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# The impact of severe nitrogen limitation and microaerobic conditions on extended continuous cultivations of *Saccharomyces cerevisiae* with cell recirculation

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**Abstract**

Continuous cultivations of *Saccharomyces cerevisiae* ATCC 96581 with severe nitrogen limitation (C/N ratios 200 and 400 g g$^{-1}$) and cell recirculation were carried out under anaerobic and microaerobic conditions for more than 300 h. With a dilution rate of 0.06 h$^{-1}$ and 90% recirculation in combination with an estimated 70% biomass sedimentation rate in the bleed flow, specific growth rates of 0.002–0.006 h$^{-1}$ were obtained. Under these conditions, ethanol yields of 0.46–0.48 g g$^{-1}$ were achieved. The biomass yields on ATP were only 1.6–2.9 g mol$^{-1}$, indicating metabolic uncoupling or high maintenance energy requirements. Viability levels, measured by FUN® staining and fluorescence microscopy, usually varied between 100 and 80%. However, under anaerobic conditions at C/N ratio 400, a reproducible drop to 25% viability occurred between 250 and 300 h of fermentation, after which the culture recovered again. Under anaerobic conditions, an increase in the C/N ratio from 200 to 400 resulted in a three-fold higher specific glycerol production, in spite of lower biomass formation and lower cellular protein and RNA content. A low oxygen addition eliminated the large drop in viability and the increased glycerol production observed at C/N 400, and caused viability and glycerol levels similar to the anaerobic C/N 200 case. A *S. cerevisiae* W303-1A *gpd1*Δ*gpd2*Δ mutant, completely deficient in glycerol production, could ferment a nitrogen-limited medium under RQ-controlled microaerobic conditions with an ethanol yield of 0.45 g g$^{-1}$, indicating that the increased glycerol production under nitrogen limitation is not necessary, as long as there is sufficient oxygen transferred to the culture.


*Keywords:* Yeast cell recycle; Fuel ethanol production; Glycerol and byproduct formation; *gpd1*Δ*gpd2*Δ mutant; Oxygen limitation; Growth energetics

## 1. Introduction

Bakers' yeast, *Saccharomyces cerevisiae*, is used worldwide for biological production of ethanol. In recent years, when many countries are searching for possibilities to cut net emissions of carbon dioxide, bioethanol as fuel for vehicles has been brought to the fore. While the use of bioethanol is considered advantageous from an environmental point of view, production economy is inevitably a critical aspect. The raw material represents a major share of the production costs [1], and any improvement in the conversion of available sugars and in the selectivity towards ethanol formation should lead to an improved process economy. Therefore, the production of byproducts should be minimized.

Quantitatively, carbon dioxide, glycerol and cell biomass are the most significant byproducts during anaerobic ethanol production by *S. cerevisiae*. $CO_2$ is an inevitable fermentation product, but the off-gas can be sold as a high-quality raw material and is, therefore, only a logistic problem. Substantial amounts of glycerol, up to 10% (w/w) of the carbon source, may be formed under anaerobic conditions for the purpose of reoxidising surplus NADH being formed in anabolism [2–6]. Glycerol is also produced as a compatible solute during osmotic stress [7]. Yeast growth during the ethanol production process essentially represents a loss of carbon from the main product. However, the yeast must retain biological activity high enough to completely convert the fermentable sugars at a reasonable rate.

One way to approach the byproduct formation is by using metabolic engineering [8]. Nissen et al. decreased the NADH formation in biosynthesis by substituting the ATP- and NADH-consuming GS-GOGAT system for the NADPH-consuming glutamate dehydrogenase [9]. This decreased the formation of glycerol by 38% in anaerobic batch cultures. It also increased the ATP requirement for biosynthesis, causing an ethanol yield on glucose of 0.41 g g$^{-1}$, an increase by 10% compared to the

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wild type [9]. Glycerol formation can also be reduced or even completely eliminated by deleting one or both of the *GPD1* and *GPD2* genes, encoding the two cytosolic glycerol 3-phosphate dehydrogenases [10,11]. However, the *gpd1*Δ*gpd2*Δ double mutant, completely devoid of glycerol production, requires low amounts of oxygen to balance the NADH formation [10].

Another approach is to modify the production process so that the intrinsic properties of the yeast can be utilized to their fullest extent. This includes, e.g. the nutrient supply and the mode of operation of the bioreactor. For example, the supply of nitrogenous compounds is of fundamental importance for biosynthesis. By limiting the availability of nitrogen, biosynthesis can be decreased [12–14]. However, complete starvation for nitrogen has many consequences for *S. cerevisiae*. It causes changes in the macromolecular composition [15–17], inactivation of the glucose transport system [18], arrest in the G1 phase of the cell cycle [19], increased turnover of proteins and production of glycerol [16,20] and reduced fermentative capacity [17,21–23]. Nitrogen limitation on the other hand, i.e. when nitrogen is being supplied continuously, but is the limiting factor for cell growth, reduces the protein content [13] and influences the intracellular pool of amino acids in *S. cerevisiae* [24]. In comparison to carbon limitation, nitrogen limitation causes a lower biomass yield and a higher ethanol yield in chemostat cultures and severe nitrogen limitation appears to uncouple the catabolic ATP generation from the anabolic demand for ATP [12–14]. An optimised nitrogen limitation therefore has the potential to increase the ethanol yield. However, despite the decreased biosynthesis, severe nitrogen limitation or starvation also causes an increased glycerol production, for reasons which are not clear [13,14,16].

Moderate addition of oxygen to the medium can eliminate the formation of glycerol without causing any respiratory sugar catabolism [10,25,26]. This can be referred to as microaerobic conditions. A broad optimal respiratory quotient of 12–20 has been reported, at which the ethanol yield was slightly higher than under anaerobic conditions [26]. Moreover, addition of oxygen has many beneficial effects on the biomass, such as increased ethanol tolerance and viability [27,28].

Earlier research suggest that an efficient process for large-scale ethanol production should be based on continuous cultivation with cell retention by either immobilization or cell separation and recirculation [29–32]. The main advantage with cell retention is that the increased biomass concentration may allow for efficient conversion of the substrate, while relatively little substrate is used in biosynthesis. A continuous system with a high degree of recirculation would thus result in very low specific growth rates, which would cause an increased energy requirement because of cell maintenance. This may affect the ethanol yield in a positive way. On the other hand, the specific and volumetric ethanol production rate may be negatively affected, since the fermentative capacity has been shown to be correlated with the specific growth rate [33].

The aim of this study was, in part, to investigate how the metabolism, macromolecular composition and viability of the specific strain *S. cerevisiae* ATCC 96581 is affected by nitrogen limitation and microaerobicity during extended continuous cultivations with cell recirculation. ATCC 96581 is very tolerant to inhibitors, present in, e.g. dilute acid wood hydrolysate, a relatively cheap, but inhibitory, raw material for bioethanol production [34–37]. Further information about this strain is therefore relevant for large-scale bioethanol production with wood hydrolysate as the main carbon source. The aim was also to investigate the effects of a deletion of the *GPD1* and *GPD2* genes on the growth and product formation under nitrogen limitation in a microaerobic bioreactor with cell recirculation.

## 2. Materials and methods

### 2.1. Yeast strains

*S. cerevisiae* ATCC 96851, kindly provided by Prof. B. Hahn-Hägerdal, Lund University of Technology, was used in experiments 1–3 (see Table 1). It was originally isolated from a spent sulfite liquor fermentation plant [36] and has been proved to be relatively tolerant to inhibitors present in wood hydrolysate [34,37]. In experiment 4 (Table 1), a prototrophic *S. cerevisiae* W303-1A *gpd1*Δ*gpd2*Δ mutant was used, which was derived from the YSH6.142-3D strain, described in ref. [38], and kindly provided by Dr. Hadi Valadi. The strains were maintained on YPD plates made from 10 g $l^{-1}$ yeast extract (Merck, Germany), 20 g $l^{-1}$ soy peptone (Merck), 20 g $l^{-1}$ agar and 20 g $l^{-1}$ D-glucose.

### 2.2. Medium composition

The preculture, startup culture and feed medium were composed as previously described [39] with slight modifications: the preculture contained 1.5 g $l^{-1}$ $(NH_4)_2SO_4$ and 1 g $l^{-1}$ glucose and did not contain any ergosterol/Tween 80. The startup culture contained 1.2 g $l^{-1}$ $(NH_4)_2SO_4$ and 11.5 g $l^{-1}$ glucose. The feed contained 40 g $l^{-1}$ glucose, and appropriate concentrations of $(NH_4)_2SO_4$ to give the carbon/nitrogen ratio in the medium for each experiment as specified in Table 1. Ergosterol and Tween 80 was added to the feed media at 10 and 420 mg $l^{-1}$, respectively.

### 2.3. Fermentation conditions

The experiments were carried out in a BIOSTAT A bioreactor (B. Braun Biotech International, Germany). The temperature was 30 °C, pH was kept at 5.0 by addition of 2 M NaOH and the stirring rate was 500 rpm. The culture was sparged with a continuous flow of gas, 450 ml $min^{-1}$, which prevented air from leaking into the reactor vessel. The gas supply rate and gas composition were held constant by mass flow controllers (Bronkhorst HI-TEC, The Netherlands). The gas was either pure $N_2$ at 450 $ml_n$ $min^{-1}$ (experiments 1 and 2) or a mixture of 440 $ml_n$ $min^{-1}$ pure $N_2$ and 10 $ml_n$ $min^{-1}$ air, giving an $O_2$ concentration of 0.5% in the inlet gas (experiment 3). Experiment 4 was carried out in a Chemap bioreactor with a feedback RQ controller [26], at an RQ of 20–25.

Yeast cells were transferred from an agar plate to a cotton-stoppered shake-flask preculture (100 ml). A start-culture (2.2 l) was inoculated from the preculture to an intitial optical density (OD) of 0.005, measured as the turbidity at 610 nm. The start-culture was grown batch-wise without nutrient limitation until OD reached 2.0. At this point, the continuous feed and cell recirculation were started.

Table 1
Carbon/nitrogen ratio and oxygen supply for experiments 1–4

| Experiment ID | C/N ratio (g $g^{-1}$) | Oxygenation | Strain |
|---|---|---|---|
| 1 | 200 | Anaerobic | ATCC 96851 |
| 2 | 400 | Anaerobic | ATCC 96851 |
| 3 | 400 | Microaerobic, 0.5% $O_2$ | ATCC 96851 |
| 4 | 300 | Microaerobic, controlled at RQ 20–25 | W303-1A *gpd1*Δ*gpd2*Δ |