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Journal of Molecular Catalysis B: Enzymatic 52–53 (2008) 82–87
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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# Bioreduction of ethyl 3-oxobutyrate by *Saccharomyces cerevisiae*: A metabolic *in vivo* study

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Available online 14 December 2007

**Abstract**

A systematic study of the bioreduction of ethyl 3-oxobutyrate by *Saccharomyces cerevisiae* under aerobic and anaerobic conditions was performed by using flow isothermal microcalorimetry. In association with this technique, other properties directly related to the metabolism of *S. cerevisiae* such as consumption of $O_2$, glucose and pH were also studied. The metabolic aspects of the bioreduction, such as compartmentalization and preferential use of the cofactors (NADH or NADPH), are discussed based on heat and ethanol production, and oxygen and glucose consumption data obtained during a period of 20 h. These data allows us to suggest the compartment in the microorganism cell where the bioreduction can occur for each experimental condition. The obtained results indicate that under aerobic conditions, the process occurs preferentially in the mitochondrial matrix and is associated with the consumption of the cofactor NADH which is regenerated by the respiratory pathway. However, under anaerobic conditions, the bioreduction occurs in the cytosol and is associated with the consumption of the cofactor NADPH, which is regenerated by the pentose phosphate pathway.
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*Keywords:* Bioreduction; *Saccharomyces cerevisiae*; Biological microcalorimetry; Metabolism; Biotransformation; Biosynthesis

## 1. Introduction

The capability of microorganisms to carry out biotransformations has led scientists to try to understand these processes and to optimize the conditions which allow living organisms and their enzymes to synthesis a wide scale of compounds. This approach is specially of great interest in the pharmaceutical industry [1–8].

*Saccharomyces cerevisiae* is perhaps the most well-known microorganism in the scientific and industrial world and is certainly the yeast most used by organic chemists to mediate enantioselective reductions of C=O bonds [9–15]. *S. cerevisiae* is also an extremely versatile microorganism with applications in asymmetric enzyme catalyzed organic synthesis with high optical yield [16,17]. *S. cerevisiae* can carry out the respiratory and fermentative catabolic concomitantly under strictly aerobic conditions. This behavior is called mixed respiratory-fermentative catabolism and is a direct consequence of the Crabtree effect or catabolic repression [18,19].

Although the bioreduction process is well studied and used for chemical synthesis on a small scale, little attention has been given to obtaining a better understanding of the biochemical and energetic aspects of this process. *In vitro* tests performed with isolated yeast enzymes and cofactors have shown that bioreduction is a reaction catalyzed by dehydrogenase enzymes that are dependent on the reduction cofactors NADH or NADPH. These cofactors are responsible for the intracellular bioreduction process in *S. cerevisiae* and they are continually regenerated by the catabolism of glucose [20–24].

The cofactor $NAD^+$ is reduced to NADH by the glycolytic pathway in the cytosol and, under ideal conditions with oxygen availability, by the oxidative phosphorylation process in the mitochondrial matrix (Krebs' cycle) [25]. However, $NADP^+$ cofactor is generally reduced to NADPH by the pentose phosphate pathway (PPP), an alternative for glucose catabolism [17,20,26]. In the complete absence of molecular oxygen, the cofactor $NAD^+$ needs to be regenerated to maintain continuity of the glycolytic pathway and the regeneration of this cofactor occurs in the fermentative pathway, while ATP is regenerated in the glycolytic pathway [27,28].

It is well known that the metabolism of living organisms always involves by heat production which results in the temperature of the medium due to the liberated or absorbed energy of all metabolic processes including the hydrolysis of ATP molecule [29–31]. Calorimetry is a highly sensitivity technique which has

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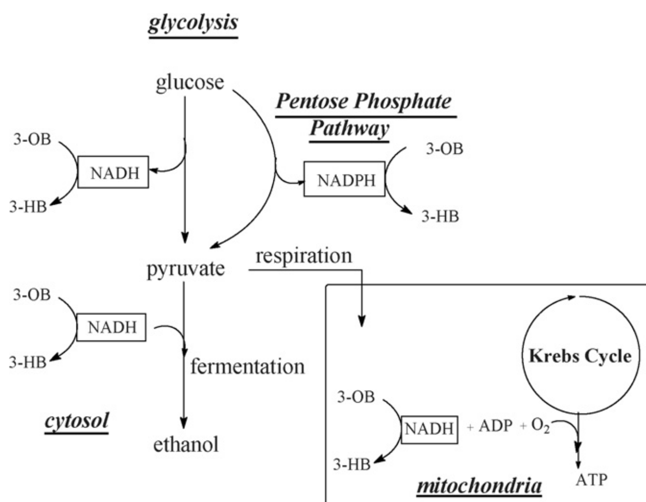
doi:10.1016/j.molcatb.2007.12.002




Scheme 1. Schematic representation of the reduction of the substrate coupled to the catabolic glucose pathway in the cytosol and the Krebs' cycle in the mitochondria.

been used for the study of biological systems [30,32,33]. For biological systems, the main advantage of this technique is that the metabolic activity can be monitored in real time. However, the heat produced in biological processes is very complex and it is not possible, using only calorimetry, to attribute it to a specific metabolic process.

Under starvation nutritional conditions, ATP molecules are not used in anabolic reactions. Under these conditions, all ATP molecules generated in the catabolic process are quickly hydrolyzed to allow metabolic continuity and the energy of the hydrolysis is liberated as heat [28,32].

In this work, we studied the metabolism of *S. cerevisiae* during the bioreduction of the pro-chiral ketone, ethyl 3-oxobutyrate (3-OB) to ethyl 3-hydroxybutyrate (3-HB), by using flow isothermal calorimetry in association with other monitoring techniques such as consumption of $O_2$ and glucose, and changes in pH. The mechanism of bioreduction of this ketone catalyzed by the yeast is shown in Scheme 1.

## 2. Experimental

### 2.1. Materials and reactional media

Glucose (Sigma) and 3-HB (Across) were of analytical grade and were used as received. 3-OB analytical grade (Carlo Erba) was distilled before each experiment. Antifoam FB-50 (ethylene polyoxide and ethylene glycol) was purchased from Dow Corning and lyophilized *S. cerevisiae* from (Fermix, DSM Bakery). 3-OB was chosen as a reduction substrate due to its solubility in the reactional aqueous media and also to the fact that it is not toxic to the microorganism.

The reaction medium for the control experiments was prepared by adding 72.00 g of glucose and 0.0500 g of antifoam to 1 L of distilled water. For the bioreduction experiments, 10.00 g of 3-OB was added to the same reaction medium as used for the control experiments.

### 2.2. Experimental conditions

The monitoring of the bioreduction of 3-OB was performed in real time using two different experimental conditions:

(a) aerobically ($2.5\ L\ min^{-1}$ of air) and in the presence of an excess of glucose ($400\ mmol\ L^{-1}$), B-AG
(b) anaerobically ($0.5\ L\ min^{-1}$ of $N_2$) and in the presence of an excess of glucose ($400\ mmol\ L^{-1}$), B-AnG

For all the studies of bioreduction of 3-OB, the experiments were always performed and analyzed in comparison with a control experiment (reaction medium with 3-OB). All the experiments were performed at least three times and the data were presented as an average value.

### 2.3. Calorimetric experiments

The reaction medium was prepared and transferred to a bioreactor (BBraun Biotech Biostat B2) equipped with pH electrode, $O_2$ electrode, Pt-100 thermocouple sensor, gas flow control, temperature control and controlled stirring. The reaction medium in the reactor was thermostated at 298 K. The $O_2$ and pH electrodes were previously calibrated. The $O_2$ electrode was calibrated with a $N_2$ flow of $0.5\ L\ min^{-1}$ (% $O_2 = 0$) and under a synthetic air flow of $2.5\ L\ min^{-1}$ (% $O_2 = 100\%$). The stirring was maintained at 400 rpm. The flow of synthetic air was kept at $2.5\ L\ min^{-1}$ (aerobic experiments, AGs) while $0.5\ L\ min^{-1}$ of $N_2$ was used for the anaerobic experiments (AnGs).

For the calorimetric experiments, an isothermal flow calorimeter (LKB-2277, Thermometric—TAM) equipped with a 0.60 mL (nominal volume) flow cell made of gold was used. This calorimetric system has two flow cells and the sample and reference cells are disposed in a twin system arrangement. Before each experiment, the calorimetric flow cell was always electrically calibrated.

For each calorimetric experiment, 10.00 g of lyophilized *S. cerevisiae* were added to the reaction media. The suspension was stirred for 5 min at 400 rpm to obtain a homogeneous cell suspension. After this period, the suspension was pumped into the microcalorimeter flow cell using a peristaltic pump (LKB Pharmacia), with a constant flow rate of $85\ mL\ min^{-1}$ and the calorimetric data acquisition was started. The inlet and outlet flow Teflon tubings were connected to the bioreactor forming a continuous flow system. Bioreduction processes were monitored for a period of 20 h.

### 2.4. Glucose and ethanol monitoring

For monitoring ethanol production and glucose consumption, aliquots from the bioreactor were collected throughout the experiments. The aliquots were centrifuged at 9000 rpm for 10 min for cells deposition. The glucose consumption was monitored by a colorimetric reaction using an enzymatic kit [34] based on the enzymatic oxidation of glucose to gluconic acid with formation of hydrogen peroxide ($H_2O_2$). The samples were measured using a UV–vis spectrophotometer (HP 8453) at $\lambda = 505$ nm.

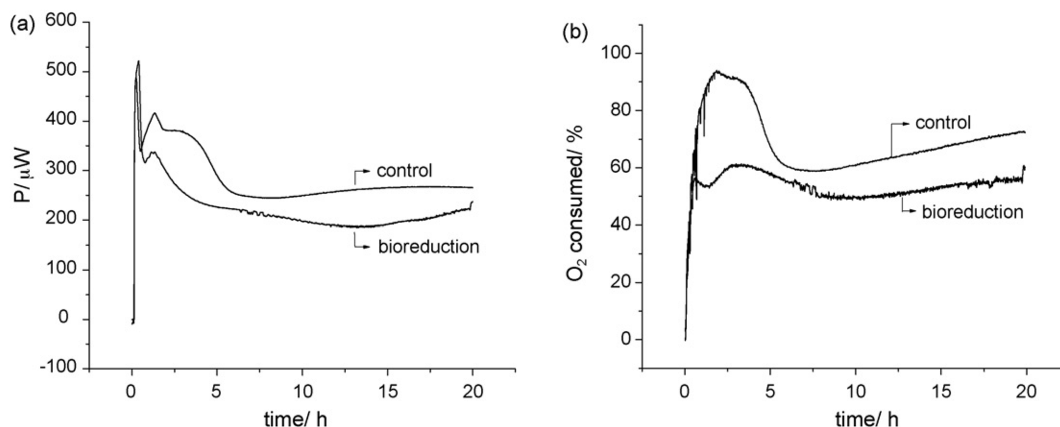



Fig. 1. Experimental data obtained for the aerobic experiments: (a) power–time curve and (b) $O_2$ curve.

For the quantification of the ethanol produced, an enzymatic kit [35] was used. The method is based on the enzymatic oxidation of ethanol to acetic acid. When the reaction is completed, the cofactor NADH produced in the media was quantified by measuring the absorption in the UV spectral region at $\lambda = 340$ nm.

### 2.5. Bioreduction monitoring

Monitoring of the bioreduction process was performed by using gas chromatography. A gas chromatograph (CG-Master 500), equipped with a packed Carbowax 20M column supported on silica, using a flow rate of dry hydrogen gas of 30 mL $min^{-1}$, was used to determine the decreasing concentration of 3-OB with time and the corresponding increase in concentration of 3-HB.

Before monitoring each bioreduction experiment, analytical curves using pure 3-OB and 3-HB were prepared.

## 3. Results and discussion

### 3.1. Aerobic experiments

The calorimetric results, presented in Fig. 1a, show that power–time curves have a multiphasic behavior, composed of three distinct phases, which were also observed previously by Lamprecht [28]. The *P*–*t* curves obtained were integrated to calculate the total heat produced by the microorganism suspension (*Q*) during the period of 20 h. Fig. 1b shows $O_2$ consumed during this same period.

Table 1 contains the heat production, ethanol produced, $O_2$ and glucose consumed by the aerobic bioreduction process (B-AG).

Both the heat produced and the consumption of $O_2$ are about 20% lower than those in the control process (C-AG). These data are a strong indication that the bioreduction process is occurring in the mitochondrial matrix and is consuming NADH cofactor. The bioreduction of 3-OB competes for the intramitochondrial NADH cofactor and, consequently, directly interferes with oxidative phosphorylation, reducing NADH availability for ATP regeneration by the electron transporting chain. Consequently, the consumption of $O_2$ is reduced in the same proportion, because oxygen is the electron acceptor of the oxidative phosphorylation process, which receives electrons from the oxidation of NADH.

In summary, the competition for the cofactor NADH by the bioreduction causes a decrease in the availability of this cofactor for oxidative phosphorylation, which leads to a decrease in the consumption of $O_2$ and production of ATP, which is hydrolyzed and its energy is liberated as heat. However, the bioreduction may not occur exclusively in the mitochondrial matrix and may also occur in the cytosol, by consuming the cofactors NADH or NADPH.

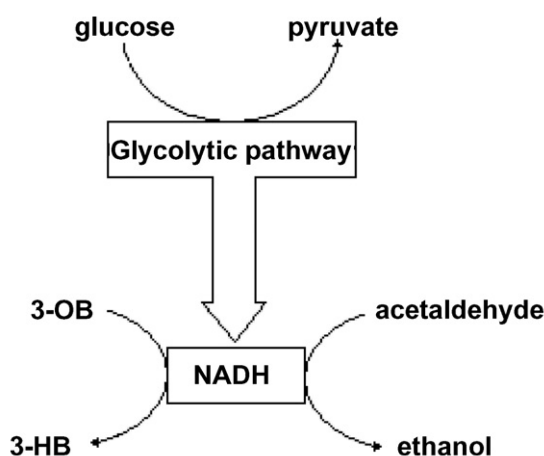
Table 1 shows that the production of ethanol is the same for the bioreduction and for the control process. The fermentative process is known to occur by the decarboxylation of pyruvate into acetaldehyde which is reduced to ethanol, using the cytosolic cofactor NADH regenerated by the glycolytic pathway. Therefore, if the bioreduction process is occurring in the cytosol, it could directly compete with the fermentative process for the cofactor NADH from the glycolytic pathway (Scheme 2).

This competition could lead to a decrease in ethanol production. However, this behavior was not experimentally observed. Furthermore, the consumption of the cofactor NADPH could cause a deviation of the glycolytic pathway flow to the PPP,

Table 1
Experimental data of the control and bioreduction metabolic process in different experimental conditions obtained during 20 h

| Experiments | $Q^b$ (kJ $g^{-1}$) | $O_2$ (mol) | Glucose consumed (mol) | Ethanol produced (mol) |
|---|---|---|---|---|
| Aerobic control (C-AG)[a] | 3.86 ± 0.02 | 0.205 ± 0.007 | 0.254 ± 0.009 | 0.206 ± 0.006 |
| Aerobic bioreduction (B-AG)[a] | 3.06 ± 0.02 | 0.162 ± 0.005 | 0.168 ± 0.007 | 0.202 ± 0.005 |

[a] C, control; B, bioreduction; A, aerobic.
[b] Heat liberated by the system (*Q*/mass of dry cells).




Scheme 2. Schematic representation of the competition between ethyl 3-oxobutyrate and acetaldehyde by the cofactor NADH.

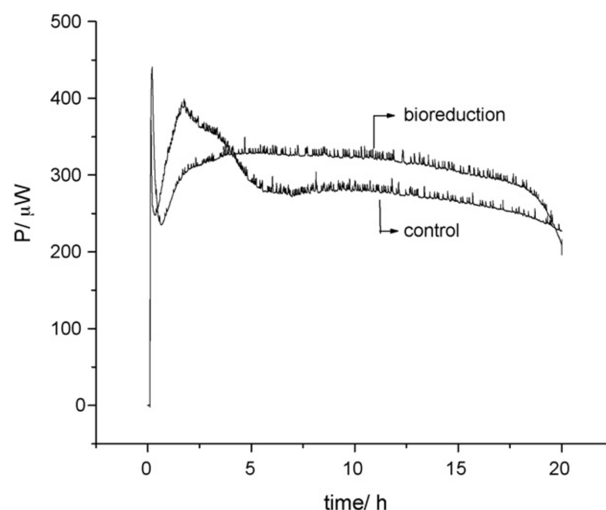



Fig. 2. Power–time curve for the anaerobic experiments.

resulting in an increase of the amount of liberated heat in comparison with the control. If this were the case, we should not observe a correlation between the decrease of the consumption of $O_2$ and the heat produced (Table 1).

Therefore, all the experimental data indicate that bioreduction under aerobic conditions and under nutritional starvation occurs compartmentalized in the mitochondrial matrix and consumes the cofactor NADH regenerated by the respiratory process. This behavior can be due to the possible presence of a homologous enzyme, β-hydroxybutyrate dehydrogenase, which is derived from the decomposition of lipids and amino acid (ketonic bodies) and is responsible for the reduction of the 3-oxobutyrate ion [27]. Due to the high structural similarity between the 3-oxobutyrate ion and 3-OB, this enzyme may promote the reduction of 3-OB with high efficiency. The conversion of 3-OB into 3-HB, under these experimental conditions was 100%.

### 3.2. Anaerobic experiments

The AnGs were performed by passing nitrogen through the reaction medium. In order to eliminate the contribution of the respiratory catabolism on the bioreduction, the same experiments were also performed under anaerobic conditions to evaluate this process without the participation of the cofactor NADH which is regenerated in the mitochondrial matrix. Therefore, under anaerobic conditions, the catabolism is expected to take place exclusively in the cytosol.

The AnGs also presented a *P–t* curve similar to those of AGs with a multiphasic behavior, with the absence of a second phase in the bioreduction process (B-AnG) (Fig. 2).

Table 2 presents the heat and ethanol produced, and glucose consumed by the anaerobic bioreduction (B-AnG). After the experimental period of 20 h, the heat production (*Q*) observed for the bioreduction was very similar to the value observed for the control process; the heat production of the control (C-AnG) was only 5% lower than that for the bioreduction (B-AnG).

As shown in Scheme 1, the regeneration of the cofactor NADH under anaerobic conditions is exclusively associated with the glycolytic pathway. Assuming that the bioreduction of 3-OB is associated with the oxidation of the cofactor NADH, this process should not influence the energetic yield of the fermentative metabolism, since the ATP regeneration in this pathway is not directly associated with the cofactor NADH, as occurs in the respiratory pathway. Consequently, the bioreduction of 3-OB and the fermentative process directly compete for the same cytosolic cofactor NADH, which is regenerated in the glycolytic pathway as shown in Scheme 2. It was also observed that the control and the bioreduction presented very similar ethanol production rates, which is a strong indication that the bioreduction, in the absence of mitochondrial activity (anaerobic condition), does not occur with the consumption of cofactor NADH of the glycolytic pathway (Scheme 1).

Another possibility of the bioreduction to occur in the cytosol would be the catabolism of the glucose by deviation of the PPP (Scheme 1) which is responsible for the regeneration of the cofactor NADPH. PPP is only a deviation of the glycolytic pathway, which occurs in the second glycolytic stage (glucose-6-phosphate). The products of the PPP, frutose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (GAP) returns to the glycolytic pathway as intermediates. F6P returns to the glycolytic pathway

Table 2
Experimental data of the control and bioreduction metabolic process in different experimental conditions obtained during 20 h

| Experiments | $Q^{b}$ (kJ $g^{-1}$) | $O_2$ (mol) | Glucose consumed (mol) | Ethanol produced (mol) |
|---|---|---|---|---|
| Anaerobic control (C-AnG)[a] | 3.88 ± 0.03 | – | 0.396 ± 0.008 | 0.591 ± 0.008 |
| Anaerobic bioreduction (B-AnG)[a] | 4.09 ± 0.02 | – | 0.390 ± 0.006 | 0.545 ± 0.009 |

[a] C, control; B, bioreduction; An, aerobic.
[b] Heat liberated by the system (*Q*/mass of dry cells).

Table 3
Molar ratio and rate constant of the bioreduction process under aerobic and anaerobic conditions

| Bioreduction | $n_{3\text{-HB}}$ (mol) | $n_{\text{Gluc}}$ (mol) | Ratio ($n_{3\text{-HB}}/n_{\text{Gluc}}$) | Bioreduction rate (mmol $h^{-1}$) |
|---|---|---|---|---|
| B-AG | 0.077 ± 0.001 | 0.168 | 0.458 | 4.37 |
| B-AnG | 0.072 ± 0.001 | 0.390 | 0.184 | 3.43 |

as a third intermediate compound, soon after the deviation point and follows the normal course of this pathway until the final product, pyruvate. The return at this point of the PPP should not promote alterations in the cytosolic ATP concentration and since all stages of the consumption and production of ATP are present, the expected energetic yield would be similar or even equal to that obtained in the glycolytic pathway, i.e., without the existence of this deviation.

However, the other intermediate compound, GAP, returns to the glycolytic pathway as the fifth intermediate. This eliminates a stage in the consumption of ATP that can positively influence the energetic yield of the metabolism. These are the theoretical foundations that we used to explain with the experimental data, i.e., the non-alteration in the ethanol production rate and the elevation in the amount of heat produced during the bioreduction process in comparison with the respective control (Table 2).

Table 3 presents the molar ratio between 3-HB produced and glucose consumed which represents the process efficiency regarding the energy source. The ratio $n_{3\text{-HB}}/n_{\text{gluc}}$ represents the amount of 3-HB produced per mole of glucose consumed by the microorganism and the values indicate that the bioreduction under aerobic conditions is about 2.5 times more efficient than that for the anaerobic one. These data supports the conclusion that the bioreduction occurs more efficiently when associated with the respiratory pathway, where the rate of regeneration of the reduction cofactor is larger than that in the glycolytic and PPP pathways. The conversion value of 3-OB into 3-HB under this condition was 93.5%. From the concentration values of 3-HB produced vs. time, we calculated the rate constant of the bioreduction reaction which are presented in Table 3.

The kinetics of the bioreduction under aerobic and anaerobic conditions gives straight lines with correlation coefficients 0.9797 and 0.9958, respectively. The rate constant values also reinforce the indication that the bioreduction occurs preferentially under aerobic rather than anaerobic conditions since, in aerobic conditions the bioreduction is 20% of that under anaerobic conditions.

## 4. Conclusion

The main objective of this study using living cells was to investigate the compartmentalization aspects of the bioreduction and to identify the cofactors that take part in this process, *in vivo*. The values of $Q$ liberated and $O_2$ consumed suggests that the bioreduction process of 3-OB can occur under aerobic conditions in the mitochondrial matrix and under in anaerobic conditions in the cytosol. This information is new, since there are no previous conclusive studies about which compartment of the cell and which cofactors take part in the bioreductions in living systems. The literature does report experiments that were performed with isolated enzymes and cofactors but these studies do not reflect *in vivo* conditions.

We believe that these data are of importance for a better comprehension of the bioreduction, since such knowledge allows the control of the bioreduction processes through the experimental conditions of the reaction media.

## Acknowledgments

The authors thank CNPq-Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasil, for financial support and Fred Yukio Fujiwara for critical comments.

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