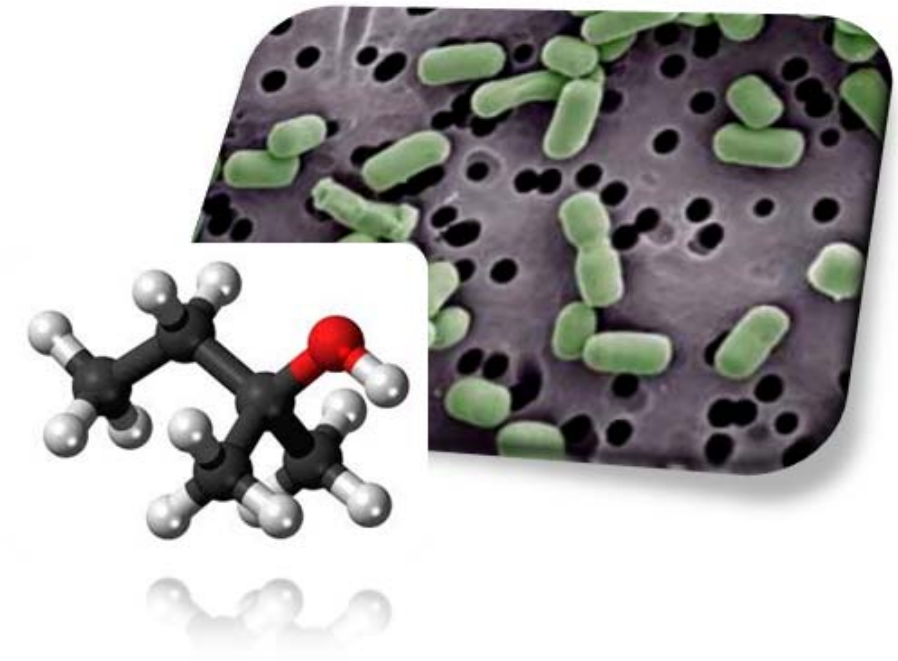
CHALMERS



# Butanol production in Lactic acid bacteria

*Master of Science Thesis in Biotechnology*

*FRANCISCA LAMEIRAS*

Department of Chemical and Biological Engineering

*Systems & Synthetic Biology*

CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden, 2012

Thesis for the Degree of Master of Science

# Butanol production in Lactic acid Bacteria

Francisca Lameiras

Examiner: Professor Christer Larson
Supervisors: PhD student Payam Ghiaci and Professor Joakim Norbeck

Department of Chemical and Biological Engineering
Systems Biology
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2012

# Preface

The present Master thesis project was carried out in Systems Biology research group, at Chemical and Biological Engineering Department of Chalmers University of Technology, as part of my ERASMUS exchange period from Minho University.

The objective of this project was to characterize proteins, shown to be present in certain lactic acid bacteria, which are able to convert a natural product of yeast metabolism, 2,3-butanediol, to 2-butanol. Diol dehydratase and alcohol dehydrogenase are two enzymes involved in this process.

The project was divided in two parts. The first part includes screening for butanol production of different *Lactobacillus brevis* strains, whereas in the second part, the enzyme activity was studied.

The ultimate goal of this project, although not part of this MSc thesis work, would be to express proteins from *Lactobacillus spp.* in yeast *Saccharomyces cerevisiae* for production of 2-butanol in this host.

Gothenburg, June 2012

*Francisca Lameiras*

# Abstract

Butanol as an alternative liquid fuel has many advantages compared to ethanol, the number one biofuel used nowadays. This 4-carbon alcohol has higher energy content, lower enthalpy of vaporization, lower water adsorption, better blending characteristics, it is less corrosive, and it can be used in conventional engines without any modification.

The aim of this project was to understand and characterize proteins involved in the 2-butanol production in *Lactobacillus brevis* strains: diol dehydratase dependent on coenzyme B12 and alcohol dehydrogenase.

Strains of *L. brevis* (SE20 and SE31) originally isolated from SEKAB E-Technology biorreactors (Örnskoldsvik, Sweden), were studied with respect to their ability to reduce 2,3-butanediol to 2-butanol. Later, enzyme activity in these strains was determinated.

Diol dehydratase was studied using the MBTH method based on the ability of ketones and aldehydes to react with it, forming azine derivatives which can be determined spectrophotometrically at 305 nm. The presence of substrate in the medium, has a positive control effect in enzyme activation, as the activity values increased about twice. Due to a limit project time, this enzyme wasn't characterized in terms of Km and Vmax.

Alcohol dehydrogenase activity was determined by measuring the reduction of $NAD^+$ to NADH at 340 nm. The parameters, maximum velocity and Michaelis constant were determined graphically by three linear methods (Lineweaver-Burk, Eadie Hofstee and Hanes-Woolf), for forward and reverse butanol reaction, showing that both reaction directions were active.

# Acknowledgements

This work was supported by Systems Biology Group in Chalmers University of Technology, by supervision, guidance and encouragement of my examiner Professor Christer Larsson, to whom I sincerely express my gratitude.

I am also grateful to PhD student Payam Ghiaci, for dedicating his time helping me and advising me with this project.

I extend my thanks to Professor Joakim Norbeck, Doctor Il-kwon Kim, Doctor George Anasontzis and all the Systems and Synthetic Biology Group for their assistance along the work.

Finally I thank my family and friends for being present and support me since the beginning.

# List of abreviations

**ADH** – Alcohol dehydrogenase

**Ado-B12 –** Adenosylcobalamin (Coenzyme B12)

**DTT** - Dithiothreitol

**HPLC** – High Performance Liquid Chromatogrphy

**Km –** Michaelis constant, substrate concentration where V = 1/2Vmax

**LbADH** – *Lactobacillus brevis* alcohol dehydrogenase

**MEK** – Methyl Ethyl Ketone or butanone

**MRS** – Man Rogosa and Sharpe medium

**$NAD^+$** - Nicotinamide adenine dinucleotide oxidized form

**NADH –** Nicotinamide adenine dinucleotide reduced form

**OD** – Optical Density

**SADH** – Secondary Alcohol Dehydrogenase

**SM2** – Synthetic Medium

**Vmax** – Maximum velocity

# Table of Contents

# 1. Introduction and Background

## 1.1. Motivation and aim

In latest years, there has been a gradual increasing of solid waste due to the increasing urbanization and population. Waste materials are being generated from different sources like manufacturing processes, industries and municipal solid wastes. However, waste-to-energy technologies can convert this waste matter into various forms of fuel that can be used to provide energy, by four major methods for conversion of organic wastes to synthetic fuels: hydrogenation, pyrolysis, gasification and bioconversion (Dermibas *et al.* 2011).

Biofuels including bioethanol, biobutanol, biodiesel, vegetable oils, biomethanol, pyrolysis oils, biogas and biohydrogen are receiving an increasing public and scientific consideration, motivated by factors such as uncertainties related to oil price as well as environmental issues. Biofuels offer one of the few options for fossil fuel replacement in the short-term, once they have the potential to offer energy security, greenhouse gas savings, foreign exchange savings, and socioeconomic issues related to the rural sector (Taylor, 2008; Dermibas, 2009 and Jin *et al.* 2011).

The major difference between biofuels and petroleum feedstocks is the oxygen content and the fact that biofuels are locally available, accessible, sustainable and reliable fuel obtained from renewable sources (Dermibas, 2009).

Biofuels are classified into first and second generation biofuels. In the first generation, sugarcane and cereal grains are used as raw materials, while in the second generation, lignocellulosic materials (e.g. agriculture and forest wastes) are used as feedstocks. It should be noted that the raw materials used for first generation are food and land competitive while second generation biofuels are non-edible biomass. For that reason, there is an increased focus on second generation biofuels due to huge availability of cheaper raw materials (Kumar and Gayen, 2011).

Biobutanol or biobased butanol fuel is one of these potential fuel replacements that have been considered as the next generation biofuel. More than a dozen companies are currently focused on developing it on commercial scale (Biobutanol.com, 2010). This fuel has little or no impact on food supply and is able to compete with $80 bbl oil (Biobutanol.com, 2010). In the future, biobutanol can potentially turn into an attractive,

economic and sustainable fuel as petroleum oil leads towards expensive fuel due to diminishing oil reserves and an increase of green house gases in the atmosphere (Kumar & Gayen, 2011).

In 2007, the US Congress passed the Energy Independence and Security Act (EISA 2007), which calls for the annual production of 36 billion gallons of biofuels by the year 2022 (Swana *et al.* 2011).

In 2009, the European Commission has approved the creation of a joint venture between the american companies BP Biofuels North America and DuPont. It was decided that the new company (Butamax Advanced Biofuels) will develop and market technologies for producing biobutanol (primarily in the US) and the company will also make and sell biobutanol in limited quantities. The technology demonstration facility located at the BP Saltend site in the UK, started its operations in 2010, and the first commercial plant is expected to be operational by 2013. Further, BP Biofuels and enzyme developer Verenium jointly own a commercial cellulosic ethanol project in Highlands County, Florida. The plant has a capacity of 36 M gallon/year, and will mainly utilize varieties of sorghum and sugar cane as feedstock (BP & DuPont, 2009a and BP & DuPont, 2009b).

US firms Gevo, Cobalt Technologies, and Butamax Advanced Biofuels expect bio-butanol to start penetrating both the biofuels and biochemicals markets by 2012 (US firms Gevo, Cobalt Technologies and Butamax Advanced Biofuels, 2010).

In this project, bacterial butanol production is studied. The aim is to characterize proteins, shown to be present in certain lactic acid bacteria, which are able to convert a natural product of yeast metabolism, 2,3-butanediol, to 2-butanol. Diol dehydratase and alcohol dehydrogenase are the two enzymes involved in this process.

Consequently, a selection of strains and isolates were tested under different growth conditions for their production of butanol. Later on, kinetic characterization of enzymes converting 2,3-butanediol to butanone and butanone to butanol were done for the bacteria that showed the highest productivity. This characterization involved different substrates, co-factors, and measurements were performed in “forward” as well as “reverse” reaction.

# 1.2. Butanol

## 1.2.1. Brief history of butanol production

Butanol production trail can be followed on table 1, since its discovery, to actual times and relevance.

Table 1 – History of the ABE fermentation
(adapted from Edinburgh Napier University, 2012).

| | |
|---|---|
| **1862** | Louis Pasteur first recorded the formation of butanol by a microorganism. |
| **1905** | Schardinger isolated organism producing acetone, ethanol and acetic acid. |
| **1910** | British company, Strange and Graham Ltd embarked on project to make synthetic rubber leading to search for solvent-producing bacteria. |
| **1911** | Fernbach and Strange issued 2 British patents for solvent fermentation using Fernbach strain and potato as a substrate. |
| **1912**<br>**1914** | Chaim Weizmann, working at Manchester University, isolated strain with high solvent yield (Clostridium acetobutylicum). |
| **1915** | Weizmann issued British patent for process using C. acetobutylicum and maize mash in addition to potato: First ABE process. |
| **1914**<br>**1918** | WWI shortage of acetone, required to produce cordite for munitions, stimulated industrial-scale application of the ABE process. Weizmann selected to head the acetone project by the British Government. Towards the end of the war, ABE plants were relocated to Canada and USA due to British food shortages. |
| **1920**<br>**1933** | US Prohibition led to shortage of amyl acetate for use as a solvent in car lacquers. With butyl acetone as a replacement there was a new demand for butanol. |
| **1939**<br>**1945** | WWII increased demand for acetone. |
| **1960** | Rise of the petrochemical industry provided a competitive source of solvents. This, coupled with increasing feedstock costs, led to the demise of the ABE industry in UK and USA. |
| **1980** | Last plants ceased operating in South Africa and USSR. |
| **1990** | First clostridia workshop on solvent and acid-forming clostridia. Continues to be held every 2 years. |
| **2005** | Butanol recognized as a biofuel when Dr David Ramey drove car across the USA fuelled exclusively on butanol. |
| **2007** | BP and Dupont announce plans to develop butanol as a biofuel. |

Traditionally, ABE fermentation, that is a bacterial fermentation process to produce acetone, n-butanol and ethanol from starch, was largely used to synthesize acetone in World War II as a solvent for preparation of nitrocellulose explosive (cordite), although this production started already with WWI (table 1).

In the meantime, butanol was gradually being more used also as a solvent in rubber production and quick-drying lacquer to render a good finish on car bodies. This way, the production of butanol in the USA increased in the 19$^{th}$ century, even though at the end of the century, microbial production of butanol and acetone declined rapidly due to availability of cheaper crude oil (Kumar and Gayen, 2011).

In recent times a greater prominence is placed on biobutanol production after recognizing its potential as biofuel in 2005 when Dr David Ramey drove car across the USA fuelled exclusively on butanol (Edinburgh Napier University, 2012).

### 1.2.2. Butanol isomers

Alcohols are classified by the occurrence of a hydroxyl group (–OH) where the alcohol molecule has one or more oxygen, which decreases the energy content; it can be said that any of the organic molecules of the alcohol family may be used as a fuel (Dermibas *et al.* 2011).

Butanol ($C_4H_9OH$) is an alcohol with 4 carbons, where the carbon atoms can form either a straight-chain or a branched structure, resulting in different properties and therefore in different isomers: n-Butanol (butan-1-ol, 1-butanol, n-butyl alcohol), isobutanol (2-methylpropan-1-ol, isobutyl alcohol), sec-Butanol (butan-2-ol, 2-butanol, sec-butyl alcohol), tert-Butanol (2-methylpropan-2-ol, tert-butyl alcohol) (Jin *et al*. 2011). The molecular structure and the main applications of butanol isomers are listed in figure 1.

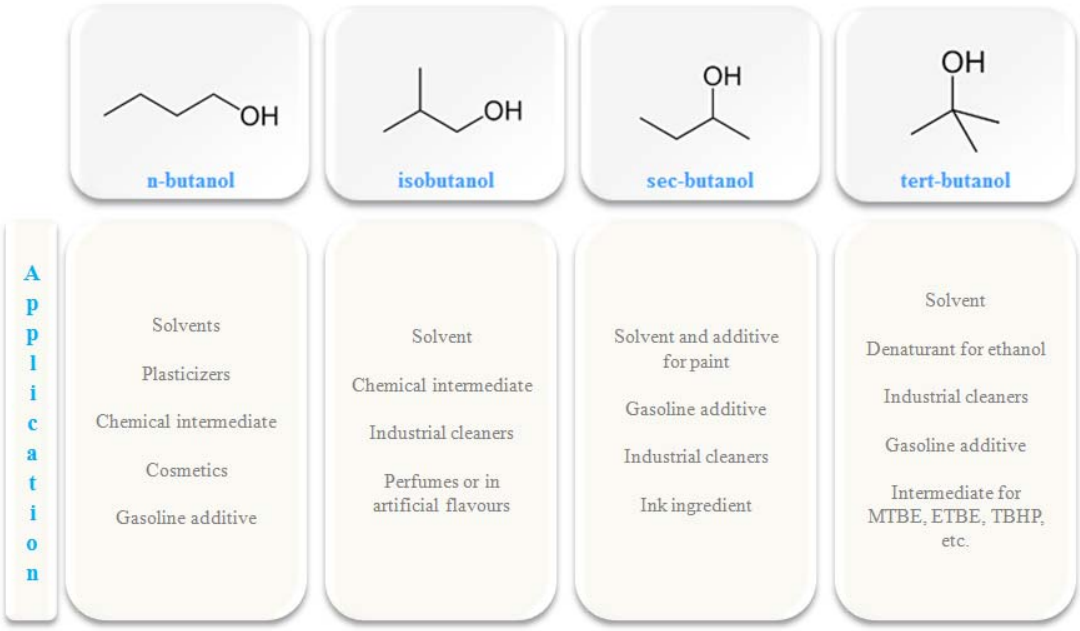



Figure 1 – Butanol isomers and main applications (adapted from Jin *et al*. 2011).

The different structures of butanol isomers have a straight impact on the physical and chemical properties, which are summarized in table 2. Even though the properties of butanol isomers are different, the main applications are similar in some aspects, such as being used as solvents or gasoline additives.

Table 2 – Butanol isomers and properties
(adapted from Jin *et al*. 2011 and Butamax$^{TM}$ Advanced Biofuels, 2010).

| | 1-butanol | Iso butanol | 2-butanol | Tert butanol |
|---|---|---|---|---|
| Density (kg/m$^3$) | 809.8 | 801.8 | 806.3 | 788.7 |
| Research octane number | 94 | 109 | 110 | - |
| Motor octane number | 81 | 90 | 93 | - |
| Boiling temperature (◦C) | 117.7 | 108 | 99.5 | 82.4 |
| Enthalpy of vaporization (kJ/kg) | 582 | 566 | 551 | 527 |
| Melting point (◦C) | -90 | -101,9 | -115 | 25-26 |
| Self-ignition temperature (◦C) | 343 | 415.6 | 406.1 | 477.8 |
| Flammability limits vol.% | 1.4–11.2 | 1.2–10.9 | 1.7–9.8 | 2.4–8 |
| Viscosity (mPa s) at 25 ◦C | 2.544 | 4.312 | 3.096 | – |

According to the previous table, tert-butanol would be an interesting isomer to explore as a biofuel, once it has the lowest enthalpy of vaporization; however this

isomer is unique among all the isomers because it tends to be a solid at room temperature, since it has a melting point slightly above 25ºC.

Thus, 2-butanol takes place at the interest of a fuel with less energy requirement, and for that reason, this isomer was explored along this project.

### 1.2.3. 2-Butanol: properties, advantages and disadvantages

Even though on biofuel's field, bioethanol is currently the leading candidate to replace gasoline as transportation fuel, biobutanol has been recently gaining favor over ethanol (Nair *et al*. 2008).

As mentioned along this section, when comparing ethanol to butanol, butanol has better properties as fuel such as higher energy content, higher octane number, lower enthalpy of vaporization, lower solubility in water, less corrosive and higher vapor pressure, thus promising a better and long-term solution to transportation fuel requirements (Nair *et al*. 2008).

Some other properties are also compared in table 3, between 2-butanol and ethanol, gasoline and diesel.

Table 3 – Butanol isomers and properties
(adapted from Jin *et al*. 2011).

| | Gasoline | Diesel | Ethanol | 2-butanol |
|---|---|---|---|---|
| Molecular formula | $C_4$-$C_{12}$ | $C_{12}$-$C_{25}$ | $C_2H_5OH$ | $C_4H_9OH$ |
| Density (kg/m$^3$) | 720-780 | 820-860 | 790 | 806 |
| Octane number | 80-99 | 20-30 | 108 | 110 |
| Boiling temperature (◦C) | 25-215 | 180-370 | 78.4 | 99.5 |
| Enthalpy of vaporization (kJ/kg) | 380-500 | 270 | 904 | 551 |
| Self-ignition temperature (◦C) | 300 | 210 | 434 | 406.1 |
| Flammability limits vol.% | 0.6-8 | 1.5-7.6 | 4.3-19 | 1.7–9.8 |
| Viscosity (mPa s) at 25 ◦C | 0.4-0.8 | 1.9-4.1 | 1.08 | 3.096 |

The enthalpy of vaporization i.e., the energy required to transform a given quantity of a substance from a liquid into a gas at a given pressure, is much lower than ethanol and close to the value for gasoline (Jin *et al*. 2011). In other words, compared

with gasoline which has a low enthalpy of vaporization, the enthalpy of vaporization of ethanol equates to a 34% loss in energy density when using ethanol in place of gasoline; however, the enthalpy of vaporization of butanol is almost 90% of the energy density of gasoline (Wu *et al*., 2008).

In addition, one other significant advantage of biobutanol against bioethanol is the ability of being used directly in the current design of internal combustion engines without any modifications (Nair *et al*. 2008, and Jin *et al.* 2011). Biobutanol can be used as 100% fuel or blended with gasoline or diesel at 10% and can be transported in the existing pipeline infrastructure (Biobutanol.com, 2010).

Moreover, the transportation of butanol is also an advantage against ethanol's transportation. The gasoline-ethanol blend in actual pipelines cannot carry it due to ethanol's high miscibility with water (Swana *et al*. 2011).

Regardless of butanol's advantages, there are two major issues on its production. First, the natural producers of butanol are anaerobic gram positive bacteria of the genus *Clostridia*, which are relatively difficult to culture; but butanol production in organisms such as *Escherichia coli* or *Saccharomyces cerevisiae* could improve some problems associated with *Clostridial* fermentation (Atsumi *et al.* 2008 and Steen *et al*. 2008). Especially *S. cerevisiae* is a more resilient organism than bacteria and consequently it may be possible to engineer it for higher tolerance to butanol than may be possible for any bacteria; moreover is used extensively in industrial fermentations (Nair *et al*. 2008). A strategy for biobutanol production has been to manipulate the amino acids biosynthetic pathways in *E. coli.* or to introduce genes from natural butanol producers like *Clostridia* into *S. cerevisiae* or *E. coli.* hosts (Atsumi *et al.* 2008 and Steen *et al*. 2008).

The second disadvantage is the toxicity/inhibition to bacteria at concentrations over 20 g/L, far below its solubility in water of 70 g/L (Nair *et al*. 2008). Butanol toxicity is mainly caused by its hydrophobic nature that increases the fluidity of the cell membrane (García *et al*. 2011). As a result, the function of the cell membrane as a controlling barrier between cell interior and exterior is affected (Dürre, 2011 and Ezeji *et al*. 2007).

These problems associated with the bioproduction of butanol result in a low butanol titer. Moreover, the economics of the biobutanol production is mainly dependent on the cost of the fermentation substrate (García *et al*. 2011 and Ezeji *et al*.

As a resume, the main challenges in biobutanol production are low butanol titer, availability of compatible feedstocks, and product inhibition. These obstacles are being addressed using several metabolic engineering strategies, and integrated continuous fermentation processes with efficient product recovery techniques like gas stripping (Kumar and Gayen, 2011).

Throughout the past two decades, research has been carried out on the use of alternative fermentation and product recovery techniques for biobutanol production (Ezeji *et al*. 2007b). These techniques involve the use of immobilized and cell recycle continuous bioreactors and alternative product recovery technique like adsorption, gas stripping, ionic liquids, liquid–liquid extraction, pervaporation, aqueous two-phase separation, supercritical extraction or perstraction (Ezeji *et al*. 2007b).

### 1.2.4. Butanol producing microorganisms

In 1862, Pasteur discovered that butanol was a natural product of anaerobic bacteria (Pasteur, 1862). Several species of Clostridium bacteria (rod-shaped, spore-forming, Gram positive and strict anaerobe) are capable of metabolizing different sugars, amino and organic acids, polyalcohols and other compounds to butanol, acetone and isopropanol; but butanol, being of relatively high value, is usually the most desired product (García *et al*. 2011, Ezeji *et al*. 2007a and Dürre, 2011).

A classic characteristic of the clostridial solvent production is the biphasic fermentation. The first phase is the acidogenic phase that occurs during the exponential growth phase in which the acid forming pathways are activated, and acetate, butyrate, hydrogen, and carbon dioxide are produced as major products (Jin *et al.* 2011). The second phase is the solventogenic phase during which acids are reassimilated and used in the production of butanol and other solvents like acetone and ethanol (Jin *et al.* 2011).

Renewable and cost-effective cellulosic materials as feedstocks for butanol production could be a progression in economic issues. *Clostridium beijerinckii that has*

*been* genetically modified to become a hyper-butanol producing strain is being explored as promising strain to produce biobutanol from cellulosic materials (Kumar and Gayen, 2011). Furthermore, high biobutanol titer is being focused through genetic modifications of other Clostridia as well as other microorganisms such as, *E. coli, S. cerevisiae, Pseudomonas putida*, and *Bacillus subtilis*, in both aerobic and anaerobic fermentation (Kumar and Gayen, 2011 and Ezeji *et al*. 2007a).

Berezina *et al.* 2010 demonstrated that recombinant *L. brevis* strains were able to synthesize up to 0,3 g/L (or 4,1 mM) of butanol on a glucose-containing medium. The butanol tolerance of *L. brevis* was the key motivation for choosing this bacterium as a host for reconstructing the clostridial n-butanol metabolic pathway.

In another study, among 19 strains of *L. brevis* and *L. buchneri*, four strains were found to produce significant amounts of 2-butanol at the expense of meso-2,3-butanediol when grow in a medium containing 2,3-butanediol (Radler and Zorg, 1986).

Similarly, in Speranza *et al.* 1997, three strains of *Lactobacillus spp.* from food were also found to have the ability to convert meso-2,3-butanediol into the secondary alcohol.

Thus, it can be understood that there are two types of microorganisms that produce butanol; the native producers like Clostridium spp. that produce butanol directly from sugar and the non-native butanol producers such as lactic acid bacteria and yeast. Lactic acid bacteria produce butanol from 2,3-butanediol, and yeast produce only 2,3-butanediol (Speranza *et al.* 1997).

In the present study, two strains of *L. brevis* were explored for the production of 2-butanol from 2-butanone and 2,3-butanediol.

A first screen of butanol forming capacity of different strains including the ones that performed best according to the literature. However, some of our own isolates turned out to be even better than the ones reported in the literature

### 1.2.5. Butanol Pathway

As previously said, bacteria from the genre *Clostridia spp.* are the natural producers of butanol; however, butanol production can also occur in other bacteria such as lactic acid bacteria.

In figure 2, part of the pathway of *Lactobacillus spp.* is shown. The two reactions presented, the reduction of 2,3-butanediol to 2-butanone and the reduction of 2-butanone to 2-butanol, are studied in the present project.

According to Speranza *et al*. 1997, 2-butanol is produced mostly, if not completely, by a two step enzymatic activity of *Lactobacilli spp.* (figure 2), on which the dehydration of meso-2,3-butanediol is much slower than the second reaction.

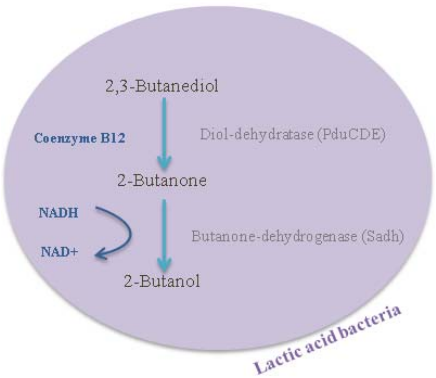



Figure 2 – Butanol pathway in lactic acid bacteria (adapted from Speranza *et al.* 1997).

According to Kumar and Gayen, 2011, the fermentation process is supported on the level of metabolic activities of the organism, and understanding this metabolic network is essential to engineer strains leading better productivity.

As referred in section 1.2.3, to solve butanol's disadvantages, pathway manipulation in resistant microorganisms like *S. cerevisiae* is being studied (figure 3).

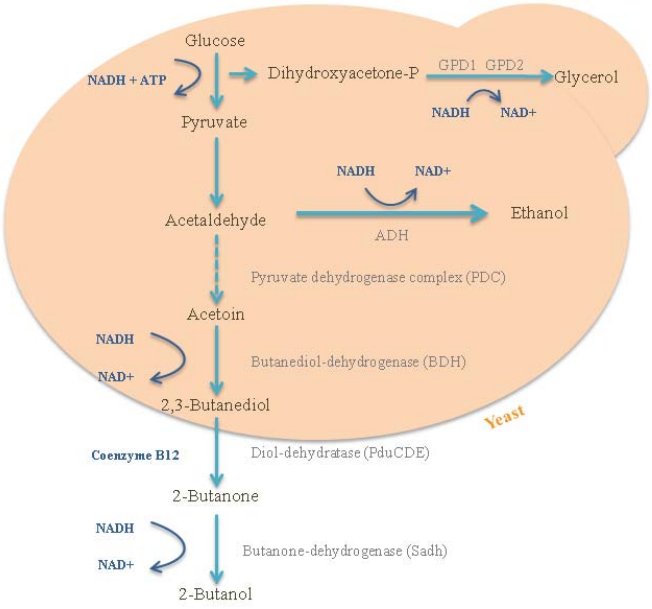



Figure 3 – Butanol pathway engineered in yeast.

The present study is incorporated in a PhD project of biobutanol production by metabolic engineering of the yeast *S. cerevisiae*, where the overall goal is to transform yeast *S. cerevisiae* into an efficient production organism for biobutanol. This will be done by introducing genes required for butanol production from 2,3-butanediol as illustrated in figure 3.

Concerning the source of 2,3-butanediol, it can be said that the pathway pyruvate-diacetyl-acetoin which is common in Saccharomyces spp. has also been demonstrated to occur in lactic acid bacteria (Axelsson, 1993).

2-butanol is formed from meso-2,3-butanediol during growth of *L. brevis* culture at the same time glucose is consumed and the fermentation products lactate, acetate and ethanol are formed (Radler and Zorg, 1986). Furthermore, butanediol is only metabolized by growing cells when glucose is present as the main substrate (Radler and Zorg, 1986).

# 1.3. Enzyme activity

## 1.3.1. Fundamentals of enzyme kinetics

Enzymes catalyze the biochemical reactions in cells of all organisms and these reactions constitute the chemical basis of life.

Most enzymes are proteins – a few are ribonucleic acids or ribonucleoproteins – and the catalytic machinery is located in a relatively small active site, where substrates bind and are chemically processed into products (Frey and Hegeman, 2007).

In the simplest form an enzyme reaction can be formulated as:

$$E + S \underset{-k1}{\overset{k1}{\rightleftharpoons}} ES \xrightarrow{k2} E + P$$

A substrate S reacts with an enzyme E to form an enzyme–substrate complex ES, which is converted to product P, releasing the free enzyme, which enters into a new reaction cycle (Cornish-Bowden, 1995).

In reality, the situation is more complicated as for most enzyme reactions, more components must be considered such as two or more substrates and products, cofactors, inhibitors and activators (Bisswanger, 2008).

Forward rate constants must on average be larger than comparable reverse rate constants for any reaction in which the equilibrium constant favors the forward direction (Cornish-Bowden, 1995).

An enzyme assay is a laboratory method for measuring enzymatic activity. In this assays, some parameters should be controlled, such as the substrate saturation and the effect of temperature and pH (Cornish-Bowden, 1995).

According to Cornish-Bowden, 1995, there are two types of enzyme assays: discontinuous and continuous. In a discontinuous assay, samples are removed at intervals from the reaction mixture and analyzed to determine the extent of reaction. On

the contrary, in a continuous assay, the progress of the reaction in monitored continuously with automatic recording apparatus.

For enzymes whose molar concentration cannot be measured, either because the enzyme has not been purified or because its molecular mass is unknown, it is often convenient to define a unit of catalytic activity (Cornish-Bowden, 1995). The traditional "unit" of enzymologists is the amount of enzyme that can catalyze the transformation of 1μmol of substrate into product in 1min under standard conditions. Enzyme activity can also be presented in katal, the SI unit (1U = 16,67 nanokatals).

Specific activity is another common unit defined as the amount of enzyme that can catalyze the transformation of 1μmol of substrate into product in 1min, per mg of total protein. In an impure sample, the specific activity is lower because some of the mass is not actually enzyme (Bisswanger, 2008).

The turnover number ($k_{cat}$), or μmol product $sec^{-1}$ $\mu mol^{-1}$ of active enzyme, is the number of times each enzyme molecule carries out its catalytic cycle per second, and can be calculated from the specific activity, as demonstrated in equation 1, where $E_T$ is the total enzyme concentration which is assumed to be constant.

$$K_{cat} = \frac{Vmax}{E_T} \qquad \text{Eq. (1)}$$

In enzymology, Michaelis-Menten kinetics is one of the most used and well-known model of enzyme kinetics.

#### 1.3.1.1. Michaelis-Menten

The Michaelis-Menten equation is the fundamental equation of enzyme kinetics (equation 2), although it was originally derived for the simplest case of an irreversible enzyme reaction, converting a single substrate into a product (Bisswanger, 2008).

$$v = \frac{Vmax\,[S]}{Km + [S]} \qquad \text{Eq. (2)}$$

Where *v* represents velocity, *V*max the maximum velocity, [S] the substrate concentration and Km the Michaelis constant. In figure 4, the Michaelis-Menten plot is shown; Km is the concentration of substrate obtained when the reaction reaches half of the maximum velocity.

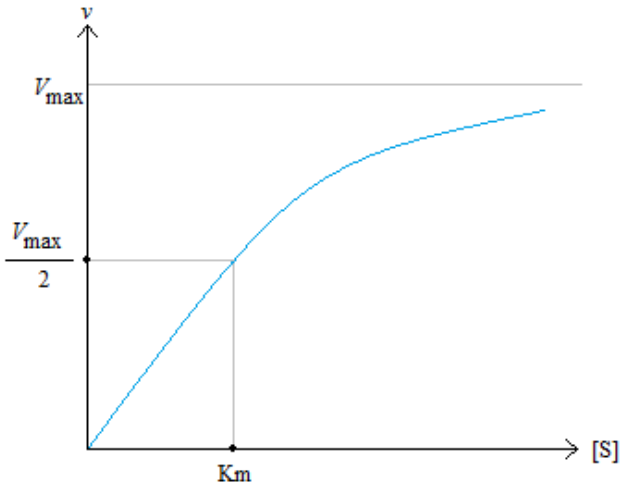



Figure 4 – Michaelis-Menten plot.

In practice, this plot is inadequate as it is difficult to draw a rectangular hyperbola accurately and to locate the asymptotes correctly (Cornish-Bowden, 1995).

Ideally one must try to find conditions in which the progress curve is virtually straight during the period of measurement, but strictly speaking this is impossible, because regardless of the mechanism of the reaction one expects the rate to change – usually to decrease – as the substrate is consumed, the product accumulates and, sometimes, the enzyme loses activity (Cornish-Bowden, 1995).

The Michaelis-Menten equation can be written in ways that allows the results to be plotted as points on a straight line, as it will be seen in sections 1.3.1.2, 1.3.1.3 and 1.3.1.4.

### 1.3.1.2. Lineweaver-Burk

The Lineweaver-Burk plot or double-reciprocal plot is the most widely used plot in enzyme kinetics, represented in equation 3.

$$\frac{1}{v} = \frac{Km}{Vmax}\frac{1}{[S]} + \frac{1}{Vmax} \qquad \text{Eq. (3)}$$

However, alike all other plots, it has some advantages and disadvantages. Lineweaver-Burk gives a misleading impression of the experimental error: for small values of v errors in v lead to enormous errors in 1/v, but for large values of v the same small errors in v lead to barely noticeable errors in 1/v (Cornish-Bowden, 1995). Lineweaver-Burk kinetics is represented in figure 5.

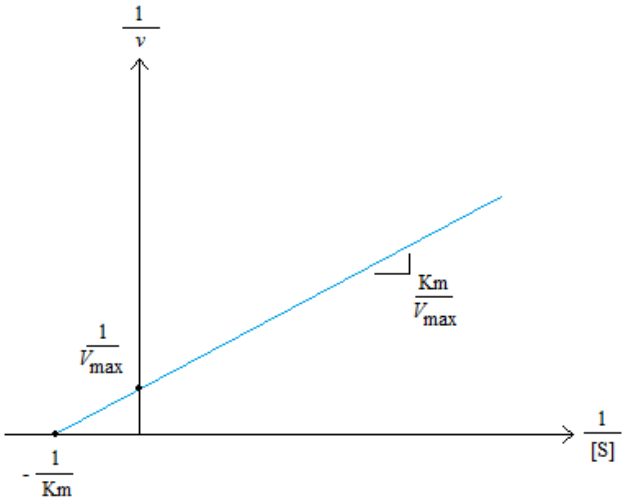



Figure 5 – Lineweaver-Burk plot.

#### 1.3.1.3. Eadie-Hofstee

Eadie-Hofstee is another straight linear plot with slope –Km (equation 4), which also derives from equation 2.

$$v = -Km\frac{v}{[S]} + Vmax \qquad \text{Eq. (4)}$$

In this case, the fact that v appears in both coordinates means that errors in v affect both of them and cause deviations towards or away from the origin rather than parallel with the ordinate axis (Cornish-Bowden, 1995). Eadie-Hofstee plot is shown in figure 6.

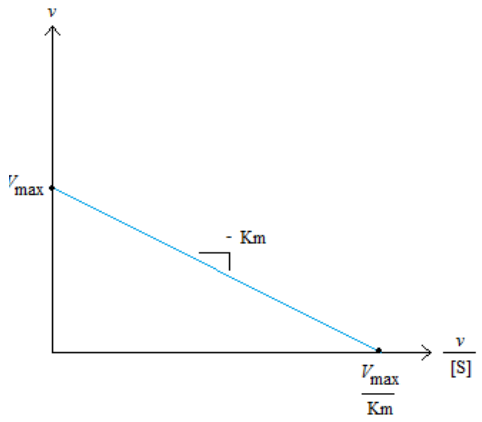



Figure 6 – Eadie-Hofstee plot.

### 1.3.1.4. Hanes-Woolf

A plot of [S]/v against [S] should also be a straight line with a slope 1/Vmax and interception Km/Vmax (equation 5).

$$\frac{[S]}{v} = \frac{1}{Vmax}[S] + \frac{Km}{Vmax} \qquad \text{Eq. (5)}$$

Over a range of [S] values, the errors in [S]/v provide a faithful reflection of those in v. This is the reason that the Hanes-Woolf plot should be preferred over the other straight line plots (Cornish-Bowden, 1995). Hanes-Woolf kinetics is illustrated in figure 7.

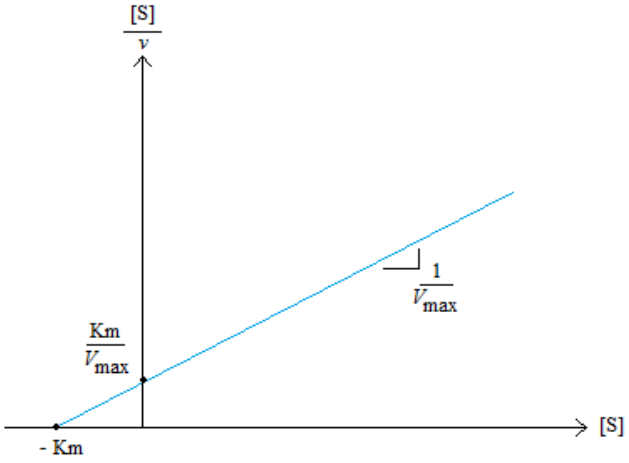



Figure 7 – Hanes-Woolf plot.

In this project, two enzymes were studied, diol dehydratase and alcohol dehydrogenase, which are responsible for the conversion of 2,3-butanediol to 2-butanone and 2-butanone to 2-butanol, respectively.

Along the following sections, then mentioned enzymes and respective coenzymes will be presented and studied.

### 1.3.2. Diol dehydratase

Diol dehydratase catalyzes the 5'-deoxy-5'-adenosylcobalamin (Ado-B12) dependent conversion of 1,2-propanediol, ethanediol, and glycerol to propionaldehyde, acetaldehyde and β-hydroxypropionaldehyde respectively – figure 8 (Toraya *et al.* 1977).

Ado-B12 or coenzyme B12, has been shown to be an intermediate hydrogen carrier in the dehydration reaction mediated by diol dehydratase (Roth *et al.* 1996). This dehydratase enzyme has a relative molecular weight of about 230000 daltons and is consisted of four different subunits (Radler and Zorg, 1986).

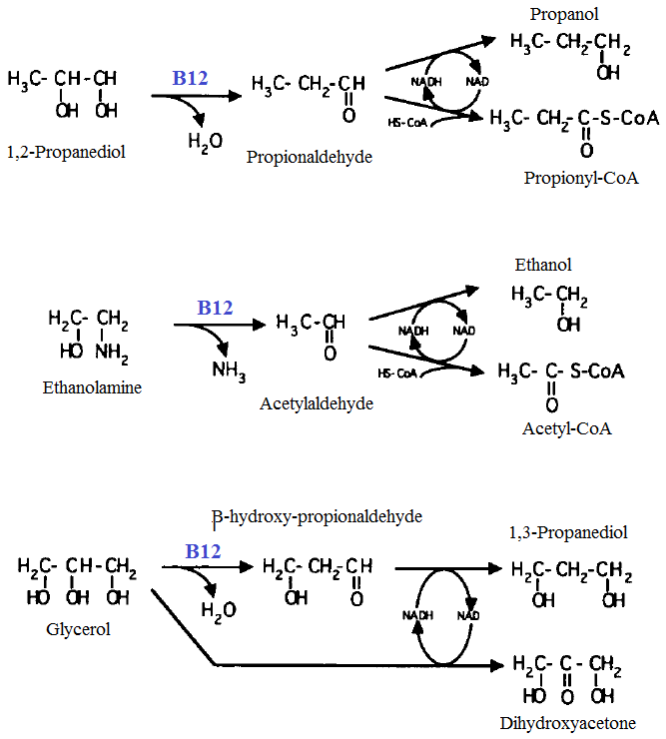



Figure 8 – Diol dehydratase reactions in different substrates (Roth *et al.* 1996).

Adenosylcobalamin is a metabolically active form of vitamin B12 (Johnson et. al 2001), and aside from the referred substrates, 2,3-butanediol is also converted by diol

dehydratase in the presence of this coenzyme (figure 9), as it will be studied in this project (Toraya *et al.* 1977).

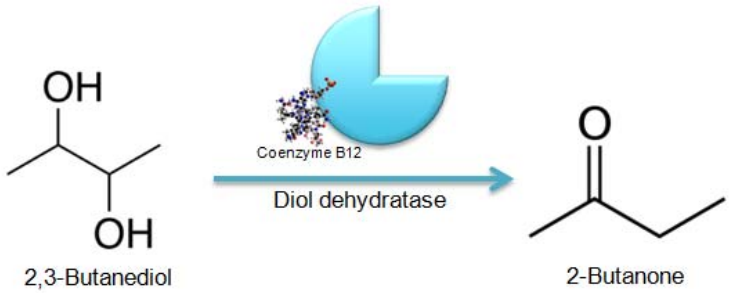



Figure 9 – Enzyme dehydratase.

According to Roth *et al.* 1996, coenzyme B12 dependent synthetic pathway may have evolved to allow anaerobic fermentation of small molecules, and its synthesis is restricted to some Bacteria and Archaea; many animals, like humans, and protists require B12 but apparently do not synthesize it – figure 10.

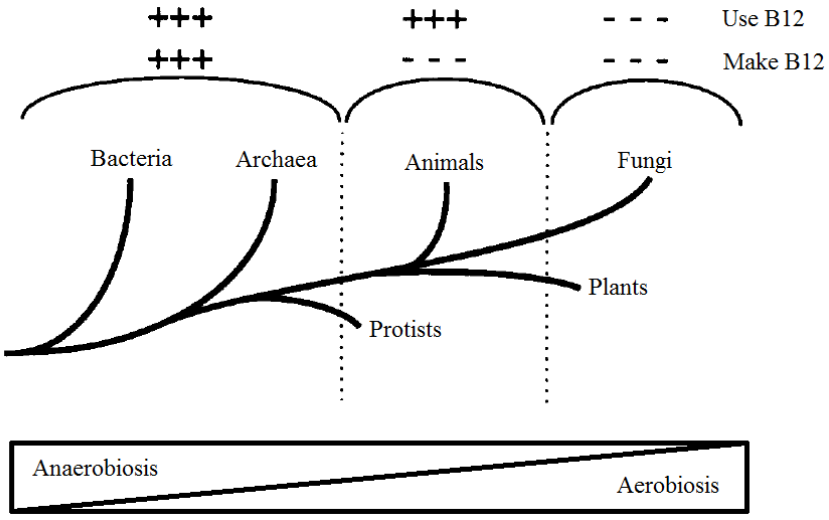



Figure 10 – Distribution of cobalamin synthesis and use among living forms (Roth *et al.* 1996).

Coenzyme B12 has a molecular weight of 1580, and at least 25 enzymes are uniquely involved in its synthesis, forming a molecule with three parts: a central ring, an adenosyl moiety, and a nucleotide loop (Roth *et al.* 1996).

The analogs investigated with the diol dehydrase system can be grouped in three categories; cobalamins type 1 that have coenzyme activity and show high affinity for

the apoenzyme; cobalamins type 2 which do not serve as coenzymes and are weak competitive inhibitors with respect to adenosylcobalamin; and cobalamins type 3 that are inactive as coenzymes but function as very efficient competitive inhibitors with respect to adenosylcobalamin (Toraya *et al.* 1977).

Adenosylcobalamin (Ado-B12) biosynthesis is encoded by the cob operon, while the neighbour operon, pdu operon, encodes 20 proteins for the B12-dependent degradation - figure 11 (Ailion and Roth, 1997).

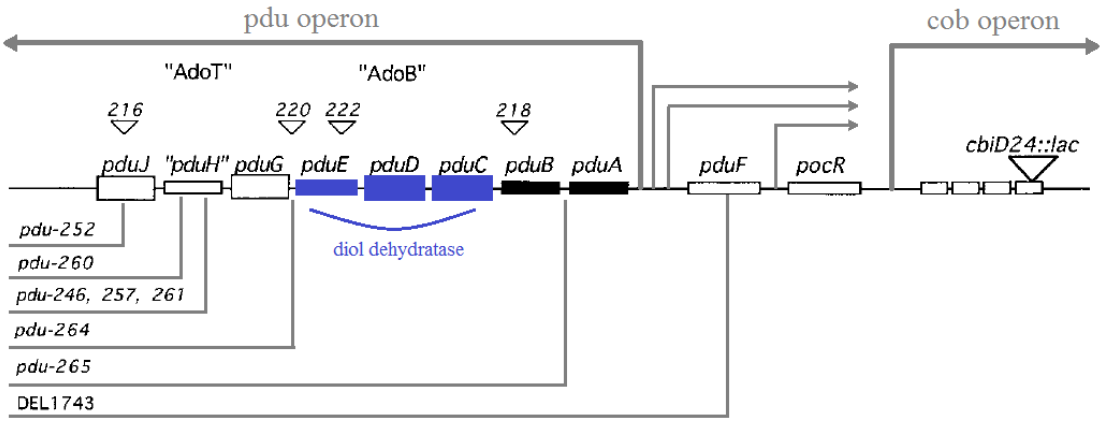



Figure 11 – Cod and pdu operons (Ailion and Roth, 1997).

The substrate, propanediol, induces the transcription of both operons (positive control), and exogenous cyanocobalamin represses the cob operon (negative control) (Ailion and Roth, 1997).

### 1.3.3. Alcohol dehydrogenase

Consistent with Burdette and Zeikus, 1994, solvent formation in anaerobic metabolism is linked to regenerating oxidized nicotinamide cofactors reduced during catabolism. The enzyme responsible for this solvent formation is alcohol dehydrogenase.

Alcohol dehydrogenase (EC 1.1.1.1) is a dimer with a mass of 80 kDa which belongs to a group of dehydrogenase enzymes that occur in many organisms and

facilitate the conversion between alcohols and aldehydes or ketones with the reduction of nicotinamide adenine dinucleotide, $NAD^+$ to NADH (Burdette and Zeikus, 1994). The inverted reaction is also occurring by re-oxidation of NADH to NAD+ which takes place when an aldehyde is reduced to an alcohol.

The alcohol dehydrogenase from *L. brevis* (LbADH) it is said to be a robust and versatile catalyst for enatioselective reduction of ketones to the corresponding alcohols (Leuchs and Greiner, 2011).

In this work, ADH is studied in the catalyses of 2-butanone into the alcohol 2-butanol (figure 12).

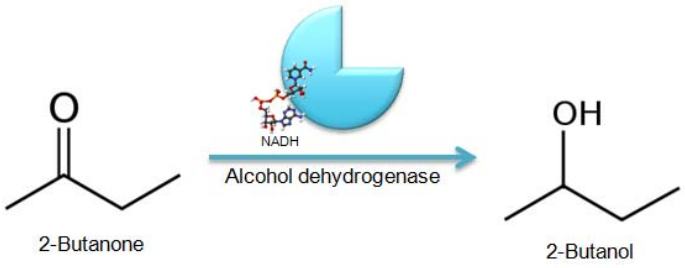



Figure 12 – Enzyme dehydrogenase.

According to Leuchs and Greiner, 2011, the application of LbADH is rising and the number of patent applications for this enzyme is also an indicative of its potential.

The objective of this project is to characterize proteins, shown to be present in certain Lactic acid bacteria (*L. brevis*), which are able to convert a natural product of yeast metabolism, 2,3-butanediol, to butanol. Diol dehydratase and alcohol dehydrogenase are two enzymes involved in this process, as seen above.

Consequently, a selection of strains and isolates will be tested under different growth conditions for their production of butanol. Later on, kinetic characterization of enzymes converting 2,3-butanediol to butanone and butanone to butanol will be done for the two strains showing the highest productivity. This characterization will involve different substrates, co-factors, and measurements will be performed in “forward” as well as “reverse” reaction.

# 2. Materials and Methods

## Experimental procedures

## 2.1. Microrganisms, culture and extraction

From the four *L. brevis* strains studied in a previous 15 credit project, the two best strains were chosen for this work. Strains SE20 and SE31 were earlier characterized from SEKAB E-Technology, Örnskoldsvik (Sweden) bioreactors, in preceding works. The first part of this master thesis consisted on these *L. brevis* strains screening.

*S. cerevisiae* genetically engineered strains were also studied in the enzyme assays, not as a main goal, but as a control in this project. Two strains were tested: strain *Δgpd1,2+sadh*, a strain with a double deletion gene and a plasmid that codifies for the protein alcohol dehydrogenase which converts 2-butanone to 2-butanol; and the control strain *Δgpd1,2+315* containing an empty plasmid; both obtained as part of a PhD program.

### 2.1.1. Culture media

*L. brevis* cells were grown sequentially in two types of media, MRS medium and SM2 medium.

The MRS medium is an improved complex growth medium that supports good growth of L*actobacilli spp*. when compared with other general medium (Man *et al.,* 1960). For that reason, pre-culture was performed in this medium.

SM2 is a modified synthetic medium described in Weiller & Radler, 1972, with some changes (table 4). In this medium, some lactic acid bacteria are able to produce butanol (Weiller & Radler, 1972).

Table 4 – SM2 composition medium (Adapted from Weiller & Radler, 1972).

| Component | Concentration | Component | Concentration |
|---|---|---|---|
| Glucose | 10 g/L | Adenine | 0,01 g/L |
| Tween80 | 1 g/L | Uracil | 0,01 g/L |
| L-ascorbic acid | 0,5 g/L | L-Cysteine | 0,1 g/L |
| Pyridoxolhydrochloride | 1 mg/L | L-Tryptophan | 0,1 g/L |
| Nicotin acid | 1 mg/L | DL-α-Alanine | 0,2 g/L |

| | | | |
|---|---|---|---|
| Ca-D(+)-Pantothenat | 1 mg/L | DL-α-Aminobutyricacid | 0,1 g/L |
| Riboflavin | 1 mg/L | L-Arginine.HCl | 0,2 g/L |
| Thiaminiumdichloride | 5 mg/L | L-Asparagine | 0,1 g/L |
| Cyano-cobalamine | 0,01 mg/L | L-aspartic acid | 0,3 g/L |
| D(+)-Biotin | 0,01 mg/L | L-glutamic acid | 0,3 g/L |
| 4-Amino benzoic acid | 0,1 mg/L | Glycin | 0,2 g/L |
| Folic acid | 0,2 mg/L | L-Histidine.HCl | 0,2 g/L |
| Sodium acetate.3H20 | 3 g/L | L-Isoleucine | 0,1 g/L |
| Tri-sodium citrate.2H20 | 1 g/L | L-Leucine | 0,2 g/L |
| K2HPO4 | 1,5 g/L | L-Lysine.HCl | 0,2 g/L |
| KH2PO4 | 1,5 g/L | L-Methionine | 0,1 g/L |
| MgSO4.7H20 | 0,5 g/L | L-Phenylalanine | 0,1 g/L |
| NaCl | 0,02 g/L | L-Proline | 0,2 g/L |
| MnSO4.1H20 | 0,02 g/L | L-Serine | 0,1 g/L |
| FeSO4.7H20 | 0,02 g/L | L-Threonine | 0,1 g/L |
| Guanine | 0,01 g/L | L-Tyrosin | 0,1 g/L |
| Xanthine | 0,01 g/L | L-Valine | 0,1 g/L |

*S. cerevisiae* cells were grown in yeast SC_leu medium (Synthetic complete medium, omitting leucine) with complete supplement mixture of amino acids minus leucine, yeast nitrogen bases and 2% glucose.

### 2.1.2. Inoculum and fermentation

Fermentation experiments (1% inoculum in MRS for bacterial strains and SC_leu for yeast strains) were carried out at 30ºC at 200 rpm, as described in Speranza *et al.* 1997.

In both microorganisms, 1 g/L of 2,3-butanediol for the diol dehydratase enzyme assay and 1 g/L of 2-butanone for the alcohol dehydrogenase enzyme assay, were added to media.

Bacterial strains were cultivated in 500 mL shake flasks and yeast strains in flasks with anaerobic loops.

Later cells were recovered still in the exponential phase, where enzymes are active (Schutz and Radler, 1984) and subsequently extracted. In table 5, a resume of the experiments and culture conditions is shown.

Table 5 – Experimental design of cultivation of different microorganisms for different enzyme assays.

| Microorganism | Enzyme Assay | Pre culture | Fermentation | Culture time |
|---|---|---|---|---|
| ***L. brevis*** | Alcohol dehydrogenase | MRS (24 h) | SM2 + 1 g/L 2-butanone | 48 hours |
| | Diol dehydratase | MRS (24 h) | SM2 + 1 g/L 2,3-butanediol | 48 hours |
| ***S. cerevisiae*** | Alcohol dehydrogenase | SC_leu (24 h) | SC_leu + 1 g/L 2-butanone | 48 hours |
| | Diol dehydratase | SC_leu (24 h) | SC_leu + 1 g/L 2,3-butanediol | 48 hours |

### 2.1.3. Extraction

The cell extraction was performed according to Schutz and Radler 1984, with some modifications. After 48 hours of growth, cells were centrifuged 10 min at 4000 rpm (Beckman high speed centrifuge) and washed twice in 10 mM potassium phosphate pH 7.2 and 1 mM dithiothreitol buffer. DTT has a role of enzyme activation, since it is a strong reducing agent (Radler and Zorg, 1986). Later the cells were suspended in 2 mL of the above mentioned buffer.

Cell disruption (Mehmeti *et al.* 2011) was carried on by adding the solution to a *lysing matrix E* (MP™) and protein extraction was obtained by using the *FastPrep®-24* (MP™). Cells were lysed by vortexing them at 6 m/s for 5 cycles of 20 seconds (figure 13). Between each cycle, the cell suspension was kept on ice for 1 min. Cell debris was removed by centrifugation at 14000 g units for 30 min at 4°C.

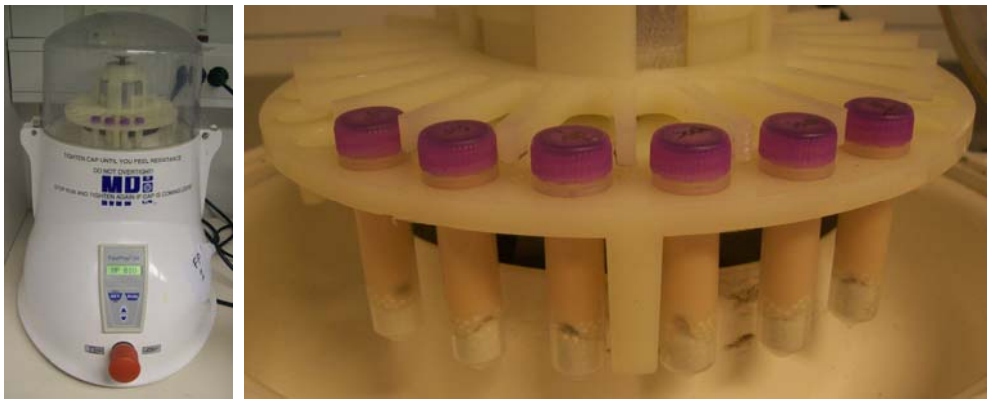


Figure 13 - *FastPrep®-24 MP™* (left) and *Lysing matrix E* (right).

To remove the *noise/background* caused by metabolites and substrates inside the cells, desalination of the samples was performed using a spinning column *amicon ultracel-4 10k device (Merk Millipore)* - figure 14. The extraction and disruption protocol described was executed at 4°C to avoid enzyme inactivation.

Figure 14 – Spinning column *amicon.*

## 2.2. Enzyme assay

Two types of enzyme assays were performed; a discontinuous assay for diol dehydratase in which samples were removed at intervals from the reaction mixture and analyzed; and a continuous assay for alcohol dehydrogenase, in which the progress of the reaction is monitored continuously.

### 2.2.1. Diol dehydratase

Diol dehydratase enzyme assay was performed according to Toraya *et al.* 1997. During 10min at 37°C, the following mixture was incubated: 0,05 M Potassium chloride, 0,035 M potassium phosphate buffer pH 8, 15 μM adenosylcobalamin (coenzyme B12), 0,05 M-0,4 M substrate (1,2-propanediol, 2,3-butanediol, glycerol and 1,3-propanediol) and crude extract obtained in section 2.1.3.

After 10 min of incubation, the reaction was terminated by adding 0,5 mL of 0,1 M potassium citrate buffer pH 3,6. Immediately 0,25 mL of 0.1 % MBTH hydrochloride was added. The activity is determined by 3-methyl-2-benzothiazolinone hydrazone method: the ability of aldehydes, ketones (like 2-butanone) and keto acids to react with MBTH forming azine derivates which can be determined spectrophotometrically (Paz *et al.* 1965)

Following, the mixture was incubated for 15 minutes at 37 °C. Finally, 0,5 mL of water was added and the absorbance was read at 305 nm in a plate reader (Fluostar Omega, BMG LabTech)

### 2.2.2. Alcohol dehydrogenase

Alcohol dehydrogenase enzyme assay followed Jo *et al.* 2008. At 30ºC, the following mixture was incubated: 50 mM potassium phosphate buffer pH 7, 10 mM dithiotreitol, 0,5 mM-100 mM substrate (butanol, propanol and ethanol), 2 mM coenzyme $NAD^+$ and crude extract obtained in section 2.1.3.

The reverse reaction was also measured with 0,5 mM-100 mM butanone as substrate and 1 mM coenzyme NADH instead of $NAD^+$.

The activity was determined by measuring the reduction of $NAD^+$ to NADH at 340 nm in a plate reader at 30ºC (*Fluostar Omega, BMG LabTech*) - figure 15.

Figure 15 – Plate reader *(Fluostar Omega, BMG LabTech).*

## 2.3. Analytical methods

During fermentation, samples were taken for further analysis of optical density, substrate and product concentrations, and total protein concentration.

### 2.3.1. Optical density

The OD was measured at 600 nm of wave length on the *ThermoScientific Genesys 20* apparatus (figure 16), to characterize the microbial growth.

### 2.3.2. High Performance Liquid Chromatography

High performance liquid chromatography to estimate the concentration of glucose, 2,3-butanediol, 2-butanone and 2-butanol was carried out in the *Dionex Ultimate 3000* apparatus (Aminex HPX-87H Ion Exchange Column, pump flow = 0,6mL/min, P = 39 bar, T = 45ºC) - figure 16. The retention time was 8,5 min for glucose, 17,5 min for 2,3-butanediol, 27 min for 2-butanone and 30 min for 2-butanol.

Figure 16 - *ThermoScientific Genesys 20* (left) and *Dionex Ultimate 3000* (right).

### 2.3.3. Total protein concentration

The protein concentration was measured in mg/mL and obtained by *NanoDrop2000 spectrophotometer (Termo Scientific).*

## 2.4. Activity measurement

During both enzyme assays, absorbance was read at different wave lengths. MBTH is measured at 305 nm for diol dehydratase and NADH is measured at 340 nm for alcohol dehydrogenase. The absorbance values obtained were converted to activity units.

### 2.4.1. Beer-Lambert Law

In optics, the Beer-Lambert law relates the absorption of light to the properties of the material through which the light is travelling according to equation 6.

$$A = \varepsilon l c \qquad \text{Eq. (6)}$$

In this equation, A represents the absorbance, ε the extinction coefficient, l the path length and c the molar concentration of absorbing species in the material.

In diol dehydratase assay, the apparent molar extinction coefficient at 305 nm for the colored product formed from propionaldehyde in the MBTH method was 13,3 x $10^3$ $M^{-1}$ $cm^{-1}$ (Toraya *et al.* 1997).

In the alcohol dehydrogenase, the amount of NADH formed is determined using an extinction coefficient (at 340 nm) of 6.22/mM/cm (Jo *et al.* 2008).

For both cases, using equation (6) it was possible to know the concentration of the product formed, and later the activity was defined as the amount of enzyme catalyzing the formation of 1 mol of product formed in one min under the standard assay conditions.

### 2.4.2. Specific activity

The specific activity (mol/min/g) was calculated by dividing the enzyme activity by the amount of total protein concentration, providing an homogenization of data of different assays, since different assays mean different extractions and therefore different protein concentration.

### 2.4.3. Enzyme parameters

Enzymatic parameters, Michaelis constant (mmol/min/g) and maximum velocity (mM), were determined by three different linear plot methods: Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf.

# 3. Results and Discussion

# 3.1. Microorganisms screening

### 3.1.1. Culture media, inoculum and fermentation

The careful and detailed preparation of synthetic medium was shown to be essential for growth of butanol producing bacteria. Butanol is not produced in a complex media such as MRS medium, however this medium is important to be used, to provide a strong pre-culture, as the growth is higher when compared to SM2 (appendice I – table 9).

Figure 17 – SM2 medium.

When bacteria strains grow first in SM2 medium, then inoculated in the same medium, there is no butanol production. However, if pre-culture is prepared in MRS medium and subsequently inoculated in synthetic medium, butanol is produced.

The SM2 medium is characterized as a light yellow transparent solution (figure 17), and one of its components, vitamin B12, has an important role in enzyme activity and conversion of 2,3-butanediol to 2-butanone, as it will be presented in the following sections.

Bacteria and yeast fermentations occurred in shake flasks as shown in figure 18, fitted with of an anaerobic loop for yeast fermentation.

Figure 18 – Bacterial fermentation (left) and yeast fermentation (right).

Strains SE20 and SE31, were the *L. brevis* strains chosen for this project, since these strains verified a higher growth and butanol production (0,4 g/L) between four strains already analyzed, in a previous 15 credit project (appendices I – figures 30-38)

Therefore, fermentation in SM2 was followed and samples were taken every hour in the exponential phase, for later analysis.

### 3.1.2. Optical density and pH

Figure 19 and 20 represents the OD and pH evolution of the two strains of *L. brevis* growing in SM2 medium in the presence of 2,3-butanediol and 2-butanone respectively.

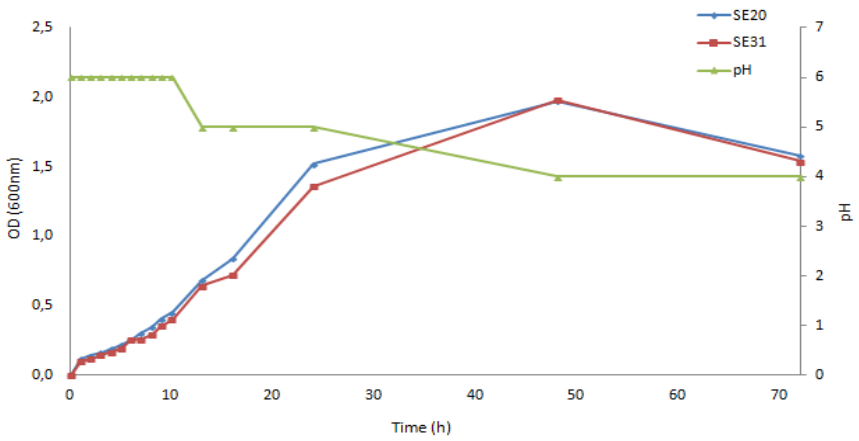



Figure 19 – Optical density at 600nm and pH measurement of *Lactobacillus spp*. strains (SE20 and SE31),
growing in 10g/L glucose and 1g/L 2,3-butanediol.

Analyzing figure 19, on the first 48 hours of fermentation, bacteria are growing exponentially, reaching a maximum OD value of 2. After 48 hours, no growth was observed.

There are no major differencies in growth between the two strains, although, strain SE20 has a somewhat higher growth rate.

Another measured parameter was the pH. At the beginning of the fermentation, the pH was adjusted to 6, but after 10 hours the pH started to decrease, and at the end of the growth phase a pH of about 4 was reached.

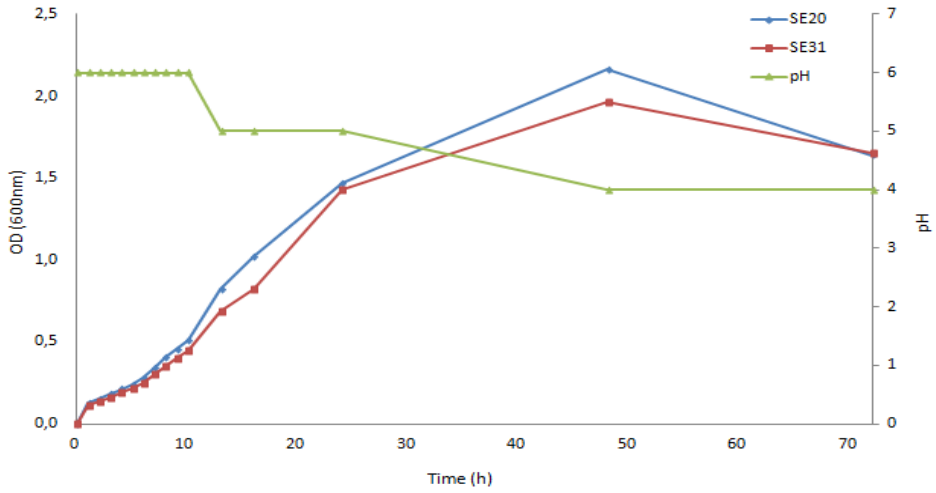



Figure 20 – Optical density at 600nm and pH measurement of *Lactobacillus spp*. strains (SE20 and SE31), growing in 10g/L glucose and 1g/L 2-butanone.

The same tendency is verified for fermentation in the presence of 2-butanone (figure 20), with both strains.

### 3.1.3. HPLC analysis

High-performance liquid chromatography was performed in order to measure the concentration of the substrates: glucose, 2,3-butanediol and 2-butanone; and the product: 2-butanol.

Figure 21 and 22 represent the variation of glucose, 2,3-butanediol and 2-butanol concentration during 90 hours of fermentation for strains SE20 and SE31 respectively.

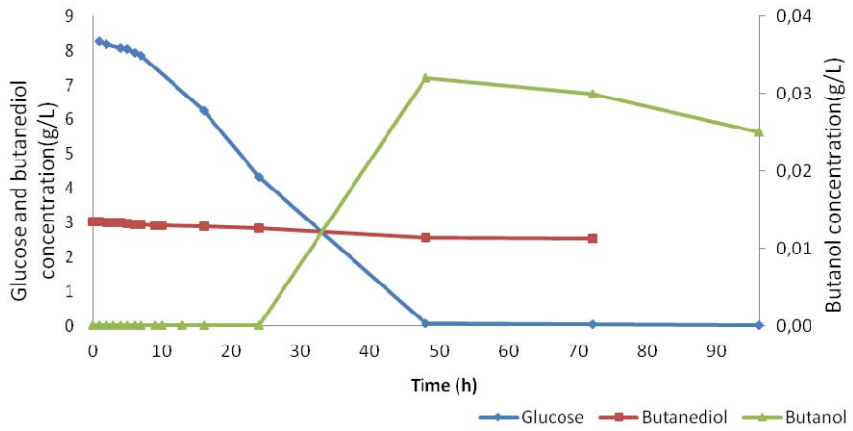


Figure 21 – Concentration of glucose, butanediol and butanol, during fermentation time, for strain SE20 growing on SM2 medium in the presence of butanediol.

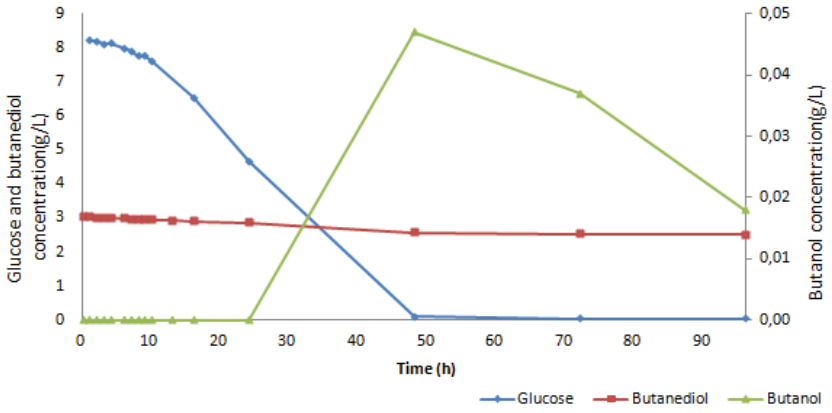



Figure 22 – Concentration of glucose, butanediol and butanol, during fermentation time, for strain SE31 growing on SM2 medium in the presence of butanediol.

Comparing strains SE20 and SE31 (figure 21 and 22), it is possible to identify a similar behavior on these strains. Glucose was totally consumed, whereas, in total, less than 1 g/L of 2,3-butanediol was consumed during the first 48 hours of growth.

Butanol achieved a higher concentration in strain SE31, around 0,047 g/L, after 48 hours of growth, when 2,3-butanediol was consumed. For strain SE20 its maximum butanol titer was around 0,032 g/L, also after 48 hours of growth.

Similarly, figures 23 and 24, represent the variation of substrates and product concentration, as glucose and 2-butanol, for strains SE20 and SE31, respectively, but this time in the presence of 2-butanone.

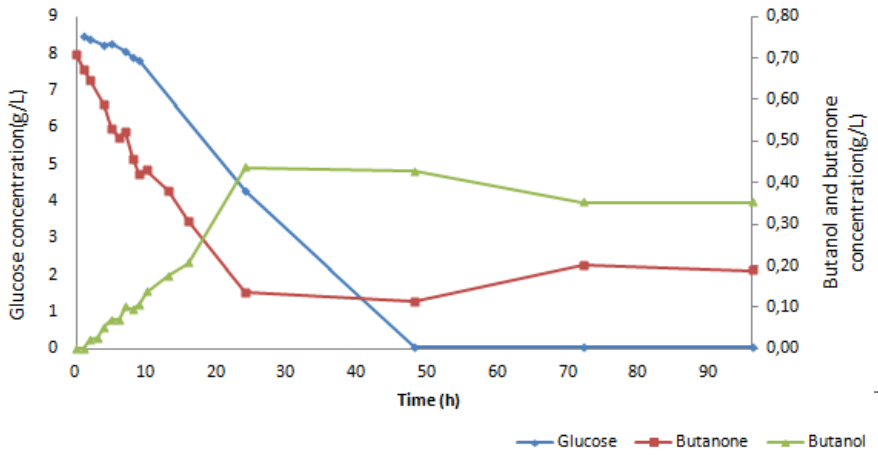


Figure 23 – Concentration of glucose, butanediol and butanol, during fermentation time, for strain SE20 growing on SM2 medium in the presence of butanone.

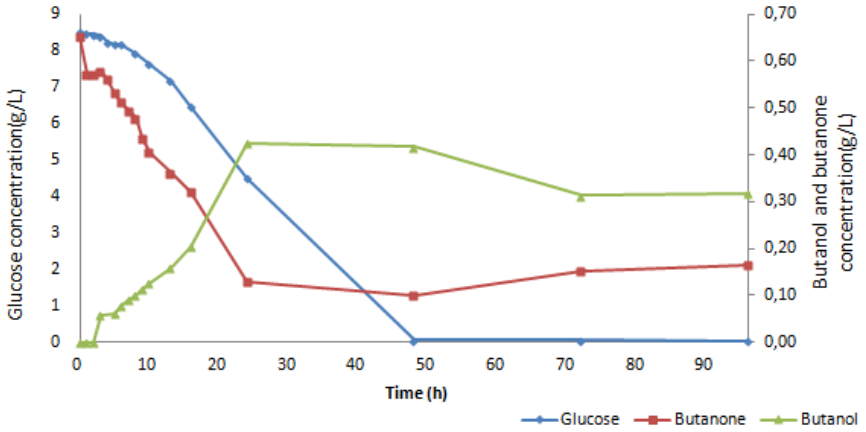



Figure 24 – Concentration of glucose, butanediol and butanol, during fermentation time, for strain SE31 growing on SM2 medium in the presence of butanone.

Glucose was completely consumed resembling the previous results for strains SE20 and SE31 in the presence of 2,3-butanediol, but in this case, 0,6 g/L of 2-butanone was used, during the first 24 hours of fermentation.

Butanol was produced in higher concentrations, 0,44 g/L for strain SE20 and 0,43 g/L for strain SE31, when comparing the growth in the presence of 2-butanone to the growth in the presence of 2,3-butanediol. In this last case, achieving butanol is a more direct step from MEK than from butanediol (figure 2), involving only one enzyme reaction catalyzed by alcohol dehydrogenase.

Moreover, butanol concentration was superior as compared to results reported by Speranza *et al.* 1997.

## 3.2. Enzyme activity

### 3.2.1. Extraction and total protein concentration

Figure 25 – *L. brevis* cells extracted after 48 hours of fermentation

After 48 hours of growth, as discussed previously, butanol is produced. Therefore, the enzymes responsible for that conversion are active in the cell, and for that reason, extraction was performed after that period (figure 25).

Total protein concentration was measured (table 6) after each extraction, before every enzyme assay, for later calculation of specific activity, turning the results of different extractions, comparable.

Table 6 – Total protein concentration determined for different enzyme assays in different conditions.

| | **Enzyme assay** | | | |
|---|---|---|---|---|
| | Diol dehydratase | | Alcohol dehydrogenase | |
| **Medium and substrate** | SM2 in the presence of 1,2-propanediol | SM2 in the presence of 2,3-butanediol | SM2 in the presence of 2-butanone | |
| ***L. brevis* strain** | SE20 | | SE20 | SE31 |
| **Protein concentration (g/L)** | 67,2 | 35,1 | 23,5 | 27,5 |

Several enzyme assays were executed for both enzymes, trying different conditions and varying parameters, in the attempt to get an optimized assay.

In the following two sections, some results of the two types of enzyme assays are presented and discussed.

### 3.2.2. Diol dehydratase

Diol dehydratase catalyzes the reaction that converts 2,3-butanediol into 2-butanone. The enzyme assay involving diol is a discontinuous assay in which samples are removed at intervals from the reaction mixture.

One first enzyme assay performed had the purpose to see the influence of the presence of substrate in the medium. Figure 26 shows the enzyme activity when cells are cultured in the presence or absence of 1,2-propanediol

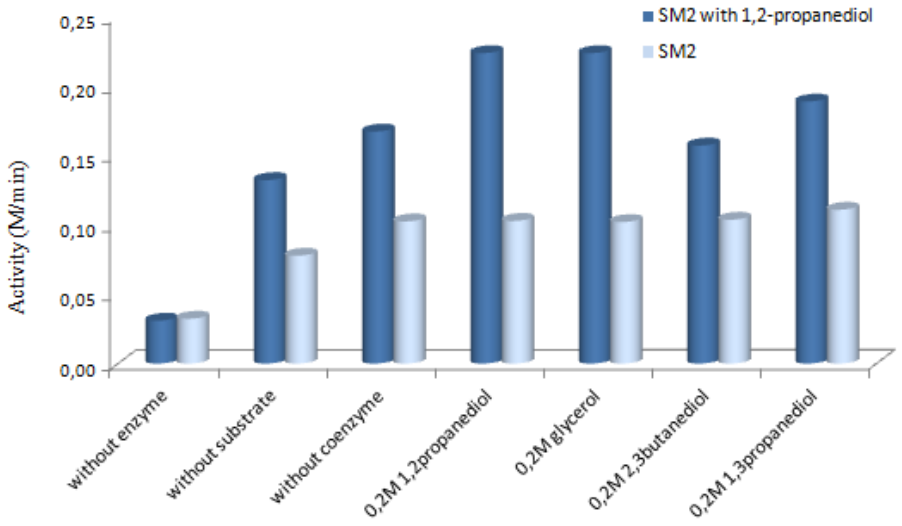



Figure 26 – Enzyme activity of *L. brevis* strain SE20, grown on SM2 medium and on SM2 medium in the presence of 1 g/L 1,2-propanediol. Enzyme assays were performed for different substrates (1,2-propanediol, glycerol, 2,3-butanediol and 1,3-propanediol) and three controls were executed.

Results show that activity is higher when cells grow in the presence of a substrate. In a way it is possible to say that the enzyme is activated by the substrate. As it was demonstrated previously, propanediol induces the transcription of both the cob and the pdu operons (Ailion and Roth, 1997).

In this experiment, three controls were also tested (without enzyme, without substrate and without coenzyme). For the controls without substrate and coenzyme activity was also comparably high. The reason probably being that no enzyme purification was performed and thus the crude extract included a lot of metabolites and enzymes that can interact with the MBTH method. For the control control without substrate, the activity measured is the same between the two samples.

In later enzyme assays, only strain SE20 was studied, but is expected a similar behavior for strain SE31. This strain was grown in the presence of two different substrates, 1,2-propanediol and 2,3-butanediol, and samples were taken at minute zero

and ten (appendice II – figure 39 and 40). samples were withdrawn at zero and 10 minutes during the enzymatic assay to determine the activity.

The values of absorbance were converted to concentration by application of equation 6, and activity was defined as the amount of enzyme catalyzing the formation of 1 µmol of product formed in one min under the standard assay conditions. Later, specific activity was calculated, i.e. the activity of the enzyme per gram of total protein (expressed in µmol $min^{-1}g^{-1}$) - figure 27.

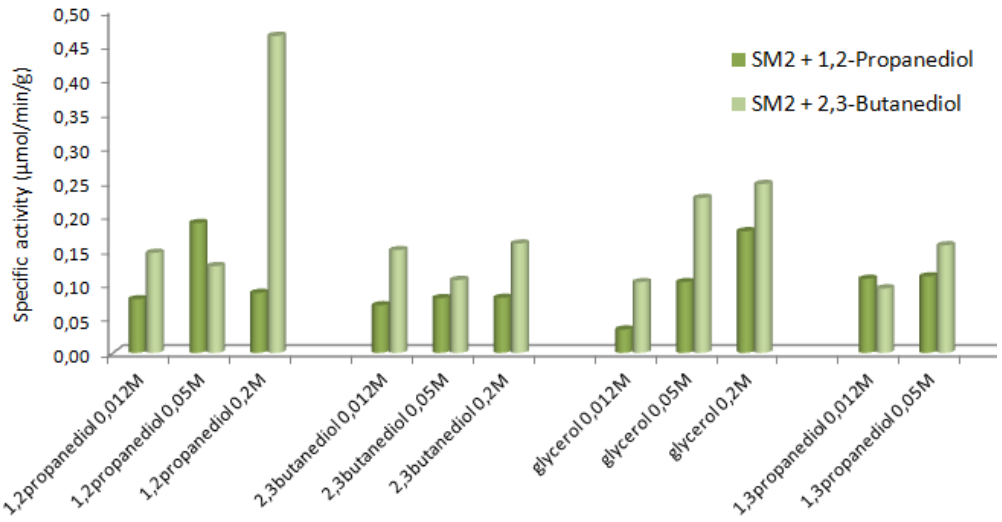



Figure 27 – Specific activity of *L. brevis* strain SE20 grown on SM2 medium in the presence of 1 g/L 1,2-propanediol and 1g/L 2,3-butanediol. Enzyme assays were performed for different substrates (1,2-propanediol, 2,3-butanediol, glycerol and 1,3-propanediol) in three different concentrations.

Samples were normalized to the control without substrate. This time the objective was to see if any difference was verified between samples growing in different substrates. Around 91% of the samples recorded a higher specific activity when growing on SM2 in the presence of 2,3-butanediol, comparing to 1,2-propanediol, showing a bigger specificity of diol dehydratase to 2,3-butanediol.

Attempts to make a kinetic characterization of the dehydratase failed, because it is not possible to conclude any difference between the four different substrates in different concentrations, as results are not conclusive and don't follow a tendency. It would be expected that when increasing the concentration of the substrate an increasing specific activity should follow but this was not the case. For this reason, it wasn't

possible to determine the enzyme kinetics of the reaction and the enzyme parameters weren't calculated for enzyme diol dehydratase.

In Radler and Zorg, 1986 Km values for 2,3-butanediol and 1,2-propanediol calculated from Lineweaver-Burk plots were 34 mmol and 0,7 mmol, showing that dehydratase has more affinity to 1,2-propanediol.

### 3.2.3. Alcohol dehydrogenase

Alcohol dehydrogenase is responsible for conversion of 2-butanone into 2-butanol. The enzyme assay involving ADH, is continuous and the progress of the reaction is monitored by following consumption or formation of NADH at 340 nm.

Enzyme assays were performed for both strains SE20 and SE31, following forward (2-butanol + $NAD^+$ → 2-butanone + NADH) and reverse reaction (2-butanone + NADH → 2-butanol + $NAD^+$) – (appendice III – figures 41, 42, 43 and 44)

For the same strains, other substrates such as ethanol and propanol were tested (appendice III – figures 45 and 46), as a comparison to the butanol reaction. However in these cases it wasn't possible to determine the enzymatic parameters, since for these substrates the Km is too small, as the affinity is high for the referred alcohols.

From figures 41, 42, 43, and 44, the change of absorbance was determined by the slope of the plot in the first seconds of reaction. Using the beer-lambert law (equation 6), the concentration of NADH formed was determined.

For each concentration of substrate tested, the specific activity was established as the amount of enzyme catalyzing the formation of 1 mmol of product formed in one min, per gram of total protein.

Finally, enzymatic parameters, Km (mM) and maximum velocity, Vmax (μmol/min/g), were determined by three different linear plot methods: Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf.

The three kinetic graphs were obtain for strains SE20 and SE31 (figures 28 and 29 respectively), for forward and reverse reaction.

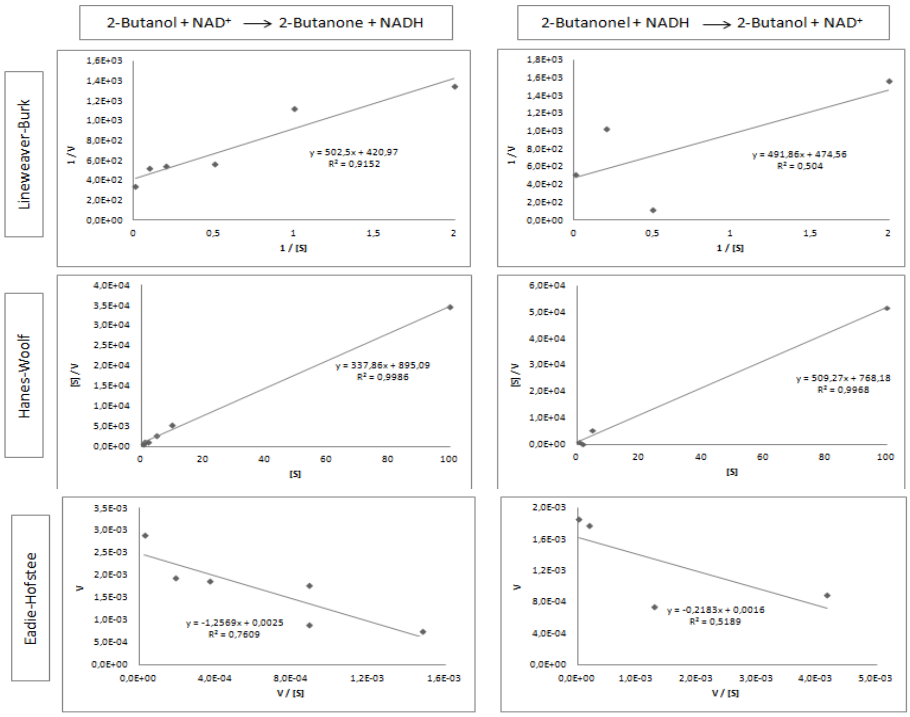



Figure 28 – Enzyme kinetics of *L. brevis* strain SE20 grown on SM2 medium in the presence of 1 g/L 1,2-propanediol. Representation of three different plots: Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee.

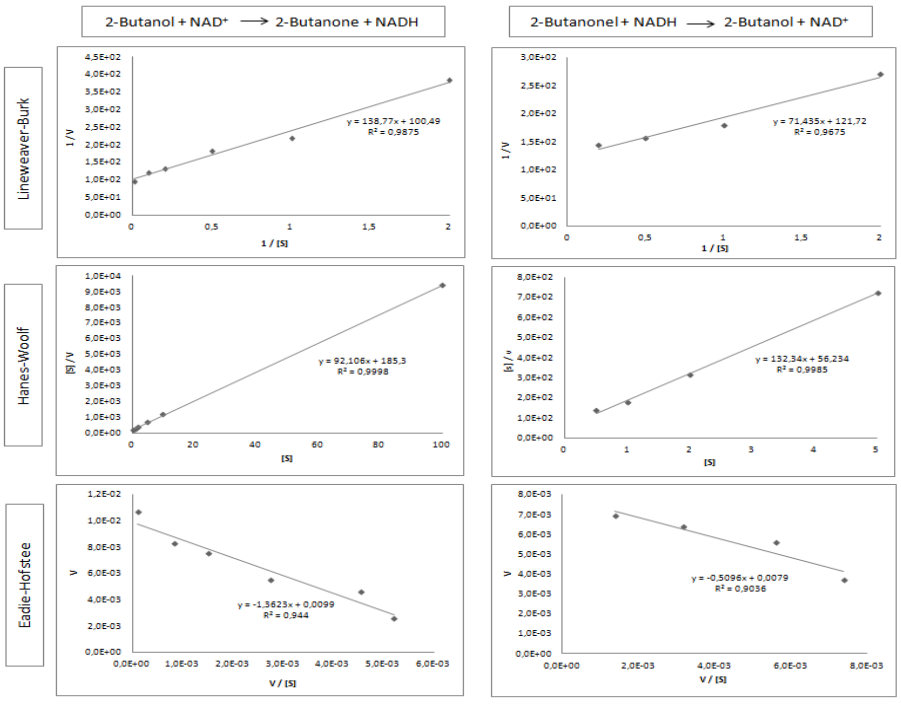



Figure 29 – Enzyme kinetics of *L. brevis* strain SE31 grown on SM2 medium in the presence of 1 g/L 1,2-propanediol. Representation of three different plots: Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee.

For further characterization of the dehydrogenase, and attending to the slopes and origin in the yy axis, the substrate affinities for 2-butanone and 2-butanol were determinated. Km and Vmax were estimated for all three methods (table 7 and 8).

Table 7 – Enzyme parameters (Km and Vmax) determination by three different plotting methods, for strains *L. brevis* SE20 and SE31, in the reaction of butanol with $NAD^+$, forming butanone and NADH.

| | **Lineweaver-Burk** | | **Hanes-Woolf** | | **Eadie-Hofstee** | |
|---|---|---|---|---|---|---|
| | Km (mM) | Vmax (µmol/min/g) | Km (mM) | Vmax (µmol/min/g) | Km (mM) | Vmax (µmol/min/g) |
| se20 | 1,19 | 2,4 | 2,65 | 3,0 | 1,26 | 2,5 |
| se31 | 1,38 | 10,0 | 2,01 | 10,9 | 1,36 | 9,9 |

Table 8 – Enzyme parameters (Km and Vmax) determination by three different plotting methods, for strains *L.brevis* SE20 and SE31, in the reaction of butanone and NADH, forming butanol with $NAD^+$.

| | **Lineweaver-Burk** | | **Hanes-Woolf** | | **Eadie-Hofstee** | |
|---|---|---|---|---|---|---|
| | Km (mM) | Vmax (µmol/min/g) | Km (mM) | Vmax (µmol/min/g) | Km (mM) | Vmax (µmol/min/g) |
| se20 | 1,04 | 2,1 | 1,51 | 2,0 | 0,22 | 1,6 |
| se31 | 0,59 | 8,2 | 0,42 | 7,6 | 0,51 | 7,9 |

According to Cornish-Bowden, 1995, it is known that some enzymes are more effective for one direction of reaction than the other, and for that reason they are called "one way enzymes". In this case, alcohol dehydrogenase is equally effective on both directions, since Km and Vmax values are in the same order of magnitude.

Comparing the results obtained between different linear plots, one can say that in most cases the determined parameters Km and Vmax are relatively similar or at least in the same order of magnitude.

Moreover it was verified that among different strains, Vmax is quite a lot higher for SE31 while Km values are similar.

# 4. Conclusion

## Outlook and prospects

In the present master thesis work, two strains of *L. brevis* were studied in their butanol production from 2,3-butanediol.

Both strains, SE20 and SE31, achieved a butanol titer of ˜0,4 g/L, growing at the same rate. Therefore, it is not possible to conclude that one strain is better than the other. In future studies, to increase butanol's titer, genetic engineering tools should be explored.

The main goal of this project was accomplished. Proteins involved in the 2-butanol production in the referred *L. brevis* were studied and characterized, as their activity was measured by two different enzymatic assays.

Diol dehydratase was studied using the MBTH method, concluding that growth in the presence of substrate resulted in a doubling of the enzymatic activity, presuming that substrate has a positive control effect in enzyme activation. Due to a limit project time, this enzyme wasn't characterized in terms of Km and Vmax.

One solution to succeed in the diol characterization would be to perform an enzyme purification. According to Radler and Zorg, 1986, cell-free extracts of *L. brevis* had shown a very weak activity, for substrate 2,3-butanediol, and only if reaction was prolonged. Therefore, purification for this enzyme should be attempted through precipitation with streptomycin, dialysis, precipitation with ammonium sulphate, and chromatography (Radler and Zorg, 1986).

Alcohol dehydrogenase activity was determined by measuring the reduction of $NAD^+$ to NADH at 340nm. The parameters, Km (mM) and Vmax (mmol/min/g) were determined graphically by three linear methods for forward (Lineweaver-Burk 1,19 mM 2,4 µmol/min/g, Eadie Hofstee 1,26 mM 2,5 µmol/min/g and Hanes-Woolf 2,65 mM 3,0 µmol/min/g) and reverse butanol reaction (Lineweaver-Burk 1,04 mM 2,1 µmol/min/g, Eadie Hofstee 0,22 mM 1,6 µmol/min/g and Hanes-Woolf 1,51 mM 7,9 µmol/min/g).

Finally, Vmax of the strain SE31 was higher than SE20. However, this was not reflected in a higher butanol production rate during growth of this strain. This could suggest that the dehydratase rather than the dehydrogenase is the rate limiting component.

# 5. References

**Ailion, M.; Roth, J. R. (1997).** Repression of the cob operon of salmonella typhimurium by Adenosylcobalamin is influenced by mutations in the pdu operon. Journal of Bacteriology Oct: 6084-6081

**Axelsson, L. T. (1993).** Lactic acid bacteria. Classification and physiology. Decker: New York

**Berezina, O. V.; Zakharova, N. V. ; Brandt, A.; Yarotsky, S. V.; Schwarz, W. H.; Zverlov, V. V. (2010).** Reconstructing the clostridial n-butanol metabolic pathway in *Lactobacillus brevis*. Applied Microbiology and Biotechnology 87: 635–646

**BioButanol.com (2010).** BioButanol, the next generation biofuel. [online] Available at: <http://www.biobutanol.com/> [Accessed 29 May 2012 ]

**Bisswanger, H. (2008).** Enzyme Kinetics. Principles and Methods. 2nd edition. Tübingen: Wiley-Vch

**BP and DuPont (2009a).** Biobutanol, a European jv for BP and DuPont. Focus on Catalysts 9: 3

**BP and DuPont (2009b).** BP-DuPont launches Butamax. Focus on Catalysts 12: 5

**Burdette, D.; Zeikus, J. G. (1994).** Purification of acetaldehyde dehydrogenase and alcohol dehydrogenases from Thermoanaerobacter ethanolicus 39E and characterization of the secondary-alcohol dehydrogenase as a biofunctional alcohol dehydrogenase-acetyl-CoA reductive thioesterase. Biochemical Journal 302: 163-170

**Butamax™ Advanced Biofuels. (2010).** California Biobutanol Multimedia Evaluation. Tier I report

**Cornish-Bowden, A. (1995).** Fundamentals of Enzyme Kinetics. London: Portland Press

**Demirbas, A. (2009).** Biofuels securing the planet's future energy needs. Energy Conversion and Management 50: 2239–2249

**Demirbas, M. F.; Balat, M.; Balat, H. (2011).** Biowastes-to-biofuels. Energy Conversion and Management 52: 1815–1828

**Dürre, P. (2011).** Fermentative production of butanol — the academic perspective. Current Opinion in Biotechnology 22: 331–336

**Edinburgh Napier University. (2012).** History of the ABE fermentation. [online] Available at: <http://www.napier.ac.uk/randkt/rktcentres/bfrc/Pages/ABEhistory.aspx> [Accessed 29 May 2012 ].

**Ezeji, T.; Qureshi, N.; Blaschek, H. P. (2007[a]).** Butanol Production From Agricultural Residues: Impact of Degradation Products on Clostridium beijerinckii Growth and Butanol Fermentation. Biotechnology and Bioengineering 97: 6

**Ezeji, T.; Qureshi, N.; Blaschek, H. P. (2007[b]).** Bioproduction of butanol from biomass: from genes to bioreactors. Current Opinion in Biotechnology 18: 220–227

**Frey, P. A. and Hegeman, A. D. (2007).** Enzymatic reaction mechanisms. Oxford University Press

**García, V.; Päkkilä, J.; Ojamo, H.; Muurinen, E.; Keiski, R. L. (2011).** Challenges in biobutanol production: How to improve the efficiency? Renewable and Sustainable Energy Reviews 15: 964–980

**Jin, C.; Yao, M.; Liu, H.; Lee, C. F.; Ji, J. (2011).** Progress in the production and application of n-butanol as a biofuel. Renewable and Sustainable Energy Reviews 15: 4080–4106

**Johnson, C. L. V.; Pechonick, E.; Park, S. D.; Havemann, G. D.; Leal, N. A.; Bobik, T. A. (2001).** Functional genomic, biochemical, and genetic characterization of the Salmonella pduO gene, an ATP:Cob(I)alamin. Journal of Bacteriology 2011: 1577-1584

**Kumar, M.; Gayen, K. (2011).** Developments in biobutanol production: New insights. Applied Energy 88: 1999–2012

**Man, J.D.; Rogosa, M.; Sharpe, M.E. (1960).** A Medium for the Cultivation of *Lactobacilli*. *Journal of Applied Bacteriology* 23: 130-135

**Nair, N. U.; Shao, Z.; Zhao, H.; Sullivan, R. P.; McLachlan, M.; Johannes, T.; Zhao, H. (2008).** Biobutanol from yeast: a synergistic genome and protein engineering approach. In: The 2008 AIChE annual meeting, Philadelphia, PA; November 16–21, 2008

**Leuchs, S.; Greiner, L. (2011).** Alcohol dehydrogenase from *Lactobacillus brevis*: A versatile robust catalyst for enantioselective transformations. Chemical and Biochemical Engineering Quarterly 25: 267-281

**Pasteur, L. (1862).** Quelques résultats nouveaux relatifs aux fermentations acétique et butyrique. *Bulletin de la Société ChimiqueMay* 1862: 52-53.

**Paz, A.; Blumenfeld, O. O.; Rojkind, M.; Henson, E.; Furfine, C.; Gallop, P. M. (1965).** Determination of Carbonyl Compounds with N-Methyl Benzothiazolone Hydrazone. Archieves of Biochemistry and Biophysics 109: 548-559

**Radler, F.; Zorg, J. (1986).** Characterization of the enzyme involved in formation of 2-Butanol from meso-2,3-Butanediol buy lactic acid bacteria. American Society for Enology and Viticulture 37: 3

**Roth, J. R.; Lawrence, J. G.; Bobik, T. A. (1996).** Cobalamin (coenzyme B12): Synthesis and Biological Significance. Annual Review of Microbiology 50: 137-81

**Speranza, G.; Corti, S.; Fontana, G.; Manitto, P. (1997).** Conversion of meso-2,3-Butanediol into 2-Butanol by *Lactobacilli*. Stereochemical and enzymatic aspects. Journal of Agricultural and Food Chemistry 45: 3476-3480

**Swana, J.; Yang, Y.; Behnam, M.; Thompson, R. (2011).** An analysis of net energy production and feedstock availability for biobutanol and bioethanol. Bioresource Technology 102: 2112–2117

**Taylor, G. (2008).** Biofuels and the biorefinery concept. Energy Policy 36: 4406–4409

**US firms Gevo, Cobalt Technologies and Butamax Advanced Biofuels. (2010).** Biofuels, biobutanol fuel prepares for lauch. Focus on Catalysts 10: 2

**Wu, M.; Wang, M.; Liu, J.; Huo, H., (2008).** Assessment of potential life-cycle energy and greenhouse gas emission effects from using corn-based butanol as a transportation fuel. Biotechnology Progress 24 (6): 1204–1214.

# 6. Appendices

## Appendice I - Screening of four Lactobacillus brevis strains

Strains, SE20 and SE31, were isolated from SEKAB E-Technology, Örnskoldsvik (Sweden) bioreactors in previous works. The two other strains, 579 and 734, were purchased from culture collection, University of Gothenburg (GCUG). Optical density and HPLC analysis were performed as a screening of these four strains.

The pre-culture in different media was also studied. After 24 hours of fermentation, the strains got an optical density above 1 when cultivated in MRS medium. Similar values were reached in SM2 medium but after one week of cultivation (table 9).

Table 9 – Optical density measured in MRS and SM2 media after 24h of fermentation.

| | OD(600nm) | |
|---|---|---|
| **Strain** | **MRS (24h)** | **SM2 (1week)** |
| **SE20** | 1,489 | 1,397 |
| **SE31** | 1,323 | 1,249 |
| **579** | 1,145 | 1,071 |
| **734** | 1,747 | 1,398 |

Figure 30 represents the optical density measurement during 132 hours, with a frequency of 12 hours.

.

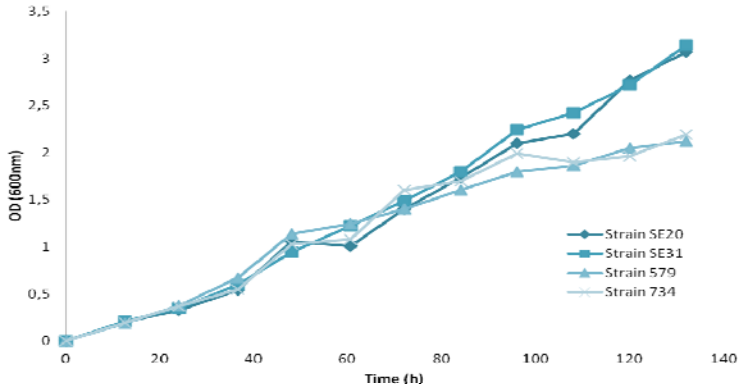



Figure 30 – Optical density measurement at 600nm of *Lactobacillus spp*. strains, growing on glucose in the presence of butanediol.

Figure 31, 32, 33 and 34 represents the variation of glucose, 2,3-butanediol and 2-butanol concentration during 12 days of fermentation for strains SE20, SE31, 579 and 734 respectively.

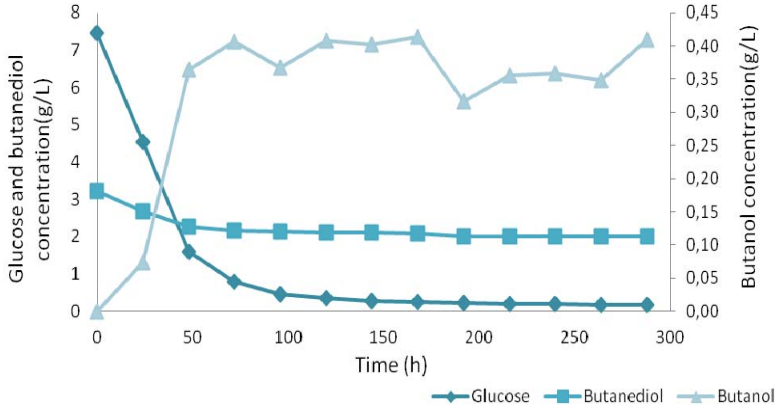



Figure 31 – Concentration of glucose, 2,3-butanediol and 2-butanol, during fermentation time, for strain SE20 growing on SM2 medium in the presence of 2,3-butanediol.

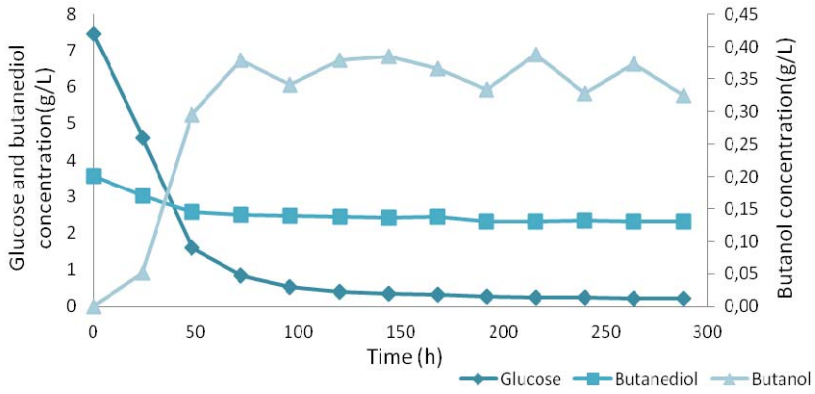



Figure 32 – Concentration of glucose, 2,3-butanediol and 2-butanol, during fermentation time, for strain SE31 growing on SM2 medium in the presence of 2,3-butanediol.

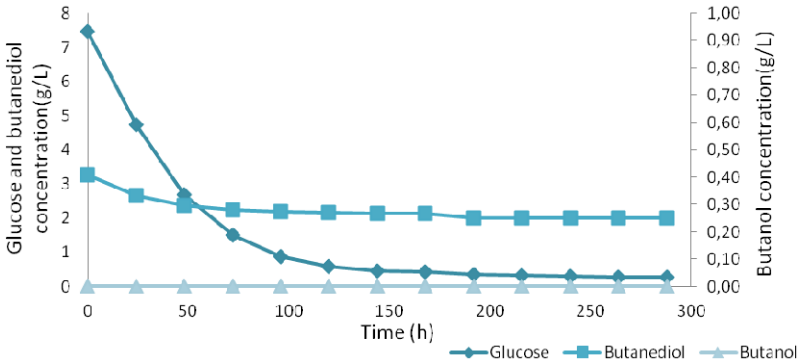



Figure 33 – Concentration of glucose, 2,3-butanediol and 2-butanol, during fermentation time, for strain 579 growing on SM2 medium in the presence of 2,3-butanediol.

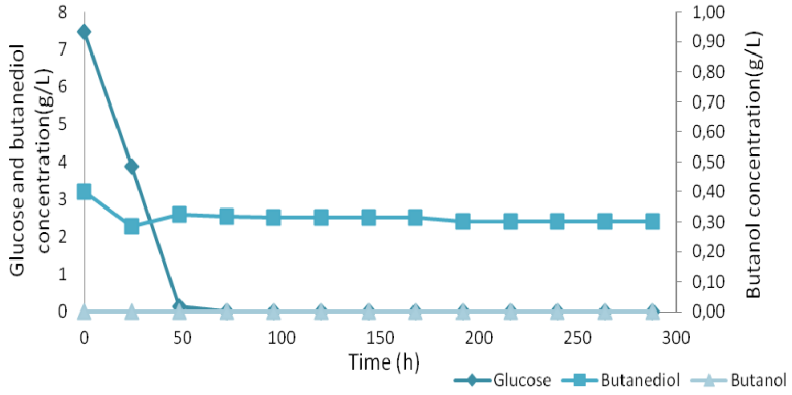



Figure 34 – Concentration of glucose, 2,3-butanediol and 2-butanol, during fermentation time, for strain 734 growing on SM2 medium in the presence of 2,3-butanediol.

Figure 35, 36, 37 and 38 represents the variation of glucose, 2-butanone and 2-butanol concentration during 12 days of fermentation for strains SE20, SE31, 579 and 734 respectively.

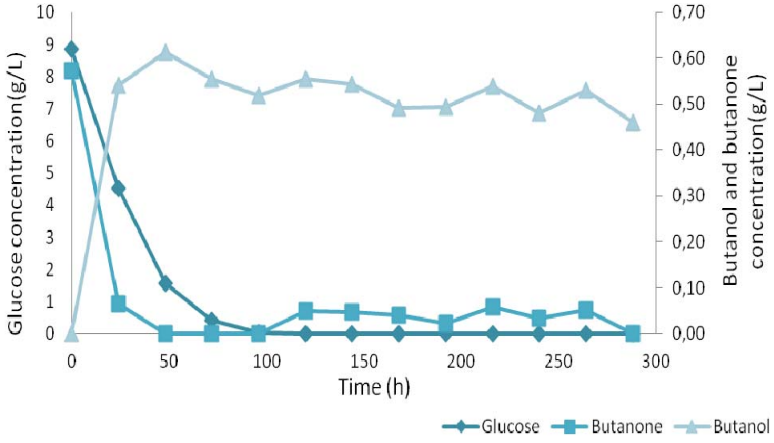



Figure 35 – Concentration of glucose, butanone and butanol, during fermentation time, for strain SE20 growing on glucose in the presence of 2-butanone as substrate.

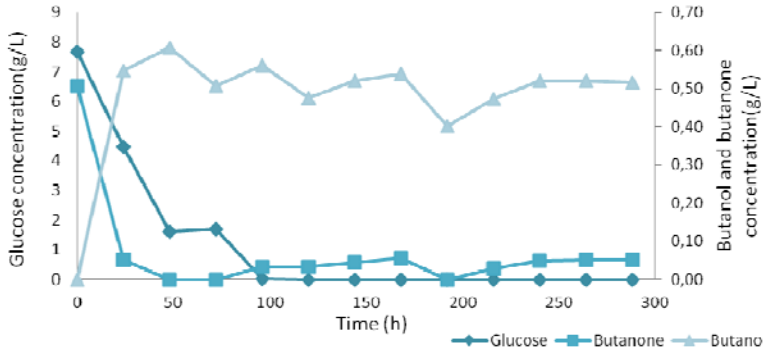



Figure 36 – Concentration of glucose, butanone and butanol, during fermentation time, for strain SE31 growing on glucose in the presence of 2-butanone as substrate.

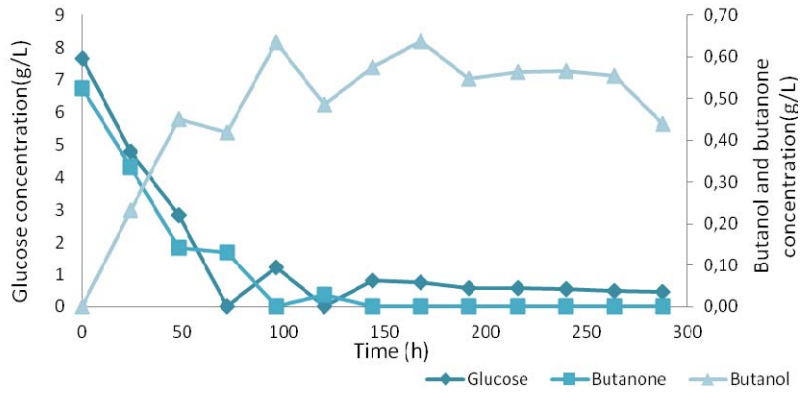



Figure 37 – Concentration of glucose, butanone and butanol, during fermentation time, for strain 579 growing on glucose in the presence of 2-butanone as substrate.

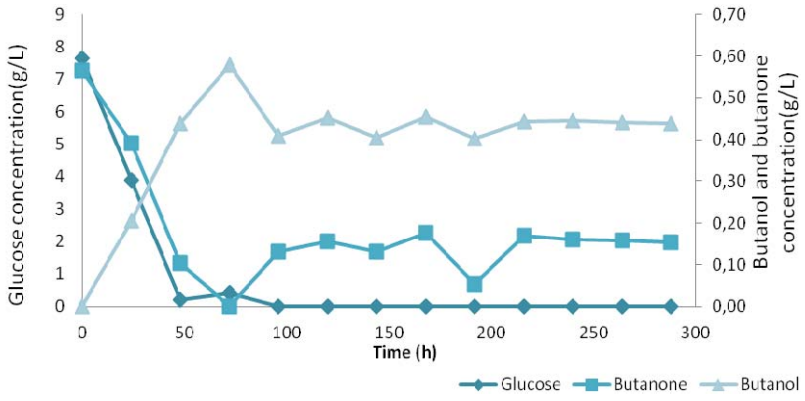



Figure 38 – Concentration of glucose, butanone and butanol, during fermentation time, for strain 734 growing on glucose in the presence of 2-butanone as substrate.

# Appendice II – Diol dehydratase activity measurement

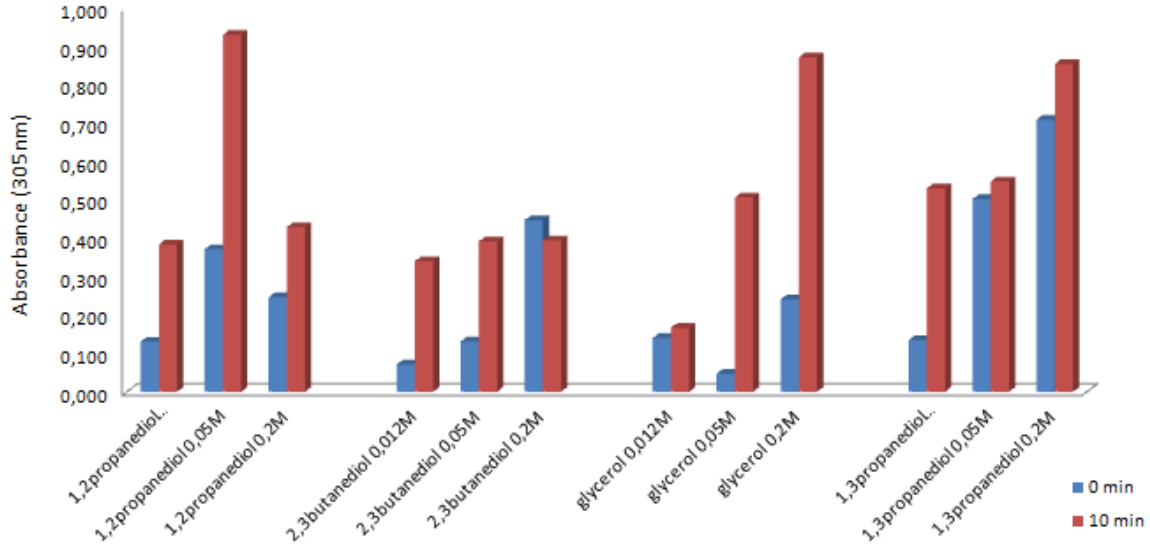



Figure 39 – Absorbance measured at time zero and ten minutes in diol dehydratase enzyme assay for strain SE20 growing on SM2 medium in the presence of 1,2-propanediol. Different substrates in different concentrations were tested.

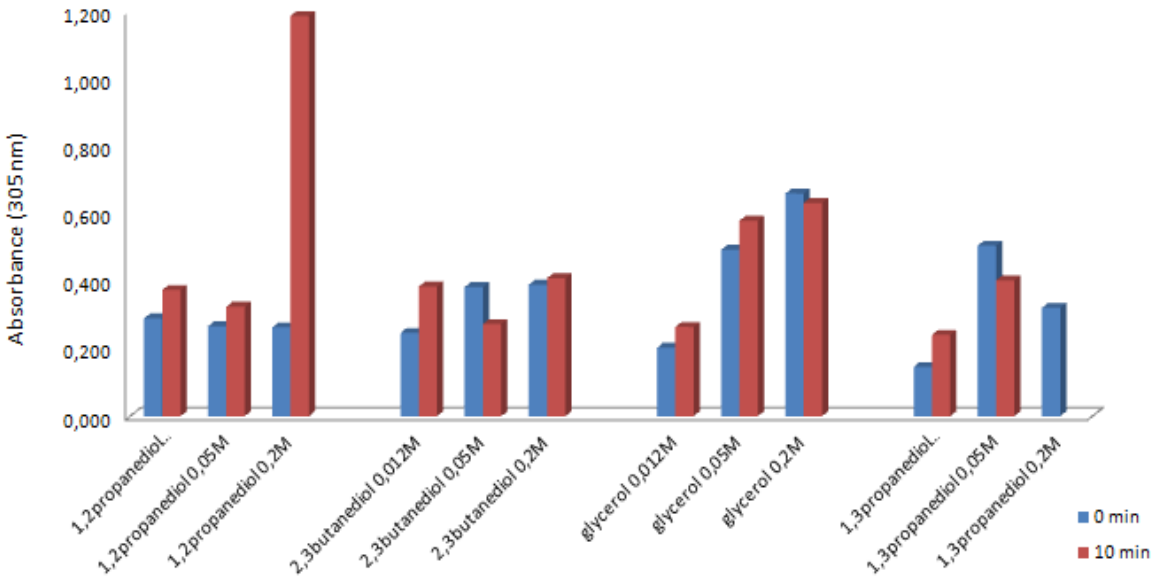



Figure 40 – Absorbance measured at time zero and ten minutes in diol dehydratase enzyme assay for strain SE20 growing on SM2 medium in the presence of 2,3-butanediol. Different substrates in different concentrations were tested.

# Appendice III – Alcohol dehydrogenase activity measurement

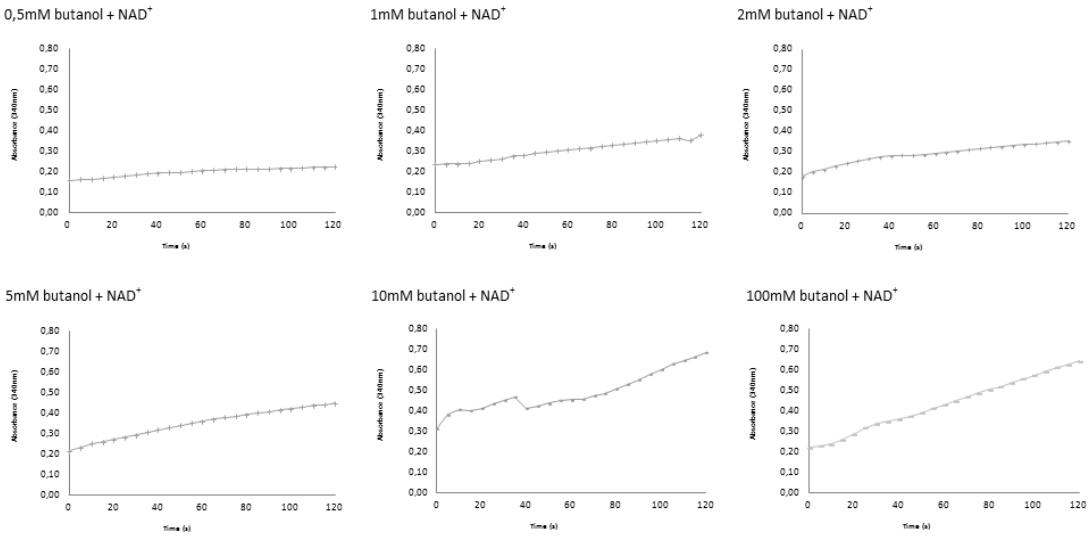



Figure 41 – Absorbance versus time (min), in alcohol dehydrogenase enzyme assay for strain SE20, in the presence of butanol as substrate, in different concentrations.

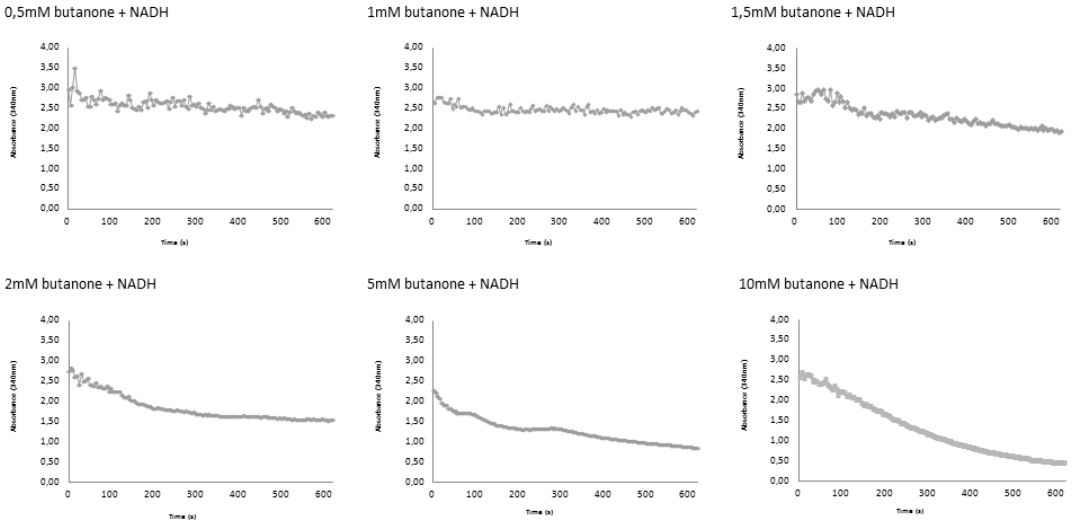



Figure 42 – Absorbance versus time (min), in alcohol dehydrogenase enzyme assay for strain SE20, in the presence of 2-butanone as substrate (reverse reaction), in different concentrations.

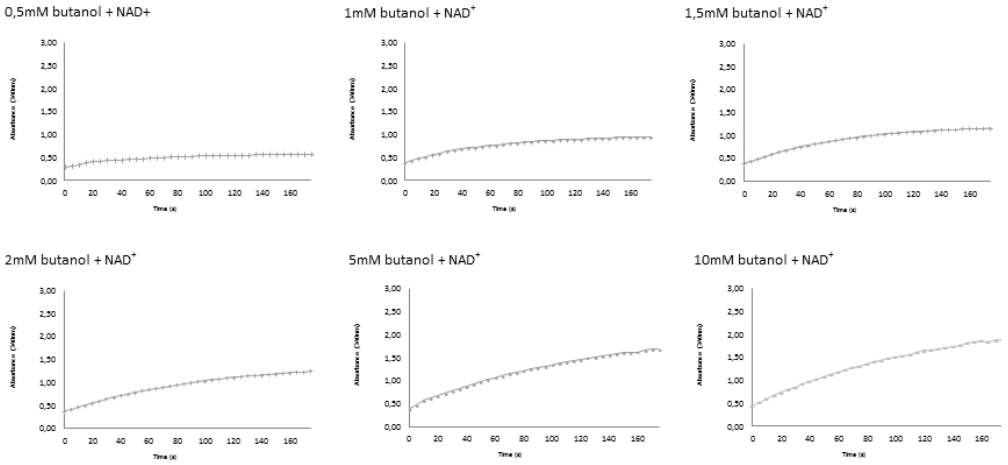



Figure 43 – Absorbance versus time (min), in alcohol dehydrogenase enzyme assay for strain SE31, in the presence of butanol as substrate, in different concentrations.

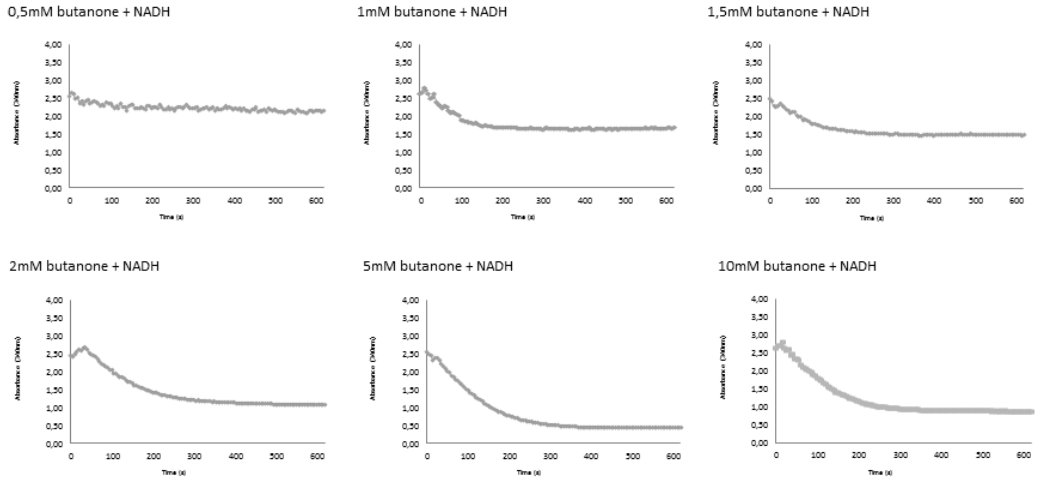



Figure 44 – Absorbance versus time (min), in alcohol dehydrogenase enzyme assay for strain SE31, in the presence of 2-butanone as substrate (reverse reaction), in different concentrations.

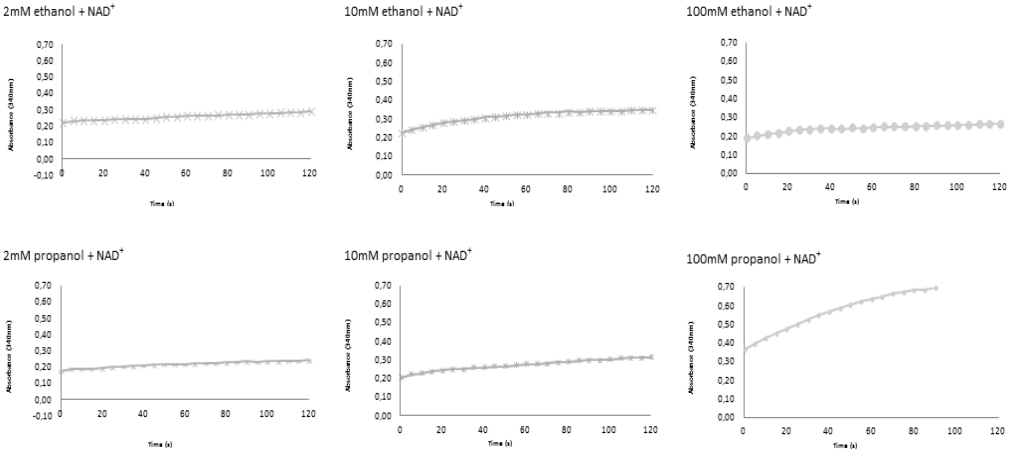



Figure 45 – Absorbance versus time (min), in alcohol dehydrogenase enzyme assay for strain SE20, in the presence of ethanol and propanol as substrates, in three different concentrations.

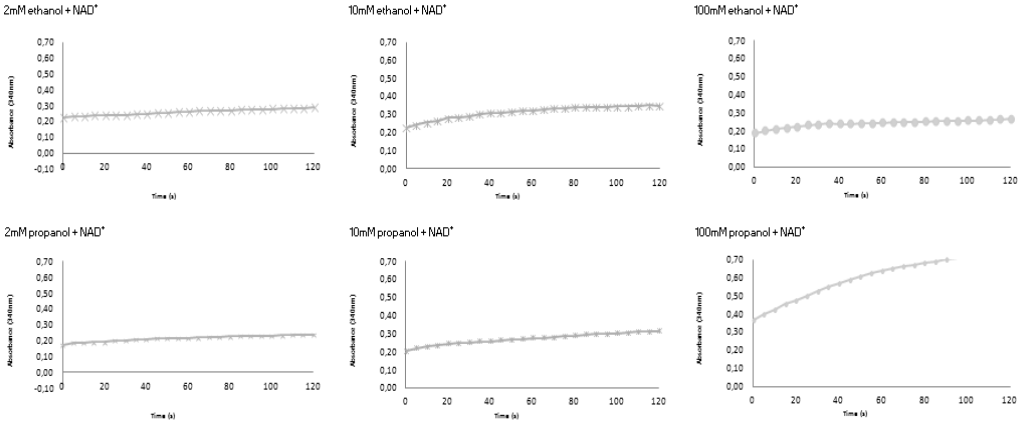



Figure 46 – Absorbance versus time (min), in alcohol dehydrogenase enzyme assay for strain SE31, in the presence of ethanol and propanol as substrates, in three different concentrations.