass of glucose consumed}}{\text{residence time in fermenter}}$$

Equation 8. Specific productivity of glycerol

$$\nu_{glyc} = \frac{\text{mass of glycerol produced}}{\text{number of viable cells present} \times \text{residence time in fermenter}}$$

4. RESULTS

4.1. Strain selection

Ethanol production over time by six distiller's strains during batch fermentation of the DFM containing 120 $g \cdot L^{-1}$ is illustrated in Figure 4.1.

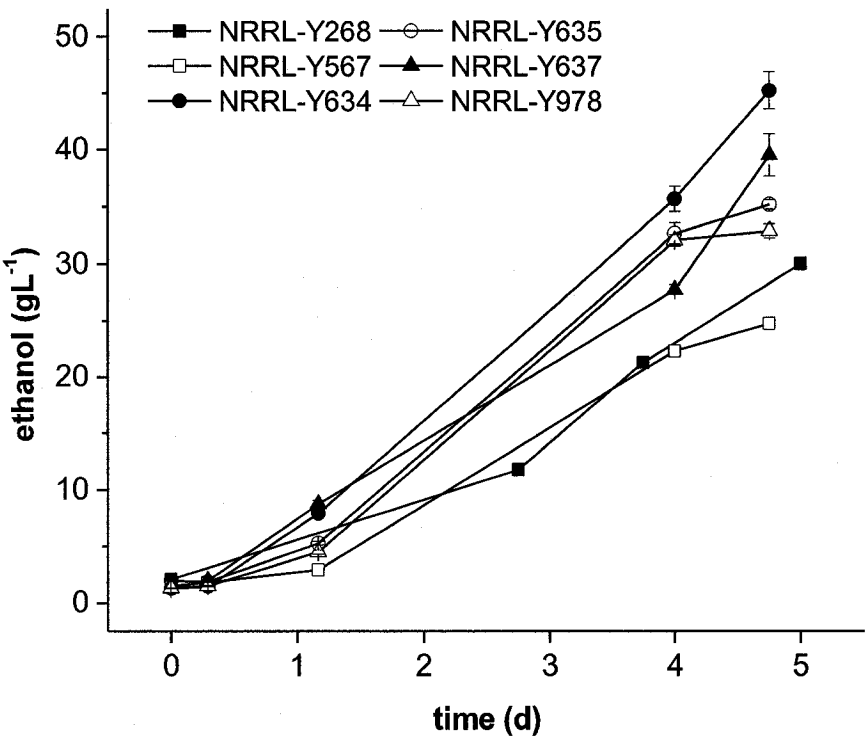

Figure 4.1. Ethanol production in batch fermentation by distiller's yeast strains (n=3)

As clearly shown, the strain NRRL Y-634 produced the highest amount of ethanol (44.2 $g \cdot L^{-1}$) in 114 h, which corresponds to a specific ethanol productivity of 0.385 $g \cdot L^{-1} \cdot h^{-1}$ (Figure 4.2). Hence, since selection criterion was such parameter, NRRL Y-634 was therefore selected to be the distiller's strain to use in the continuous fermentation experiments.

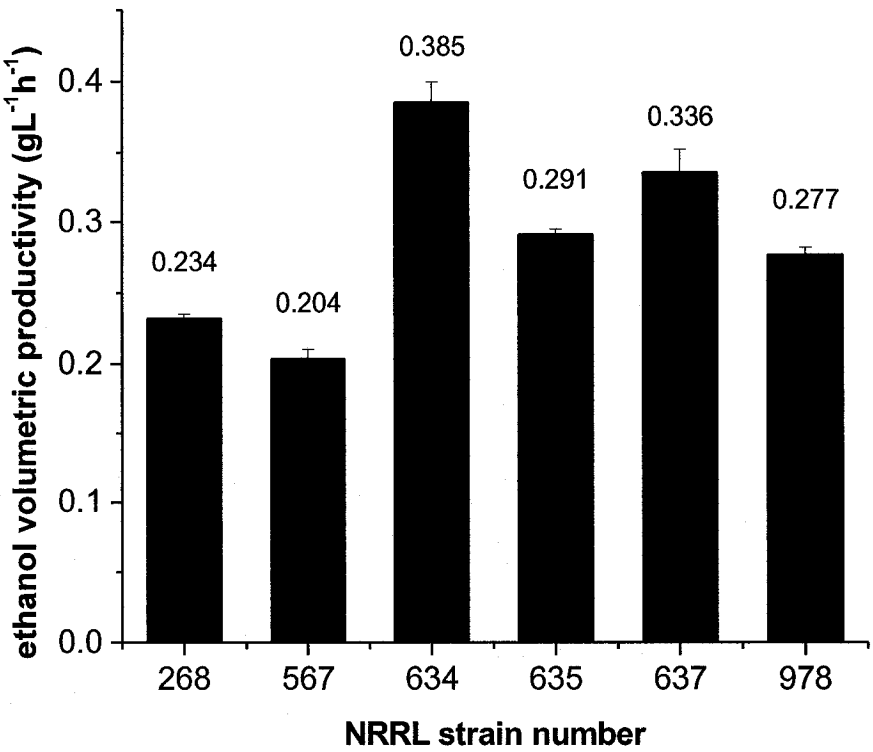

Figure 4.2. Ethanol volumetric productivity on defined fermentation medium (batch fermentation, 120 g·L^{-1} glucose) of six different distiller's yeast strains (n=3)

4.2. Single-stage continuous fermentation results

Two different oxygenation states were experimented in the SSCF system: an anaerobic fermentation (no oxygen supplied) and a micro-aerobic environment (only limited amounts of oxygen were supplied).

4.2.1. Evolution of metabolites over time

The main compounds of interest were monitored and are plotted over the time of the experiments in Figure 4.3 and Figure 4.4.

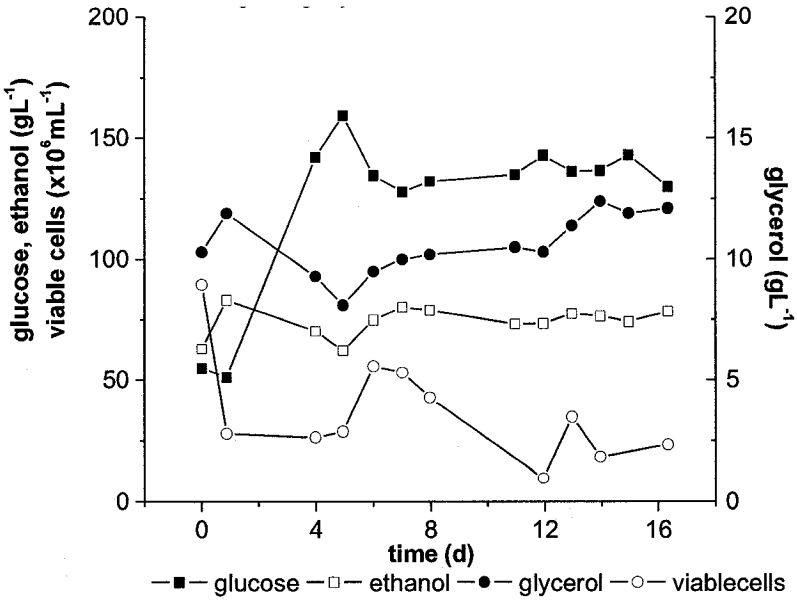

Figure 4.3. Fermentation parameters over time. Anaerobic conditions

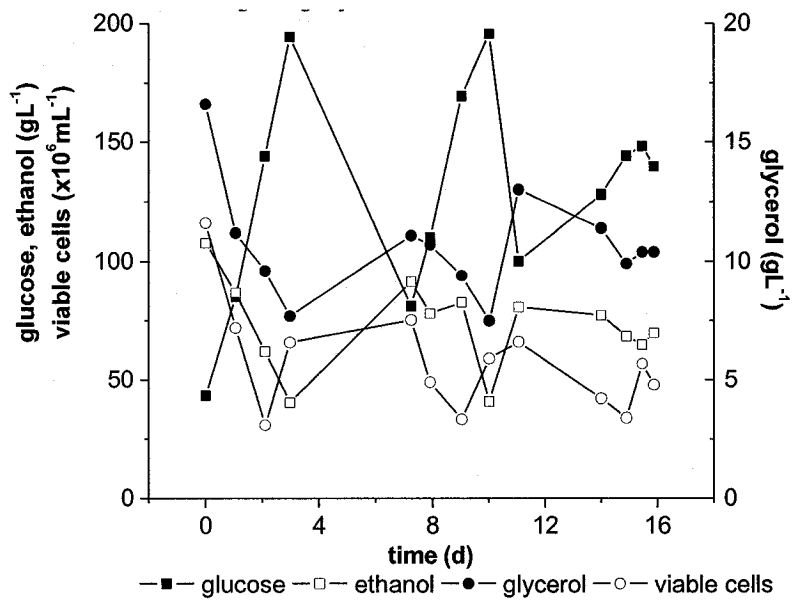

Figure 4.4. Fermentation parameters over time. Micro-aerobic conditions.

Their respective averages are tabulated below, in Table 4.1. These were averages of all the data collected over the 16 days of the experiments.

Table 4.1. Mean fermentation parameters measured in the SSCF system (n=14)

	Viable cells ($\times 10^7 \cdot mL^{-1}$)		Glucose ($g \cdot L^{-1}$)		Glycerol ($g \cdot L^{-1}$)		Ethanol ($g \cdot L^{-1}$)	
	Mean	St.dev.	Mean	St.dev.	Mean	St.dev.	Mean	St.dev.
anaerobic	3.575	2.185	121.857	33.937	10.827	1.489	75.977	8.624
micro-aerobic	5.755	4.559	135.104	56.625	10.484	3.470	70.679	28.714

4.2.2. Fermentation yields

The fermentation yields were calculated based on the averages obtained in Table 4.1. The equations 2, 3 and 4 were used. Very good yields on ethanol ($Y_{etoh/S}$) were obtained for both conditions. Considering a maximum theoretical yield of 0.51 $g \cdot g^{-1}$ (100% efficiency), anaerobic and micro-aerobic oxygenation strategies gave 83.3 % and 84.1 % overall efficiencies respectively, which can be considered excellent in the fuel ethanol industry, where efficiencies in the vicinity of 75 % are typical. Similarly, volumetric productivity of ethanol (Q_{etoh}) was considered to be above industry standards.

Table 4.2. Yields and overall volumetric productivities of the various metabolites of interest

		anaerobic experiment	micro-aerobic experiment
$Y_{X/S}$	(10^6 cells$\cdot g^{-1}$)	0.213	0.349
$Y_{glyc/S}$	($g \cdot g^{-1}$)	0.0606	0.0636
$Y_{etoh/S}$	($g \cdot g^{-1}$)	0.425	0.429
Q_{glu}	($g \cdot L^{-1} \cdot h^{-1}$)	3.64	3.44
Q_{gly}	($g \cdot L^{-1} \cdot h^{-1}$)	0.221	0.218
Q_{etoh}	($g \cdot L^{-1} \cdot h^{-1}$)	1.55	1.47

4.2.3. Influence of oxygen on glycerol formation

As depicted in Figure 4.5, a clear linear relationship between ethanol and glycerol is demonstrated. At ethanol concentrations below 100 $g \cdot L^{-1}$, glycerol formation is slightly more important: limited amounts of oxygen are supplied, whereas above 100 $g \cdot L^{-1}$, anaerobic conditions are favouring more the production of glycerol.

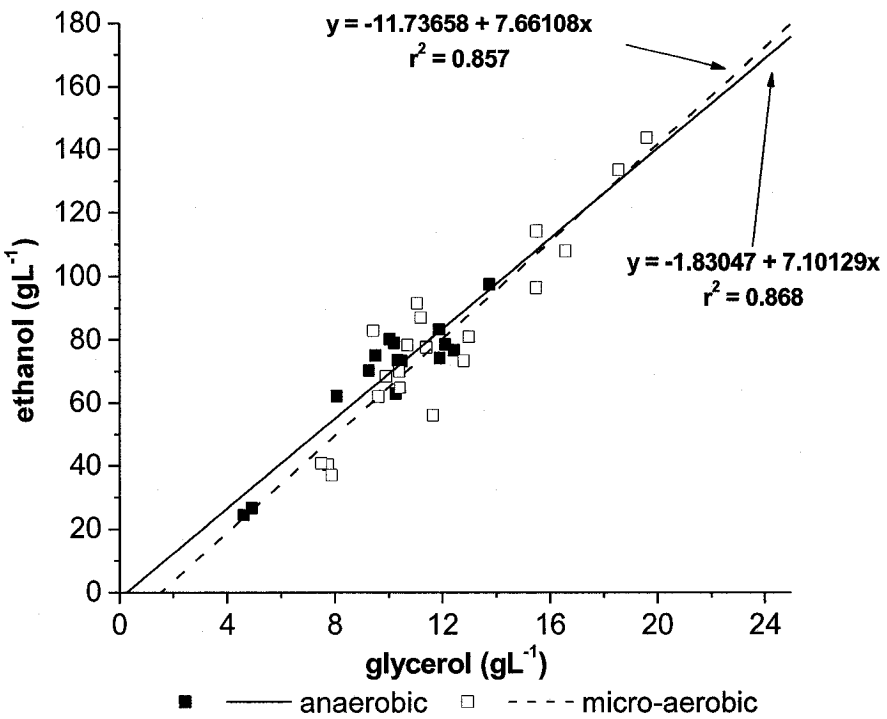

Figure 4.5. Linear relationship between ethanol and glycerol formation.

Although the difference is not clear according to the linear relationship between ethanol and glycerol presented in Figure 4.5, computation of the specific productivity of glycerol (ν_{glyc}) demonstrates a reduction of 48.0 % in the amount of glycerol excreted per cell in

anaerobiosis versus micro-aerobic conditions of the culture (Table 4.3). This difference is however not significant.

Table 4.3. Specific consumption of glucose, and specific productivities of glycerol and ethanol in a SSCF system (n=14)

	ν_{gluc}		ν_{glyc} ($\mu g \cdot 10^{-6} \cdot h^{-1}$)		ν_{etoh}	
	Mean	St.dev.	Mean	St.dev.	Mean	St.dev.
anaerobic	131.428	80.772	8.209	5.646	57.548	38.170
micro-aerobic	67.336	21.768	4.266	1.392	29.541	11.516

4.2.4. Influence of oxygen on succinate formation

Considering the fact that standard deviations around 80 % were obtained for succinate concentration changes over time, the results obtained can hardly be interpreted solidly. Nonetheless, an average of 0.609 gL^{-1} of succinate was measured in the anaerobic system, while 0.345 gL^{-1} was quantified in the micro-aerobic SSCF system. This difference cannot be seen as significant in the present case.

However, similarly to the analysis done on glycerol (Section 4.2.3), the specific productivity of succinate (ν_{succ}) determined in anaerobic conditions, expressed as micrograms of succinate per million viable cells per hour, was computed. A significant difference was determined (Table 4.5 and Figure 4.6). Indeed, a reduction in 69.8 % of the specific productivity of succinate by yeast was observed with micro-aerobic conditions.

Table 4.4. Specific productivity and mean concentration of succinate during the SSCF experiments (n=14)

	succinate conc. ($g \cdot L^{-1}$)		ν_{succ} ($\mu g \cdot 10^{-6} \cdot h^{-1}$)	
	Mean	St.dev.	Mean	St.dev.
anaerobic	0.609	0.143	0.460	0.277
micro-aerobic	0.345	0.305	0.139	0.050

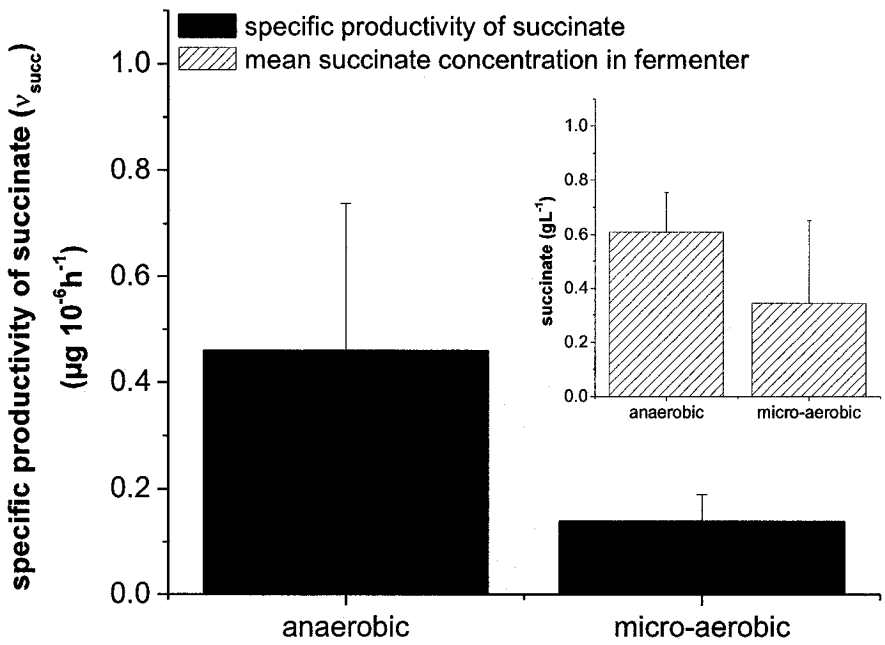

Figure 4.6. Specific productivity and mean succinate concentration during the SSCF experiments (n=14)

4.3. Multi-stage continuous fermentation results

In order to test various aeration strategies in a more industrially relevant context, a MSCF system was built, using three fermentation stages. Three different oxygenation levels were applied to the first stage: no oxygen (anaerobic conditions), saturated oxygen conditions (aerobic) and minimal oxygen (micro-aerobic). The results are presented below.

4.3.1. Evolution of metabolites over time

Three main metabolites and viable cell concentration were monitored daily in order to assess the performances of the MSCF systems. These were then plotted over time. The average concentrations of all the data collected over the 16 days of the experiments are tabulated below.

Table 4.5. Mean concentrations from daily measurements of glucose, glycerol, ethanol and viable cells counts measured during the MSCF experiments (n=16)

	Stage 1		Stage 2		Stage 3	
	Mean	St.dev.	Mean	St.dev.	Mean	St.dev.
anaerobic						
glucose	155.561	23.459	123.947	22.484	78.351	15.525
glycerol	9.947	1.244	10.821	1.452	12.748	1.161
ethanol	53.564	9.502	64.629	8.488	85.562	6.505
cell count	7.956×10^{7}	3.479×10^{7}	6.532×107	2.992×10^{7}	5.335×10^{7}	2.680×10^{7}
aerobic						
glucose	125.355	29.847	120.227	20.629	69.895	17.881
glycerol	15.132	1.674	13.007	0.957	13.719	0.626
ethanol	61.994	7.661	70.626	9.350	89.369	5.030
cell count	2.025×10^{8}	5.928×10^{7}	1.260×10^{8}	2.999×10^{7}	1.266×10^{8}	2.763×10^{7}
micro-aerobic						
glucose	141.067	44.172	120.577	33.122	75.251	23.010
glycerol	10.460	1.012	11.997	1.066	12.710	1.620
ethanol	52.239	9.235	63.051	5.164	81.710	5.640
cell count	7.156×10^{7}	3.513×10^{7}	7.376×10^{7}	1.970×10^{7}	6.556×10^{7}	2.428×10^{7}

[1] expressed in $g\cdot L^{-1}$

[2] expressed as viable cells per mL

4.3.1.1. Anaerobic conditions

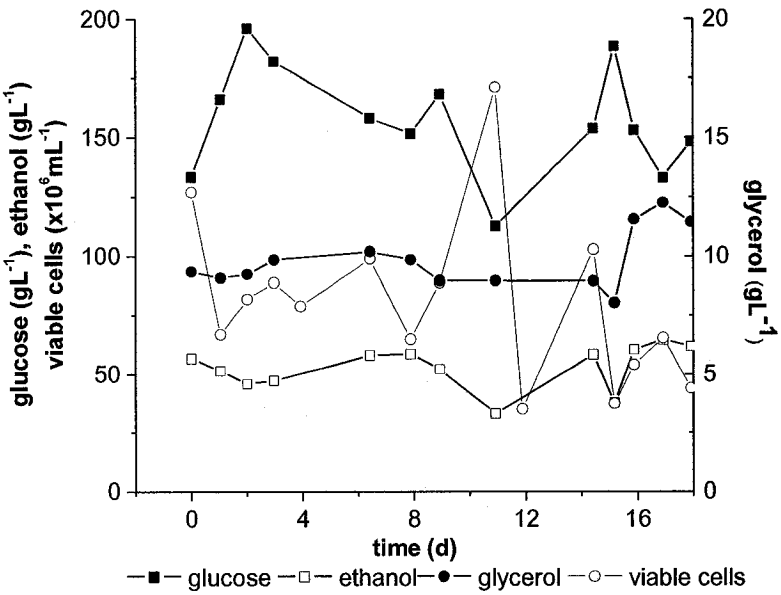

Figure 4.7. Fermentation parameters over time in stage 1. Anaerobic conditions were kept in the three stages

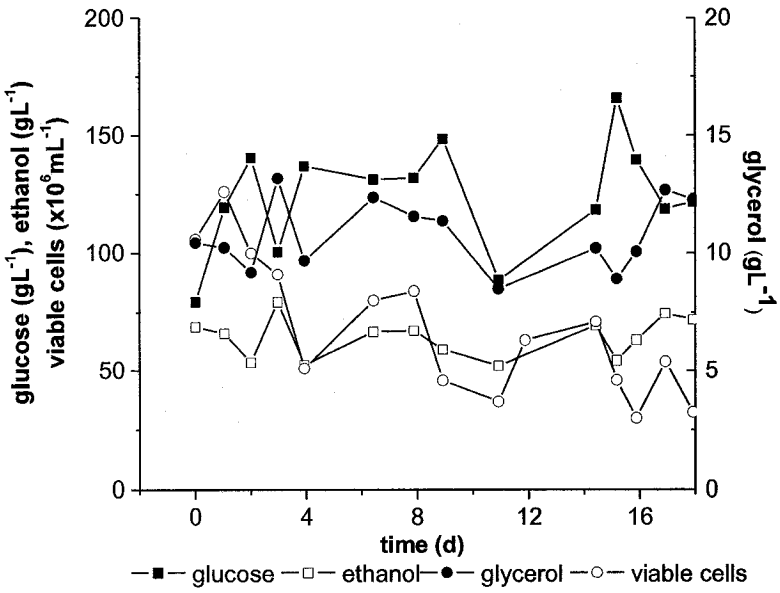

Figure 4.8. Fermentation parameters vs. time in stage 2. Anaerobic conditions were kept in the three stages

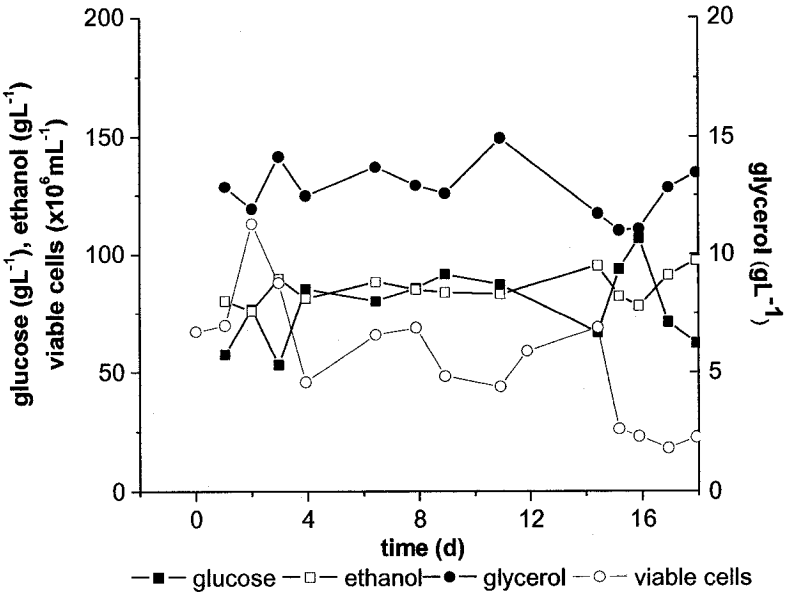

Figure 4.9. Fermentation parameters vs. time in stage 3. Anaerobic conditions were kept in the three stages

4.3.1.2. Aerobic conditions

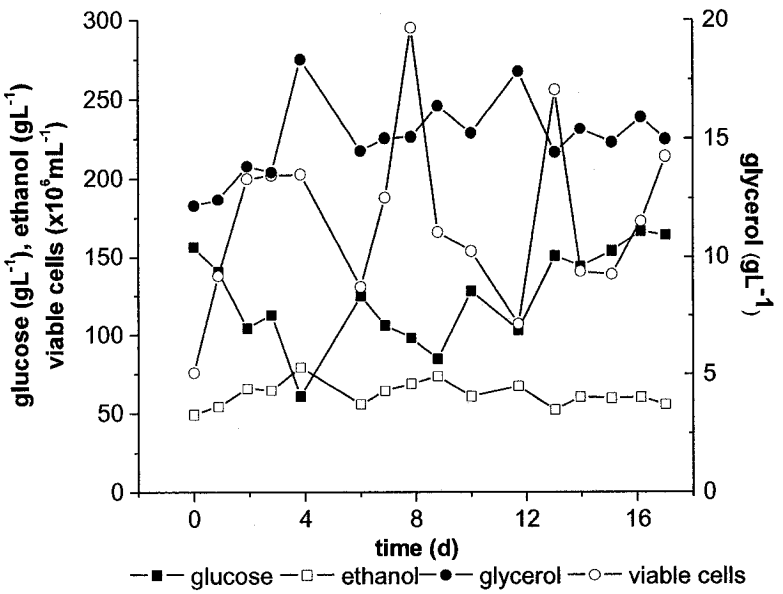

Figure 4.10. Fermentation parameters vs. time in stage 1. Aerobic conditions were kept in stage 1

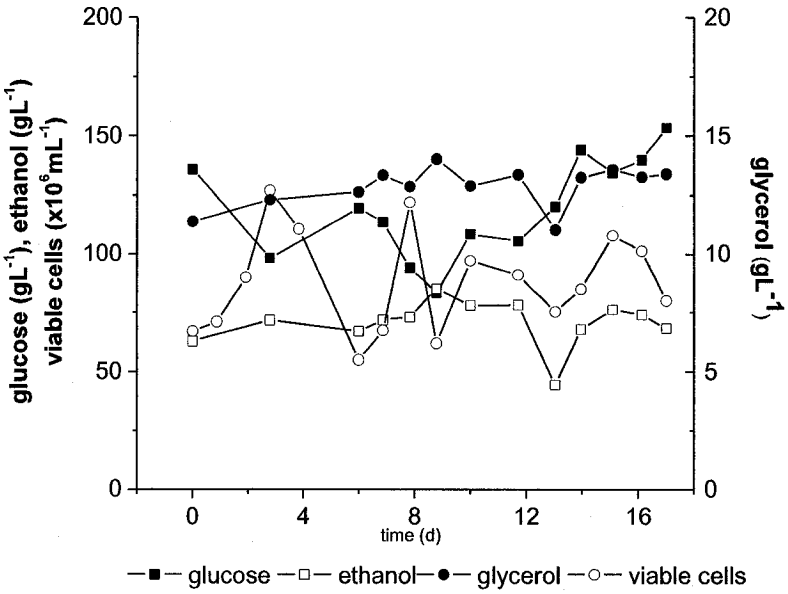

Figure 4.11. Fermentation parameters vs. time in stage 2. Aerobic conditions were kept in stage 1

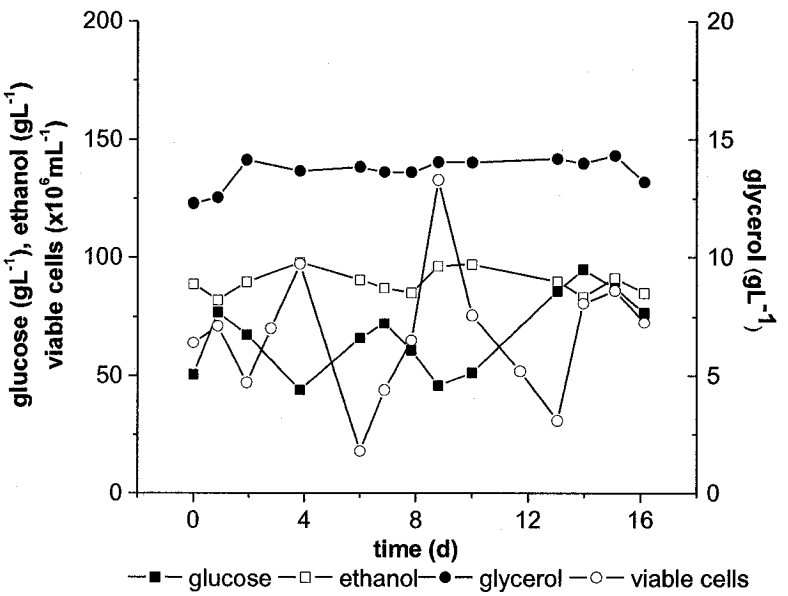

Figure 4.12. Fermentation parameters vs. time stage 3. Aerobic conditions were kept in stage 1

4.3.1.3. Micro-aerobic conditions

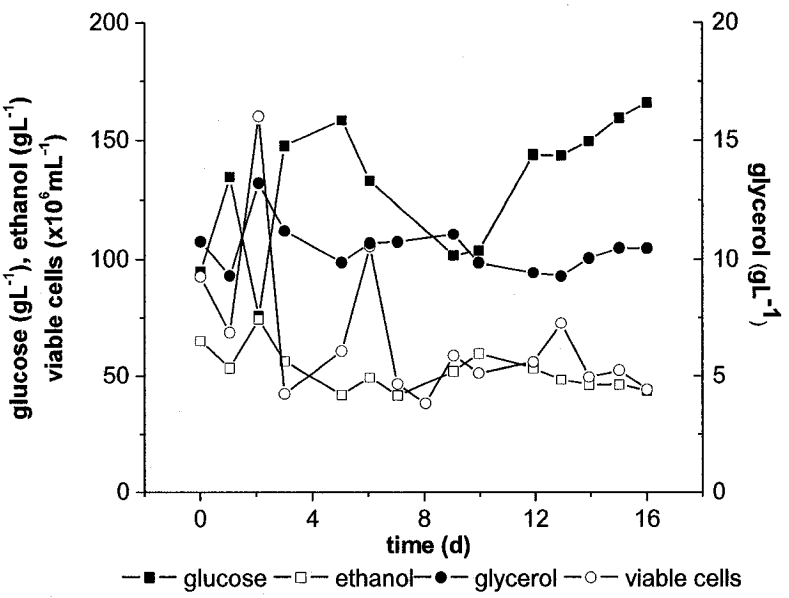

Figure 4.13. Fermentation parameters vs. time in stage 1. Micro-aerobic conditions were kept in stage 1

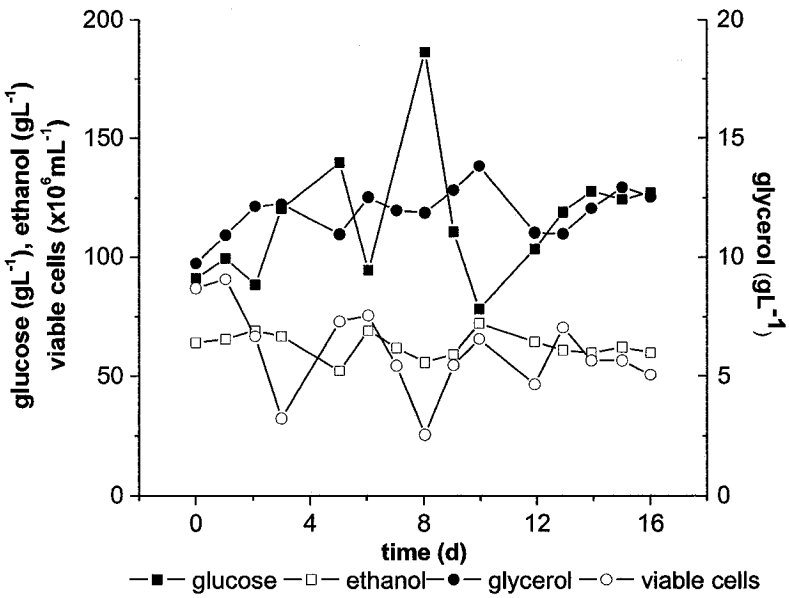

Figure 4.14. Fermentation parameters vs. in stage 2. Micro-aerobic conditions were kept in stage 1

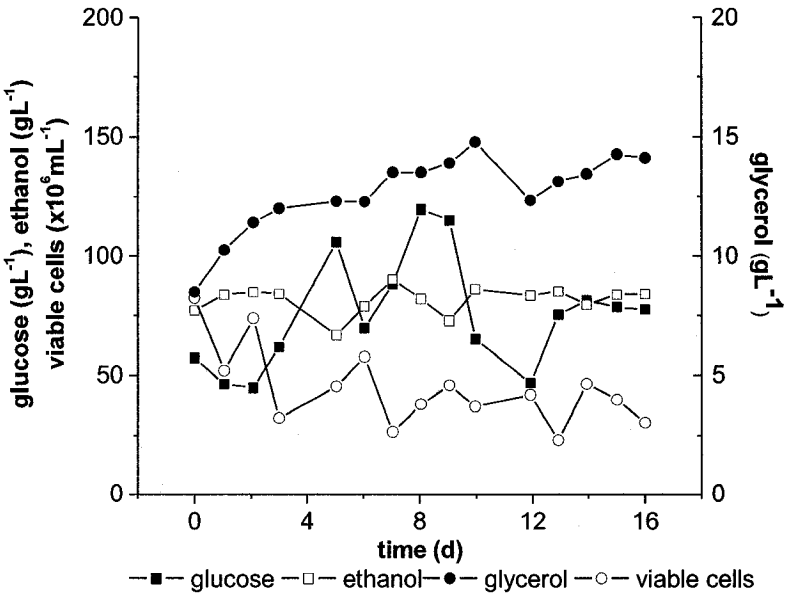

Figure 4.15. Fermentation parameters vs. time in stage 3. Micro-aerobic conditions were kept in stage 1

4.3.2. Fermentation yields

Table 4.6. Yields and volumetric productivities of the various metabolites of interest

		anaerobic experiment	aerobic experiment	micro-aerobic experiment
$Y_{X/S}$	(10^6 cells·g^{-1})	0.259	0.285	0.202
$Y_{glyc/S}$	($g \cdot g^{-1}$)	0.0575	0.0596	0.0566
$Y_{etoh/S}$	($g \cdot g^{-1}$)	0.386	0.388	0.364
Q_{glu}	($g \cdot L^{-1} \cdot h^{-1}$)	2.96	3.07	3.00
Q_{gly}	($g \cdot L^{-1} \cdot h^{-1}$)	0.170	0.183	0.169
Q_{etoh}	($g \cdot L^{-1} \cdot h^{-1}$)	1.14	1.19	1.09

4.3.3. Influence of oxygen on glycerol formation

Glycerol concentration varied over the course of the fermentation, and its formation in the first stage was affected by the amount of oxygen present (Figure 4.16).

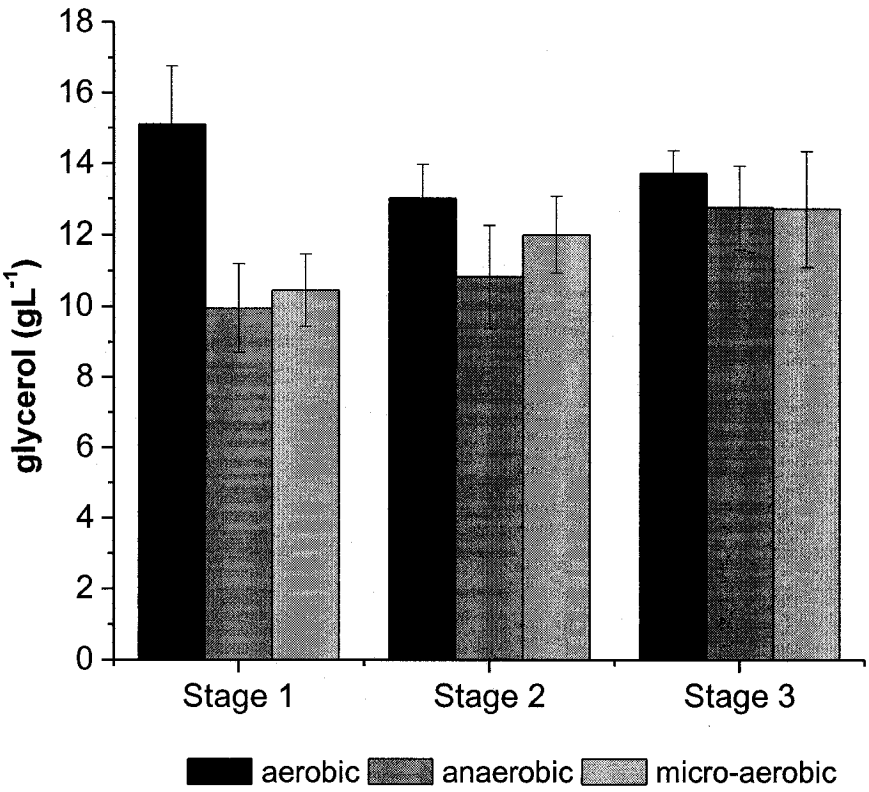

Figure 4.16. Glycerol concentration in the different stages of the MSCF system for the three different aeration strategies (n=16)

Indeed, an average of 9.95 $g \cdot L^{-1}$ was found when no oxygen was supply to the first fermenter, while 15.13 $g \cdot L^{-1}$ was measured when a saturated oxygen fermentation broth was used. The slightly higher concentration of glycerol found in the first stage when it was micro-

aerated, 10.36 $g \cdot L^{-1}$, cannot be considered significantly different from when no oxygen was supplied.

However, when the specific productivity of glycerol (ν_{glyc}, µg of glycerol per million cells per hour) is computed, it is found that ν_{glyc} in F1 is much higher when the micro-aerobic strategy was employed. In contrast to the observed concentration of glycerol, it is when the broth was fully aerated that less glycerol was produced per viable cell present.

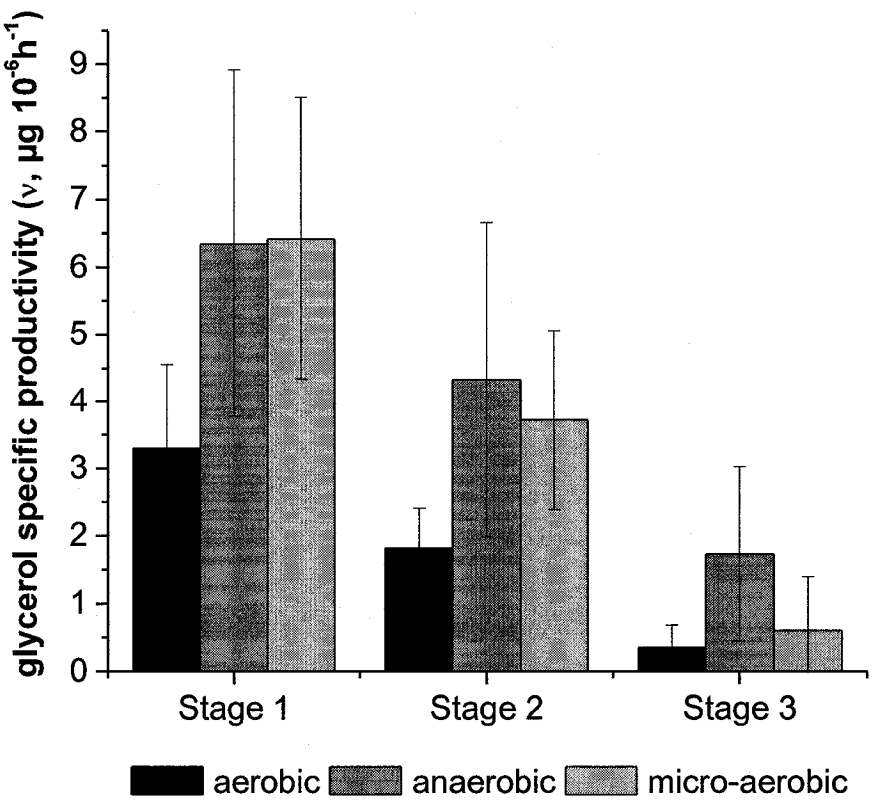

Figure 4.17. Specific productivity of glycerol in each fermenter for the three conditions tested (n=16)

Another way of evaluating the effect of oxygen on the production of glycerol is by computing the ratio of the concentrations of ethanol over

glycerol. As shown in Figure 4.5, this relationship should be linear for a particular set of fermentation conditions.

However, the attempt to establish such relationship with the data obtained during the MSCF experiments failed. Points were too scattered to obtain a valuable trend line, with a reasonable correlation factor. Nonetheless, ratios of average ethanol and glycerol concentrations were calculated. The anaerobic conditions gave a ratio of 6.71 $g \cdot g^{-1}$, the aerobic conditions gave a ratio of 6.51 $g \cdot g^{-1}$ and the micro-aerobic conditions gave a ratio even slightly lower, 6.43 $g \cdot g^{-1}$. It can therefore be observed that more glycerol was produced per amount of ethanol when some oxygen was present.

5. DISCUSSION AND CONCLUSION

5.1. Strain selection

The selection of the proper strain for ethanol production was based on the criterion that the best strain would produce ethanol at the fastest rate among a group of pre-selected strains. The strain provider, the National Center for Agricultural Utilization Research (USDA) did the first selection based on historical data obtained from its database: previous research done with the strain, origin of the strain etc.

In the fuel ethanol industry, it is important to use a robust strain that will tolerate high ethanol concentrations and high osmotic pressure. Moreover, often bacterial contaminants are present and the yeast strain needs to be able to compete and to tolerate high organic acids (lactate, acetate) concentrations. Nevertheless, the most important characteristic will be the rapidity to produce ethanol. This will ensure high plant throughput.

The batch fermentation of DFM at low glucose concentration (120 $g \cdot L^{-1}$) gave an excellent idea about the performance of the six strains in terms of ethanol productivity (Figure 4.2). Indeed, they were all submitted to the same osmotic shock (high glucose) and to the relatively poor nutrients conditions (no free amino acids available). The six strains were inoculated at the same initial viable concentration. NRRL Y-634 showed the most efficient conversion of glucose to ethanol in the given

conditions, which gave an acceptable volumetric productivity in batch mode. Effectively, its ethanol production rate was 17 % higher than the next fastest ethanol producer.

In brief, the strain finally selected offered adequate performance in both SSCF and MSCF systems where even higher initial substrate concentration was present.

5.2. Influence of oxygen on glycerol formation in single-stage continuous fermentation systems

It is well known that most glycerol is produced mainly in the first stage of fuel ethanol multi-stage continuous fermentation systems due to high osmotic shock due to the presence of very high glucose concentration (17,22,25,47,48,96). A single-stage continuous fermentation system, where the first stage was reproduced, was hence constructed in order to study the formation of this metabolite.

Two aeration strategies were tested in the SSCF system. It was initially hypothesised that some oxygen in the fermentation broth would slow down the production of glycerol by favouring the re-oxidation of NAD^+. The latter reaction is normally performed anaerobically via the reduction of dihydroacetone phosphate as an outlet for surplus NADH. Therefore, offering micro-aerobic conditions would support respiratory mechanisms that naturally recycle the NAD^+ to NADH.

As a basal setup, an anaerobic SSCF system was initiated and major metabolites were determined regularly, once steady-state was

reached, over eight changes in working volumes (eight residence times), in order to have an adequate amount of data. The fermentation profile exhibited in Figure 4.3 shows the relative stability of the system and demonstrates clearly the relationships between glucose and ethanol.

Likewise, good correlation was obtained between glycerol and ethanol concentration (Figure 4.5). This laid down the basis for the analysis of glycerol yields on glucose and ethanol/glycerol ratios. Although both curves demonstrate very similar slopes, meaning similar ethanol to glycerol ratios, linking the glycerol concentration to the viable cells in suspension gave a more precise indication of how much ethanol, and how much glycerol is produced per unit of cell population.

On the other hand, the small amount of oxygen supplied ($15\ mg \cdot min^{-1}$) did have an effect on the whole system. When looking at the fermentation profile in Figure 4.4, an obvious instability can be noticed. Although initially difficult to interpret, these oscillatory behaviours have been observed in earlier studies (6,7) where very high gravity feeding medium ($280\ g \cdot L^{-1}$ glucose) was also utilised. It has been suggested that yeast cells tend to exhibit a lag in their response to high ethanol stress, which causes a delay in cell synthesis as a result of intracellular disturbances caused by the ethanol toxicity. Cells will acclimatise themselves to the new environment, but the lag will be too long to compensate for the continuous supply of glucose. Consequently, constant shifts from high to low glucose, and low to high ethanol concentrations are

observed. This will be especially true in the presence of oxygen where cells will be further favoured to activate their aerobic metabolism.

Glycerol, indirectly, will be affected in the same way as glucose and ethanol. It can therefore be difficult to compare the data only based on average concentrations found in the two SSCF systems due to high standard deviations. The glycerol specific productivity (ν_{glyc}), expressed as micrograms per million cells (viable) per hour of fermentation, can be a better was of assessing the effect of oxygen on the production of this metabolic by-product.

Indeed, a reduction in ν_{glyc} of 36 % was observed when the SSCF system was subjected to trace amounts of oxygen. This interesting result is however counterbalanced by a reduction in ν_{etoh} of 38 %, which is linked to considerably higher viable cell concentrations under the presence of oxygen in the fermentation broth. Nonetheless, yield of ethanol on glucose remained virtually unchanged to 0.43 $g \cdot g^{-1}$ (Table 4.2) and despite a higher cell concentration, less glucose was required to produce the same amount of cells in the micro-aerobic fermenter.

Therefore, glucose was not sacrificed for biomass production, ethanol yield remained similar and less glycerol was produced per unit of cell concentration present in the SSCF system. This promising result, yet not of industrial relevance, was then challenged in a MSCF system.

5.3. Influence of oxygen on glycerol formation in multi-stage continuous fermentation systems

The idea behind the MSCF system was to evaluate the hypothesis in an industrially relevant process. Based on personal communications with the industry, a three-stage continuous fermentation system having an overall residence time of 75 h was designed and operated under three different aeration strategies (complete anaerobiosis, full aerobiosis and micro-aerobiosis). The idea of saturating the fermentation broth with oxygen was to investigate if an excess of oxygen would enhance the effect previously observed in the SSCF system. The first stage of the train was used for oxygenation since most glycerol tends to be produced at that point.

Figures 4.6 to 4.14 illustrate the changes in metabolites concentration over the course of each experiment in each of the three fermenters. The data obtained in the first stage enables some comparison with what has been obtained using a SSCF whereas data from the third stage illustrates what would be coming out of an industrial MSCF system.

In contrast with the SSCF experiments, all the MSCF experiments exhibited some oscillatory behaviour in their metabolites concentrations over time. It was in F3 that less variability was observed. This may be explained by the higher dilution rate (0.040 h^{-1} instead of 0.021 h^{-1}) which does not let the cell population to acclimatize properly to its cultivation

conditions. Moreover, feeding DFM in both F1 and F2 certainly reduced the stability of the system.

According to the average glycerol concentrations obtained at the end of the MSCF system, no difference was noted between the anaerobic and the micro-aerobic aeration strategies: both provided about 12.7 $g \cdot L^{-1}$ of glycerol in F3. On the other hand, the saturation of the fermentation broth in F1 with oxygen has caused an increase in the glycerol concentration by almost 8 %. This difference remains insignificant due to the variability of the glycerol concentration across the time of the experiments (see Figure 4.16 for error bars representing the standard deviations).

When looking at the specific productivity of glycerol for the three aeration conditions, both the anaerobic and the aerobic conditions gave rise to relatively low overall ν_{glyc} (around 2.88 $\mu g \cdot 10^{-6} \cdot h^{-1}$). On the other hand, the ν_{glyc} calculated for the MSCF micro-aerobic experiment was very similar to the data obtained in the similarly aerated SSCF system.

5.4. Concluding remarks

It is therefore difficult to conclude that the supply of oxygen really has a significant effect on glycerol formation in an industrially relevant MSCF system. Oscillations, although inherent to the systems, make the interpretation of the data difficult. Indeed, small improvements in specific

productivity of glycerol via full aeration through a MSCF system are of weak significance when considering the variations.

Nevertheless, promising results were obtained in SSCF systems where the amount of glycerol produced per unit of viable cell population was reduced by 36 %. Further investigation should be done in order to relate oxygen concentrations with glycerol specific productivities in very high gravity conditions, which are now the norm in fuel ethanol production.

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