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TITOLO TESI

Development and optimization of a fermentation process for the production of 2,3-butanediol

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Abstract

The Ph.D. project aims to develop and optimize the fermentative production of diols from biowastes. In particular 2,3-butanediol (2,3-BDO), a promising platform chemical from which valuable derivatives such as the monomer 1,3-butadiene can be produced. The biotechnological production of 2,3-BDO has been mainly studied with pathogenic bacteria under microaerobic conditions by using pure sugars. However, the pathogenicity of such microorganisms, the high cost of sugars, and the not optimized fermentation conditions make this process not industrially competitive.

During the first year, a nonpathogenic bacterial strain (*Bacillus licheniformis* ATCC-9789) was selected and its capability to ferment different sugars commonly occurring in biomass hydrolysates (hexoses and pentoses) and raw agroindustrial biowastes (sugar beet molasses and cheese whey) was assessed. The selected microorganism is able to ferment hexoses and sucrose into 2,3-BDO. Furthermore, cheese whey could be potentially partially converted in 2,3-BDO, and molasses as well represent an interesting feedstock to produce 2,3-BDO with the selected strain.

Since 2,3-BDO is produced preferentially under microaerobic conditions, i.e., with aeration but in absence of measurable oxygen in solution, the second year activities have been focused on the optimization of the oxygen supply conditions in batch fermentation mode using glucose as substrate. Experimentally, the application of a statistical design of the experiment allowed the identification of an optimal stirring/aeration combination, and the identification of a range of oxygen transfer rate values inside which the fermentation performances are maximized.

The possibility of producing 2,3-BDO at high concentration and productivity was evaluated carrying out fed-batch experiments. Since complex media, composed of protein-rich substrate (Beef extract and Peptone), and the optimal oxygen supply conditions are both mandatory to obtain the best performances, different feed and oxygen supply strategies, were assayed.

Another activity focused on the evaluation of alternative media instead of the complex media (Beef extract and peptone). Flasks and bioreactor experiments have been carried out using a mineral media completely devoid of organic nitrogen, and meat and bone meal (protein-rich substrate derived from rendering processes), whereas ,molasses and glucose were used as carbon sources. In conclusion, animal flour and molasses seem to be suitable alternatives to the commercial substrates to produce 2,3-butanediol.

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1. Introduction

Elastomers are polymers with high molar mass which when deformed at room temperature reverts quickly to their original size (Hanhi et al., 2007). These characteristics make them suitable for several applications among which tires is the most important application area. For this reason, more than 12.6 million tons per year of elastomers are consumed worldwide (Market Study Synthetic Rubber, Ceresana, June 2013). At present, the production process follows a wholly chemical route: the macromolecules, occurring in the elastomer, derive from monomers obtained from non-renewable natural gas and petroleum resources.

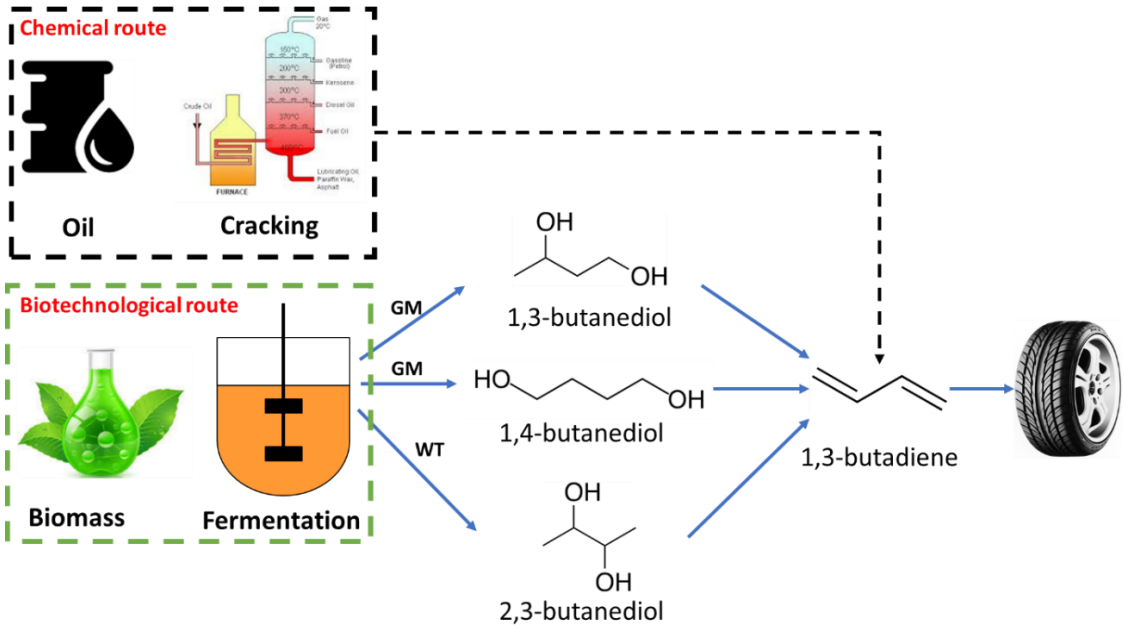


Figure 1.1 Scheme of 1,3-butadiene production, through chemical or biotechnological routes, as an intermediate for the production of elastomers for tires. Butadiene could be a by-product of the steam cracking or a product of the catalytic disidratation of butanediols which could be produced from biomasses. 1,3 and 1,4-butanediol are produced only by genetically modified microorganisms (GM), 2,3-butanediol is produced by wild type microorganisms (WT)

1,3-Butadiene is one of the most appreciated monomers, its simple chemical structure with low molecular weight and high chemical reactivity makes it a useful building block for synthesizing a wide variety of rubbers, elastomers, and polymers upon polymerization of itself or in conjunction with other monomers (Makshina et al., 2014). Currently, it is mainly produced from petroleum as a byproduct of the steam cracking process (Lynch, 2001). However, the not environmentally friendly processes that lead to the generation of greenhouse gas, the

apparently progressive depletion of the fossil fuels, and the rising demand for greener products have made it necessary to look for alternative production methods from renewable biomass feedstocks. A sustainable way for producing building blocks is the biotechnological conversion of carbon sources such as biomasses. In this sense, butadiene could be produced from the chemo catalytic conversion of diols i.e. 1,3-butanediol, 1,4-butanediol, and 2,3-butanediol (2,3-BDO) (Makshina et al., 2014) which in turn could be produced biotechnologically from sugars and agro-industrial biomasses. Thus, the production of such a butanediols from biomasses has attracted more and more interests in the last few years. Indeed, several companies, such as Versalis, Novamont, BASF, LanzaTech DuPont Tate & Lyle Genomatica, M&G (Gustavsson and Lee, 2016; Jong et al., 2012; Sanford et al., 2016) are investing in the development of a fermentative process for the production of butanediols from biomasses. Being produced by a fermentation process, several microorganisms are able to produce such a butanediols. Nerveless, only genetically modified microorganisms can produce 1,3-butanediol and 1,4-butanediol (Figure 1.1), since in nature there aren't any microorganisms which naturally possess the gene responsible for the production of these such a butanediols (Gustavsson and Lee, 2016; Kataoka et al., 2014). On the other hand, 2,3-BDO can be produced from wild type microorganisms (Celińska and Grajek, 2009; Xiao Jun Ji et al., 2011). For this reason, it could be interesting to develop an industrial production of 2,3-BDO. .

1.1. 2,3-butanediol

2,3-butanediol (also known as 2,3-butylene glycol, IUPAC name: butane-2,3-diol) is a chemical compound containing two hydroxyl group (-OH). It is characterized by the molecular formula $C_4H_{10}O_2$, whose structural formula is shown in Figure 1.2.

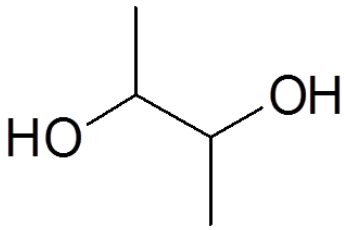
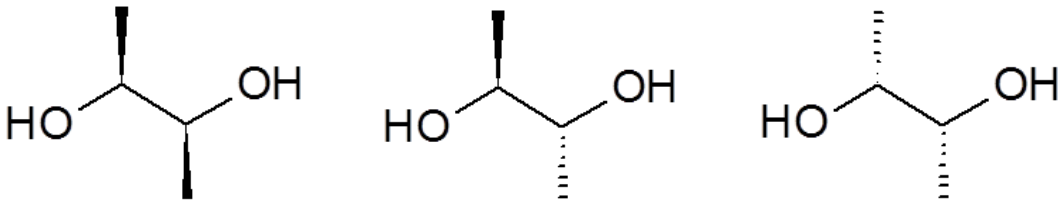


Figure 1.2 2,3-butanediol structural formula

It is an oily liquid or crystal strongly hygroscopic without color and odor, miscible in water and easily soluble in lowmolecular mass alcohols. Its molecular weight is 90.121 g/mol, and it can

structurally exist in three stereoisomeric forms: *meso*- optically inactive, (2R,3R)-(-)- levorotary isomer and (2S,3S)-(+)- dextrorotary isomer (Figure 1.3).



(2R,3R)-(-)-butanediol (2R,3S) –butanediol (2S,3S)-(-)-butanediol

Figure 1.3 2,3-butanediol stereoisomers

Its chemical and physical properties depends by the stereoisomeric form, in fact it's boiling points are between 179 and 182 °C.

1.1.1. Applications

Due to its properties, it could be applied in different industrial areas, ranging from the manufacture, chemicals, perfumes, additives for foods, moistering agents, and plasticizer. Furthermore, it is considered a platform chemical thanks to the presence of two hydroxyl groups which makes it a good substrate for different reactions. In Figure 1.4 the main products which could be obtained from the chemical conversion of 2,3-butanediol are shown.

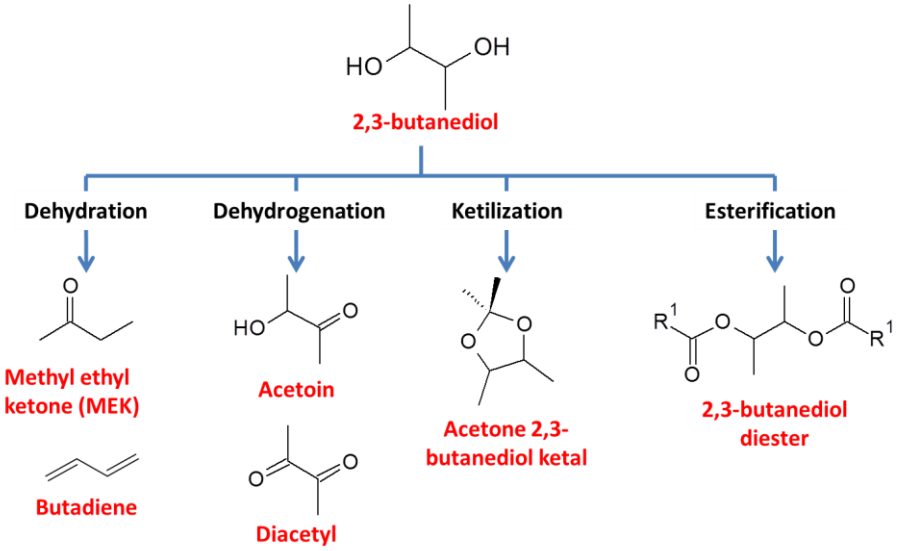


Figure 1.4 Derivatives produced from 2,3-butanediol conversion. (modified, based on Ji et al., 2011)

The dehydration of 2,3-butanediol leads to the production of two products. First is methil ethyl ketone (MEK) which is industrially used as solvent for resins and varnishes (Multer et al., 2013). The other main product of the dehydratation is butadiene (Duan et al., 2016). Such compound is an important monomer for the production of a wide variety of synthetic rubbers such as styrene-butadiene rubber used as the raw material for tires of automobiles, and styrene-butadiene latex used for foam rubbers, adhesives, and sealants.

The dehydrogenation of 2,3-butanediol leads to the production of acetoin which is a compound widely used in foods, plant growth promoters, biological pest controls, and in addition it can also be used as a precursor for a variety of chemical compounds (Zhang et al., 2016). The other product of the dehydrogenation of 2,3-butanediol is diacetyl used in the food industry as flavor (it is responsible for the buttery aroma and many dairy products) (Zhang et al., 2015).

The ketilization of 2,3-butanediol leads to the formation of the acetone 2,3-butanediol ketal who is structured with four methyl radicals. This feature of the 2,3-butanediol ketal makes it a potential agent for the formulation of gasolines thanks its chemical structure similar to the widely used methyl tert-butyl ether. Finally, the esterification of 2,3-butanediol leads to the production of precursors of different thermoplastic polymers, such as nitrate cellulose, cellulose acetate butyrate, polyvinyl chloride, polyacrylates and polymethylacrylates (Rodwell and Lafayette, 1963).

1.1.2. Production

Due to the broad spectrum of industrial application, the production of 2,3-butanediol has undergone changes over the years. 2,3-butanediol is produced chemically by a reaction sequence starting from a C_4 hydrocarbon fraction deriving from the crack gases which are composed of butenes and a mixture butane and isobutane (Butanediols, Butenediol, and Butynediol) . These reactions lead to a mixture of 2,3-butanediol isomers production and the application of high temperature and pressure. Such extreme operating conditions makes the upstream and downstream process expensive, hence an evaluation of an alternative cheap process has been investigated over the years.

Since 2,3-butanediol is a product of the metabolism of different microorganisms, biotechnological production could be a feasible alternative, also due to the mild operating condition which makes the process more suitable.

The interest in the 2,3-butanediol biotechnological production started during the Second World War. However, since less expensive petroleum-based routes became available, this interest was subsequently abandoned. Nowadays, with the shortage of fossil fuel supplies and rising petroleum prices, the idea of bio-based compounds and biorefineries is receiving significant interest. In fact, fermentation of biomasses or wastes from agricultural and agro-industrial residues has gained considerable attention (Celińska and Grajek, 2009). Thus, the biotechnological production of 2,3-butanediol become more attractive during the last decade. This shift and the increasing interest of the scientific community seems to be particularly evident considering that the publications per year related to the 2,3-butanediol have tripled during the last 5 years(Figure 1.5).

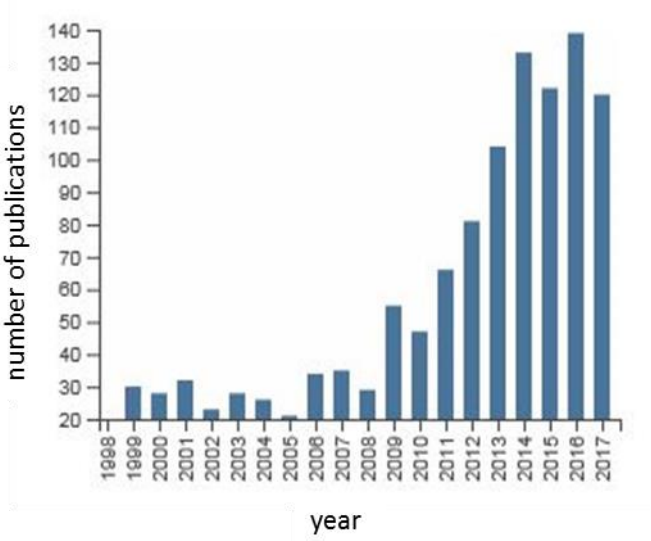


Figure 1.5 Number of publications per year 2,3-butanediol. Source: https://apps.webofknowledge.com/CitationReport.do?product=WOS&search_mode=CitationReport&SID=V2HnxQXUKw3jz986tMm&page=1&cr_pqid=1&viewType=summary&colName=WOS

According to literature data, 2,3-butanediol bioproduction is currently performed by Global Bio-Chem Technology from corn (http://www.globalbiochem.com/html/index.php) and LanzaTech from steel mill gases (Dürre and Eikmanns, 2015). This means that much more efforts could be done to optimize and make the process both cheaper and industrially achievable.

1.1.3. Metabolic pathway and microorganisms

2,3-butanediol can be produced from the fermentation of simple sugars hexose (glucose, maltose) or pentoses (xylose, arabinose, etc.). The pathway involved in 2,3-butadiol and shown

in Figure 1.6 starts from the glycolysis in which sugars are converted to pyruvate. In the second step, 2,3-butanediol is produced following three metabolic steps: i) decarboxylation of α-acetolactate pyruvate catalyzed by α-acetylated synthetase enzyme; ii) conversion of alpha-acetolactate to acetoin catalyzed by α- acetylated decarboxylase, and iii) reversible reduction of acetoin to 2,3-butanediol, catalyzed by the butanediol dehydrogenase enzyme (Biswas et al., 2012; Celińska and Grajek, 2009). Depending on the microorganism and the fermentation conditions, ethanol, acetate, lactate and acetoin are the main byproducts. In general, when anaerobic conditions are present, acetic acid, 2,3-butanediol, lactate and ethanol are the main byproducts (Celińska and Grajek, 2009; Xiao-jun Jun Ji et al., 2011). Moreover, glycerin can also be produced as a branch of the Emden Meyerhof glycolytic pathway in anaerobic conditions (Wang et al., 2001), a behavior that has been observed in Bacillus microorganisms (Jurchescu et al., 2013). On the other hand, aerobic conditions lead to the production of biomass and acetoin which is the 2,3-butanediol precursor. 2,3-butanediol should be produced in anaerobic conditions, however, oxygen limited conditions (microaerobic conditions) are mandatory to yield high amounts 2,3-butanediol and limit the byproducts formation.

This behavior can be explained by the pathway involved in the 2,3-BDO production that is part of the regulation of the NADH/NAD^+ ratio. The scares availability of oxygen, i.e., the final electron acceptor, impedes the regeneration of NAD+ which is associated to the reducing power that is linked to glycolysis and is not consumed by aerobic respiration (Celińska and Grajek, 2009; Converti et al., 2003; Rodwell and Lafayette, 1963).

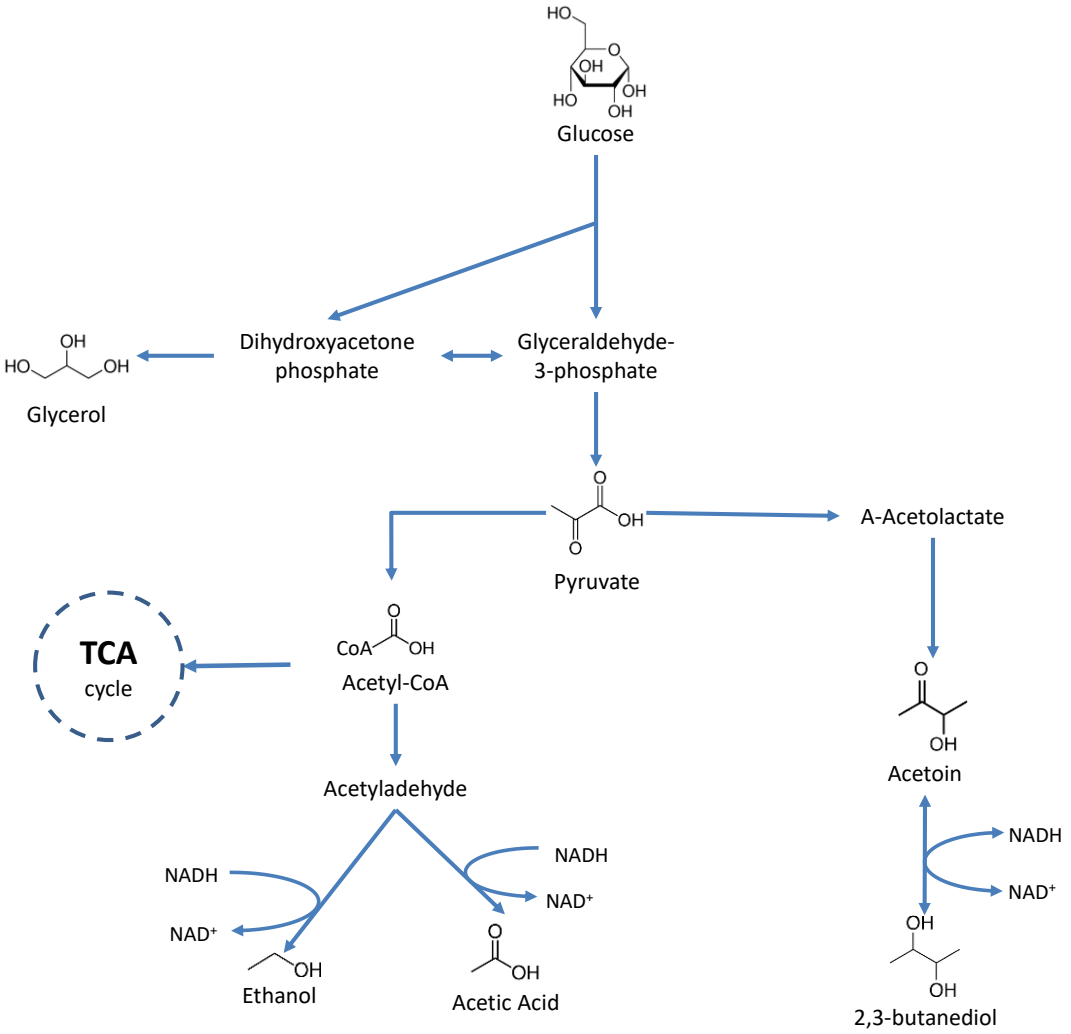


Figure 1.6 2,3-butanediol fermentation pathway (modified based on Celińska and Grajek, 2009; Ji et al., 2011)

Several microorganism species are able to produce 2,3-butanediol, but only a few of them are able to produce enough 2,3-BDO to be considered interesting for industrial applications. The main species belong to *Klebsiella*, *Enterobacter*, *Bacillus* and *Serratia* genera.

Klebsiella's microorganisms are considered as the major producers, particularly those belonging to *K. pneumoniae* and *K. oxytoca* species. These microorganisms are able to accumulate quickly high amounts of 2,3-butanediol, moreover they grow and convert most of the sugars present in cellulose and hemicellulose hydrolysates to 2,3-butanediol. However, the pathogenicity of these species is generally considered an obstacle for their large-scale use (Ma et al., 2009; Qureshi and Cheryan, 1989; Ramachandran et al., 1990).

Microorganisms of the genera *Enterobacter* can be promising 2,3-butanediol producers; they have a broad spectrum of substrates (including glucose, xylose, mannitol and various hydrolysates of biomass) and can grow rapidly under both aerobic and anaerobic conditions. However, they are classified as pathogenic bacteria like *Klebsiella* species (Hazeena et al., 2016; Zeng et al., 2004).

Another microorganism known for its ability to produce 2,3-butanediol is *Serratia marcescens*, a pathogenic gram-positive bacterium. It is widely present in nature and it can be isolated from water, soil, air, milk and clinical specimens (Shi et al., 2014). In addition, it grows rapidly on a wide range of substrates and some strains can be considered good candidates for industrial production of 2,3-butadiene. However, its pathogenicity is an obstacle like in the cases of the *Klebsiella* and the *Enterobacter* genres.

Another genus of microorganisms able to produce 2,3-butanediol is *Bacillus*. Recently, the species *Paenibacillus polymyxa* (*Bacillus polymyxa*), *B. amyloliquefaciens* and *B. licheniformis* have been studied. These microorganisms can produce 2,3-butanediol at concentrations even greater than 100 g / L, but use xylose typically less efficiently than glucose (Jurchescu et al., 2013; Raspoet et al., 1991; Yang et al., 2011). Furthermore, such microorganisms are generally recognized as safe since they belong to the biosafety level 1, a fact that makes them much more attractive for a possible industrial application.

1.1.4. Substrates

In the development of industrial fermentation processes, the substrate is usually the cost that affects the most the final product. For this reason, using substrates deriving from an agro industrial process, such as waste or by products, could help reducing the economic aspects of the production. Furthermore, the application of such biowastes for the production of other products and molecules is in line with the concept of bioeconomy and biorefinry (Koutinas et al., 2014; Philp et al., n.d.; Scoma et al., 2014).

The production of 2,3-butanediol from agro industrial deriving substrates has been widely studied. Agro industrial biowastes such as molasses, whey or glycerol and hydrolysates from vegetable biomasses, were the main substrates evaluated thus far.

Plant-derived biomass consists approximately of 40-50% cellulose, 25-30% hemicelluloses and 15-20% lignin, although its composition may vary greatly in different plant types. Cellulose and

hemicellulose are natural polymers in which simple sugars are the monomers, in particular, cellulose in composed of glucose while hemicellulose is composed of pentoses (xylose and arabinose) and hexoses (mannose, glucose, and galactose). However, a pretreatment is mandatory in order to entrap the sugars in the polymer matrix available for the microorganisms (Agblevor et al., 2007; Fitzpatrick et al., 2010). In this manner , the vegetal biomasses could be considered a source of sugars for producing 2,3-butanediol. Several works on the bioconversion of different biomass hydrolysate have been studied. Palm frond hydrolysate was used as carbon source with the microorganism *Enterobacter cloacae* SG-1 and resulting in 7.67 g/l 2,3-butanediol (Hazeena et al., 2016). Acid hydrolysate of *Jatropha hulls* was used as raw materials for the production of 2,3-butanediol with *Klebsiella oxytoca* (Jiang et al., 2012). In another study, a thermophilic *Bacillus licheniformis* strain was used for 2,3-butanediol production from corn stover hydrolysate, resulting in the production of 74.0 g/L of 2,3-butanediol with a productivity of 2.1 g/L h and a yield of 94.6% (Li et al., 2014b).

Whey is a liquid waste originating from the transformation of milk into cheese. It has an important lactose content (44-52 g/L) which promotes a high chemical oxygen demand (COD), and can also cause severe pollution problems if discharged in the environment (Domingos et al., 2016; Fernández-Gutiérrez et al., 2017). Whey can be valorized by biological processes based on lactose fermentation into biochemicals such as 2,3-butanediol. *Klebsiella pneumoniae* has been proven to be the most promising microorganism for the conversion of lactose (that can be found in whey permeate) into 2,3-butanediol, thus obtaining a concentration of 7.5 g/l 2,3-butanediol representing a yield of 0.46 g/g (Lee and Maddox, 1984). In another study, two strains of *Klebsiella* were selected for their ability to produce 2,3-butanediol from cheese whey powder. A two-stage pH control strategy with pulsed fed-batch fermentation resulted in the maximal 2,3-BD production of 57.63 g/L (Guo et al., 2017).

Molasses is a byproduct of the sugar industry, it consists of a syrup with a density of about 1.4 g cm3. Due to strictly defined processing methodology, molasses do not have a very complex biochemical composition and are mainly made of extremely high sugar concentrations, substantially due to sucrose. Given the high sugars concentration, it is considered a good fermentation substrate from which different biochemicals could be produced, i.e. citric acid, lactic acid, isoamyl acetate, oxalic acid, D-glucaric acid, Rhamnolipids and fructo-oligomers (Scoma et al., 2016). However, only a limited number of studies have applied molasses as substrate for the production of 2,3-butanediol. Under a fed-batch operation mode, 99.5 g/L of

2,3-butanediol and acetoin was obtained byusing sugarcane molasses as carbon source and *Enterobacter cloacae* (Dai et al., 2015). The microorganism *Enterobacter aerogens* have been used to convert molasses in 2,3-butanediol resulting in the production of 0.41 g/L of 2,3-butanediol (Perego et al., 2000).

Glycerol is the waste of the fatty acids esterification reaction for the production of biodiesel. Such waste is produced in huge amounts, in fact, it is estimated that 1000 thousand tons has been produced in Europe in 2013 (Ripoll et al., 2015). Various authors have applied glycerol as carbon source for the production of 2,3-butanediol. In the work of Petrov and Petrova (Petrov and Petrova, 2010) the influence of aeration and pH were investigated in a fedbatch processes for glycerol fermentation by *Klebsiella pneumoniae* which led to the production of 70 g/l 2,3-butanediol. In another work, a wide screening of microorganisms was carried out in order to evaluate their potential as 2,3-butanediol producer. The microorganism, *Raoultella terrigena*, a non-pathogenic bacteria, was selected for its ability to produce 2,3-butanediol (Ripoll et al., 2015).

1.1.5. Effect of the operating conditions

The aim of the present section is to describe the operating variables whichaffect most the 2,3-butanediol biotechnological production process.

1.1.5.1. Temperature

The operating temperature is a critical parameter for the success of every bioprocess in which microorganisms or enzymes are involved since the activity of the participating enzymes and the cellular functions depend on it. Although the optimum operation temperature depends on the microorganism used, it can generally be argued that in each case it will be close to the optimum temperature of growth of the bacterial species. In the production of 2,3-butanediol, for most of the 2,3-butanediol producing species the temperature range is 30-37°C (Celińska and Grajek, 2009).

1.1.5.2. pH

pH is another parameter that strongly influences the 2,3-butanediol production. As for the temperature, the pH depends on the microorganism used. In 2,3-butanediol biosynthesis, the pH is closely linked to physiological functions. In fact, 2,3-butanediol is related to the prevention

of intracellular acidification. The production and accumulation of products of the fermentative acid metabolism, such as succinic, formic, lactic and acetic acid, cause the Ph to drop drastically. This medium acidification induces the deviation of the metabolic path to neutral compounds, such as 2,3-BD, thus avoiding the accumulation of more toxic compounds (Maddox, 1996).

1.1.5.3. Medium composition

Other than the essential nutrients, the production of 2,3-butanediol is influenced by some exogenous additives, such as amino acids, vitamins and growth factors usually occurring in culture medium composition (Xiao-jun Jun Ji et al., 2011). The application of organic nitrogen sources, yeast extract, beef extract and peptone, usually leads to high fermentation performances. Several works show the effect of the organic nitrogen sources and its concentration on the production of 2,3-BDO (Li et al., 2013; Ripoll et al., 2015; Yang et al., 2011).

In the work of Nilegaonkar (Nilegaonkar et al., 1992) a microorganism of the species *Bacillus licheniformis* was used to produce 2,3-butanediol from glucose, using a medium containing 10 g/L of peptone from soy bean and 10 g/L of beef extract at pH 6.0 and 37°C. This had led to the optimum yield of 0.47 g/g glucose, close to the theoretical one of 0.5 g/g, after 72 h of growth.

In another work, a medium composition for the production of 2,3-butanediol by a *Serratia marcescens* species microorganism from sucrose was optimized in shake flask fermentations using the response surface methodology (RSM). Results indicated that yeast extract and sodium acetate had significant effects on the 2,3-BD production. The optimal medium was used to perform fermentation experiments leading to the production of 139.92 g/l of 2,3-butanediol with a productivity of 3.49 g/l/h and the yield of 94.67% (Zhang et al., 2010).

The effect of the yeast extract concentration was studied in the work of Häßler et al., 2012. The concentration of 60 g/L of yeast extract facilitated the production of 111 g/L of 2,3-BDO from sucrose with the microorganism *Paenibacillus polymyxa*.

Notwithstanding the common use of traditional organic nitrogen sources, such as beef extract, peptone from soy bean, yeast extract, etc., these protein sources are rather expensive and hence effect negatively the overall cost of production. Therefore, the application of a nutrient source which originates from waste of an agro industrial process could be an alternative. The next paragraph describes the effect of corn steep liquor (by-product of the corn wet-milling industry) in the production of 2,3-butanediol.

The effects of organic nitrogen sources on 2,3-butanediol production with the microorganism *Bacillus amyloliquefaciens* was studied in the article Yang et al., 2012. Corn steep liquor, soybean meal and ammonium citrate were found to be the fermentation's key factors. A response surface methodology allowed for the identification of the optimal concentration of nitrogen source wich led to the production of 62.7 g/l of 2,3-butanediol.

The effect of the initial concentration of corn steep liquor on 2,3-butanediol production from glucose by the microorganism *Bacillus subtilis* was studied by Yang et al., 2013. Corn steep liquor affects not only 2,3-butanediol production, but also the ratio of 2,3-BD to acetoin. When a high concentration of CSL was supplemented, the ratio of 2,3-BD to acetoin increased.

1.1.5.3.1. Meat and bone meal

An interesting substrate rich in proteins and amino acids is meat and bone meal, a product of the rendering industry. The latter converts the animal tissue (i.e. the waste of the animal slaughter industry) in value added products, fats and a protein rich substrates called meat and bone meal also shown in Figure 1.7 (Mekonnen et al., 2014).

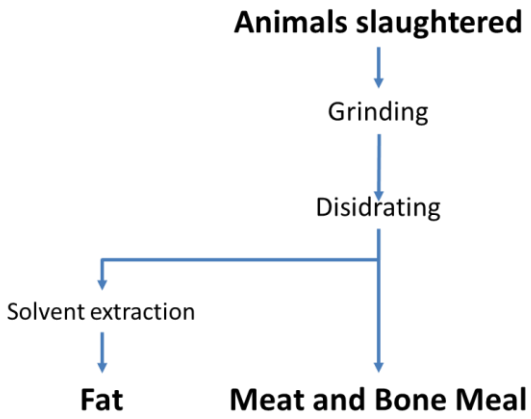


Figure 1.7 Meat and bone meal production process, figure rielaborated from Mekonnen (Mekonnen et al., 2014).

High content in proteins and high heating value makes meat and bone meal suitable for pet feeding (Liang et al., 2011) and production of energy (Cascarosa et al., 2012). However, the recent emergence of bovine spongiform encephalopathy resulted in a drastic decrease of the use of this byproduct as animal feed. Moreover, the high content in protein makes such substrate interesting as organic nitrogen source in fermentative process (Pleissner and Venus,

2016). To date, only two works have applied this substrate in biotechnological processes, the production of omega-3 polyunsaturated fatty acids (Liang et al., 2011) and cyanophycin (Solaiman et al., 2011).

1.1.5.4. Oxygen supply

Oxygen supply is undoubtedly the most influencing parameter for the biotechnological production of 2,3-butanediol. As described in the paragraph on the influence of the pH, 2,3-butanediol has a cell physiological role, in fact, it prevents acidification and participates in the regulation of the intracellular NADH/NAD^+ ratio. Likewise, oxygen supply plays a key role in the regulation of the NADH/NAD^+ ratio (Xiao-jun Jun Ji et al., 2011).

In general, bacteria involved in the 2,3-butanediol production are anaerobic facultative, in fact, such microorganisms are able to produce energy from the metabolism via respiration when in the presence of oxygen, and do the same via fermentation when in the absence of oxygen. Furthermore, they are able to produce several byproducts depending on oxygen availability (Figure 1.8).

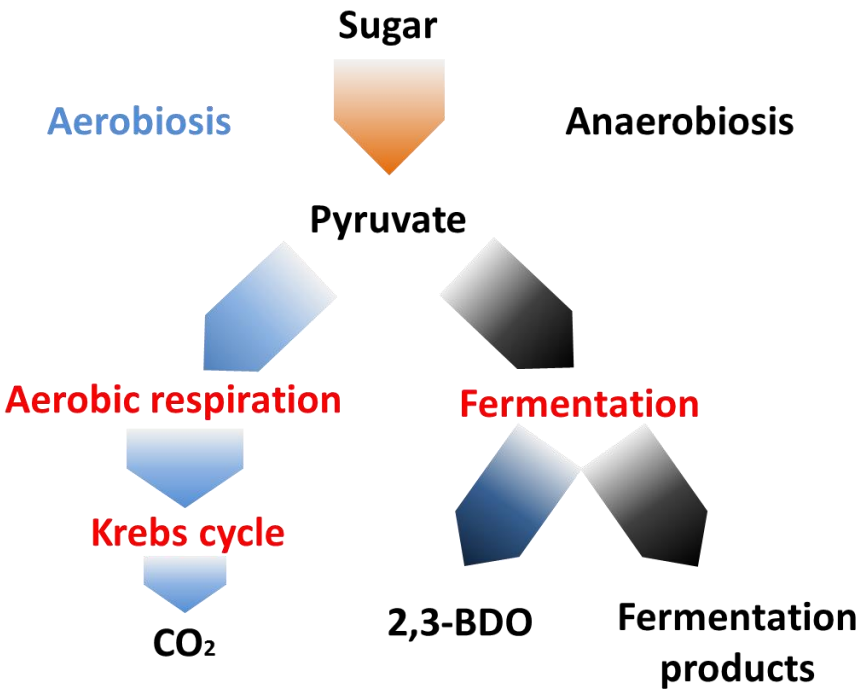


Figure 1.8 Schematic representation of facultative anaerobes respiration pathway (modified from (Rodwell and Lafayette, 1963)

In terms of regeneration of NAD^+ from NADH, 2,3-butanediol is a product of the anaerobic fermentative metabolism. However, if oxygen is absent, the production of fermentation byproducts, i.e. acetic acid, lactic acid, etc. is much more significant than 2,3-butanediol. On the other hand, under aerobic conditions, the complete substrate oxidation through respiration occurs, thus leading to the deviation of the metabolism towards the production of carbon dioxide and biomass (Celińska and Grajek, 2009). Furthermore, an excess of oxygen leads to the deactivation of the enzyme α-acetolactatosintetase, an enzyme involved in the synthesis of 2,3-BDO from pyruvate (Celińska and Grajek, 2009).

These conflicting effects indicate that the production of 2,3-BDO present a maximum for certain oxygen availability conditions, as can be seen in Figure 1.9. Moreover, not only the production of 2,3-BD but also the distribution of the other fermentative route products depends directly on the availability of oxygen (Rodwell and Lafayette, 1963).

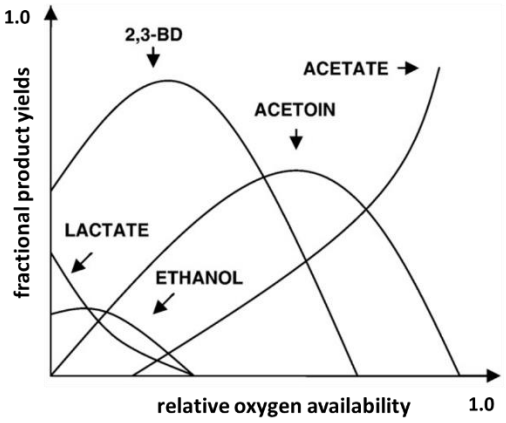


Figure 1.9 Effect of the oxygen availability on the product distribution in *P ploymyxa* (Figure based on Celińska and Grajek, 2009)

Therefore, the condition required to maximize the production of 2,3-BDO is characterized by the supply of oxygen in the broth. In fact, the concentration of dissolved oxygen is close to zero and lower than the detection limit of the electrode. Such condition is called microaerobiosis.

Being the oxygen supply the parameter that affects the process the most, and considering that 2,3-butanediol is mainly produced under microaerobic conditions, it is fundamental to study the oxygen mass balance in such a conditions.

1.1.5.5. Oxygen Mass transfer in microaerobic conditions

The following section aims to explain the theory of oxygen mass balances in aerobic and microaerobic fermentation.

The oxygen mass balances, if the perfectly mixed fluid dynamic model can be assumed both in the liquid (equation 1) and the gas (equation 2) phases, can be represented in the following manner::

$$V_L \cdot \frac{dC_L}{dt} = OTR \cdot V_L - OUR \cdot V_L \quad (1)$$

$$V_G \cdot \frac{dC_G}{dt} = Q_G^{in} \cdot C_G^{in} - Q_G^{out} \cdot C_G^{out} - OTR \cdot V_L \quad (2)$$

where C_L and C_G are the oxygen concentrations in the liquid and gas phase respectively, V_L and V_G are the volume of liquid and gas phase respectively, Q_G is the gas volumetric flow rate and the index in and out refers to the inlet and the outlet stream. OUR and OTR are respectively the oxygen uptake rate in the liquid phase, and the oxygen transfer rate from the gas to the liquid.

The OTR can be calculated as:

$$OTR = k_L a \cdot (C_L^* - C_L) \quad (3)$$

where $k_L a$ is the oxygen volumetric mass transfer coefficient, C_L^* is the value of dissolved oxygen in equilibrium with the gas phase, and C_L is oxygen concentration in the liquid. The OUR can be expressed as:

$$OUR = q_{O_2} \cdot X \quad (4)$$

where q_{O_2} is the specific oxygen uptake rate and X is the biomass concentration in the bioreactor.

Microaerobic conditions are characterized by a concentration of dissolved oxygen close to zero and lower than the detection limit of the electrode. If $C_L = 0$ and the pseudo steady-state condition are assumed, equations (2) and (3) become:

$$OUR = OTR = k_L a \cdot C_L^* \quad (5)$$

$$OTR = Q_G^e \cdot C_G^e - Q_G^u \cdot C_G^u \quad (6)$$

Hence, in the pseudo steady-state described by equations 5 and 6, being oxygen uptake rate equal to the oxygen transfer rate, the latter controls the actual bioprocess rate. The OTR can be controlled through the $k_L a$, which in turn depends on rheological characteristics of the fermentation broth, on the stirring, and on the aeration intensities.

A limited number of studies investigated the effects of aeration and agitation, and therefore of $k_L a$ and OTR, on 2,3-BDO production (Qureshi and Cheryan, 1989; Ramachandran et al., 1990; Silveira et al., 1993; Yang et al., 2011). Ramachandran (Ramachandran et al., 1990) tested the effect of different $k_L a$ values on 2,3-BDO production from lactose with a *Klebsiella oxytoca* microorganism by varying the stirring rate at a constant air flow rate. High $k_L a$ values (120 h^{-1}) led to negligible 2,3-BDO concentrations, whereas lower $k_L a$ values (78 h^{-1}) led to an increase in 2,3-BDO productivity. However, at very low $k_L a$ (47 h^{-1}), BD yield and productivity dropped again. The effect of the air supply on the production of 2,3-BDO by *Klebsiella pneumoniae* from sucrose was studied by Silveira (Silveira et al., 1993). The maximum BD productivity (1.5 g L^{-1} h^{-1}) was obtained at a $k_L a$ equal to 120 h^{-1}, whereas lower (8-55 h^{-1}) and higher (320-620 h^{-1}) $k_L a$ values led to lower productivities. The effect of agitation on the production of BD from glucose by a *Bacillus amyloliquefacien*s was studied by Yang (Yang et al., 2011), who obtained a high 2,3-BDO yield (0,42) at low rpm (100), and high values of 2,3-BDO productivity (0.86) at high rpm (200).

1.2. Bibliography review

Table 1.1 shows the results published in recent years regarding the production of 2,3-BDO, in terms of maximum concentration reached, yield and productivity. In particular, this bibliografic research took into consideration different fermentation conditions such as:

- Microrganism;
- Carbon substrate (pure sugars or from agro industrial biomasses);
- Nutrients added (medium composition, organic nitrogen sources added to the medium);
- Operation mode (batch, fed-batch, ….).

Table 1.1 Summary of the major results found in the literature for the biotechnological production of 2,3-BDO.

Microorganism	Substrate	Nutrients added	Operation mode	2,3-BDO final Concentration (g/L)	Productivity (g/Lh)	Yield (g/g)	References
Klebsiella oxytoca	Molasses	yeast extract, tryptone	fed-batch	98.2	1.0	0.48	(Afschar et al., 1991)
Klebsiella pneumoniae	Glucose	Corn Steep Liquor	fed-batch	150	4.21	0.43	(Ma et al., 2009)
Klebsiella oxytoca	Glucose	-	fed-batch	130	1.64	0.48	(Ji et al., 2009)
Serratia marcescens	Sucrose	Yeast extract	fed-batch	139.92	3.49	0.41	(Zhang et al., 2010)
Bacillu subtilis		Corn Steep Liquor	fed-batch	39.2	0.68	0.39	(Yang et al., 2013)
Bacillus amyloliquefaciens	Glucose	Corn Steep Liquor, Soybean meal	Batch	66.5	2.22	-	(Yang et al., 2012)
Bacillus licheniformis	Glucose	Yeast extract triptone	fed-batch	144.7	1.14	0.4	(Jurchescu et al., 2013)
Bacillus spp.	glucose	Yeast extract triptone	fed-batch	100	0.6	0.4	(Yan et al., 2017)
Enterobacter aerogenes	glucose	Yeast extract	fed-batch	93.75	1.74	0.4	(Jun et al., 2017)
Serratia marcescens	Sweet sorgum juice	Yeast extract	fed-batch	109.44	1.40	0.4	(Yuan et al., 2017)

Table 1.1 continued

Microrganism	Substrate	Nutrients added	Operation mode	2,3-BDO final Concentration (g/L)	Productivity (g/Lh)	Yield (g/g)	References
Klebsiella pneumoniae	cheese whey powder	-	fed-batch	48.9	-	0.43	(Guo et al., 2017)
Klebsiella variicola	Glycerol	Yeast extract peptone	fed-batch	82.5	-	0.62	(Charles et al., 2017)
Enterobacter cloacae	Sugarcane molasses	Urea, Corn steep liquor	fed-batch	90.8	1.51	0.39	(Dai et al., 2015)
Klebsiella oxytoca	Glucose	Yeast extract Casamino acids	fed-batch	142.5	1.47	0.42	(Cho et al., 2015)
Bacillus licheniformis	Apple pomace hydrolysate	Yeast extract	fed-batch	113	0.69	0.49	(Białkowska et al., 2015)
Enterobacter cloacae	glucose	-	fed-batch	85	1.7	0.48	(Priya et al., 2016)
Klebsiella oxytoca	maltodextrin	-	fed-batch	88.1	1.13	0.42	(Chan et al., 2016)
Serratia marcescens	Glucose	Yeast extract peptone	fed-batch	87.8	1.6	-	(Shi et al., 2014)
Bacillus licheniformis	Inulin	Yeast extract, Corn steep liquor	fed-batch	103	3.4	-	(Li et al., 2014a)
Bacillus licheniformis	corn stover hydrolysate	Yeast extract, Corn steep liquor	fed-batch	74	2.1	0.47	(Li et al., 2014b)

2. Aim of the work

This work was supported by the Italian project ALBE (ALternative Biomasses for Elastomers) which is a part of the Italian cluster for the green chemistry (Cluster Spring). The final objective of the project was the development of the entire chain for the production of rubber made from renewables, in which biobutadiene would be a real breakthrough in the industrial production of building blocks for rubbers. Biobutadiene is currently produced from oil based feedstock, however, it could be produced from the chemocatalytic conversion of some diols i.e. 1,3-butanediol, 1,4-butanediol, and 2,3-butanediol which in turns could be produced biotechnologically from sugars and agro-industrial biomasses.

2,3-butanediol is a platform chemical which could be converted into different molecules beside butadiene. Unlike 1,3 and 1,4 butanediol, which could be produced only by genetically modified microorganisms, 2,3-butanediol could be produced by wild-type microorganisms. However, its industrial production is still limited, indeed different problems make its production not industrially competitive.

As a project partner, the Industrial and Environmental Biotechnology Group of the Department of Civil, Chemical, Environmental and Material Engineering (DICAM) of the University of Bologna had the aim of developing a competitive fermentative process for sugar (or renewable sources) conversion to butanediols suitable to be further converted to bio-butadiene. In particular, my PhD project focused on the optimization of 2,3-butanediol production from sugars and biowastes using a wild-type microorganism.

The process has been subdivided into five main objectives:

Microorganism preselection and evaluation of the growth and production conditions (culture media, temperature, and pH). In any pure culture fermentation process, the selection of a microorganism able to produce the target molecule is the first mandatory aim. 2,3-butanediol is preferentially produced by potentially pathogen microorganisms which make its production not industrially feasible. Thus the first aim of the project was the identification of a wild-type nonpathogenic bacteria able to produce 2,3-butanediol available in microbial public cultures. Afterwards, the selection of the microbial growth and production conditions was carried out experimenting different literature conditions for the selected microorganism.

Evaluation of the possibility of producing 2,3-BDO from different sugars and biowastes. Now a day, the application of pure sugars for the fermentative production of biochemical is preferential. However, the application of such compounds increases the production costs. Thus, the evaluation of sugars or biomasses coming from the agroindustrial process for the production of 2,3-butanediol was evaluated.

Optimization of the oxygen supply conditions. The microorganisms able to produce 2,3-butanediol are facultative anaerobic bacteria which means that energy can be produced by bacteria when oxygen is present or not. Depending on oxygen availability, the metabolism is shifted towards different products. In particular, 2,3-butanediol is preferentially produced when oxygen is supplied but it is not measurable in solution. Thus, the aim of this stage is the identification of the suitable oxygen supply conditions that allow the optimization of the fermentation performances.

Study of the fed-batch operation mode. In order to make the process more industrially competitive by increasing the product concentration, the application of fed-batch fermentation mode has been tested.

Replacement of the complex media. The fermentative 2,3-butanediol production is also influenced by the organic nitrogen source, indeed it brings amino acids, vitamins or growth factors that help microbial metabolism. Usually, commercial nitrogen organic sources such as yeast extracts, Beef extract, and soy peptone are used. However, the price of such compounds makes the fermentation process industrially not competitive. Thus, in this work, the possibility of replacing the commercial organic nitrogen sources with a cheap one coming from the agroindustrial process has been studied.

3. Materials

3.1. Chemicals

All the chemical compounds were supplied by Sigma Aldrich S.r.l. (Milan. Italy).

Molasses was kindly provided by Coprobi (Italy). The tested molasses had the following composition: glucose 30%, fructose 24%, ashes 7%, humidity 13%.

Meat and bone meal used in this work was kindly supplied by the group of the Professor Giacomo Biagi (Department of Veterinary Medical Sciences – University of Bologna). Meat and bone meal used in this work, originated from poultry slaughterhouse, had the following composition: proteins 65.7%, fats 14.1%, carbohydrates 4.5%, ashes 15.5%, humidity 0.7%. In this thesis, they are called chicken meat and bone meal (CMBM)

3.2. Culture medium

In this paragraph, culture medium employed in each stage of this work is listed. Table 3.1 describes the composition of the medium used for the preparation of the precoltures. Table 3.2,Table 3.3Table 3.4Table 3.5 describes the compositions of the medium used during the selection of the fermentation medium. Such mediums were selected form the literature. The medium described in Table 3.2 is the same used in the work of Jurchescu (Jurchescu et al., 2013). The medium described in 3.3 is the same used in the work (Perego et al., 2003), finally, the medium described in table 3.4 is the same used by Nilegaonkar Nilegaonkar et al., 1992b.

Table 3.1 Pre inoculum medium composition

Compound	Concentration (g/L)
Beef extract	3
Peptone	5

Table 3.2 Medium composition 1 (Jurchescu et al., 2013)

Compound	Concentration (g/L)
Yeast extract	5
Tryptone	5
K_2HPO_4	7
KH_2PO_4	5,5
$(NH_4)_2SO_4$	1
$MgSO_4 \cdot 7H_2O$	0.25
$Na_2MoO_4 \cdot 2H_2O$	0.12
$CaCl_2 \cdot 2H_2O$	0.021
Glucosio	20

Table 3.3 Medium composition 2 (Perego et al., 2003)

Compound	Concentration (g/L)
Yeast extract	5
KH_2PO_4	6
K_2HPO_4	14
KH_2PO_4	6
$(NH_4)_2SO_4$	2
$MgSO_4 \cdot 7H_2O$	0.25
sodio citrato diidrato	1
Glucosio	20

Table 3.4 Medium composition 3 (Complex media) (Nilegaonkar et al., 1992b)

Compound	Concentration (g/L)
Beef extract	10
Peptone	10
NaCl	5
Glucosio	20

Table 3.5 Mineral salt medium (MSM)

Compound	Concentration (g/L)
KH_2PO_4	0,7
Na_2HPO_4	0,9
$NaNO_3$	2
$MgSO_4$ x $7H_2O$	0,4
$CaCl_2$ x$2H_2O$	0,1
Trace element solution (mL/L)	2

3.3. Microorganism

The microorganism employed in this work is the *Bacillus licheniformis* ATCC 9789, obtained from American Type Culture Collection (ATCC). In this section, the techniques of conservation and preparation of the inocula employed in this thesis are described.

3.3.1. Conservation

The strain, delivered as a lyophilized form, was resuspended in the rich medium Nutrient Broth (Table 3.1) as recommended by ATCC, and incubated at 30°C and 150 rpm. After 24 h growth, the culture was suspended in glycerol in order to obtain a solution 20% v/v of glycerol. The solution obtained in this way was aliquoted in 2 ml tubes and maintained at -80°C as a stock solution.

3.3.2. Precultures

The preparation of the inocula is a crucial stage in a fermentation process. In this work all the inocula were prepared starting from a -80 stock solution which was slowly thawed until ambient temperature, then, working under the laminar flow hood, 0.5 ml cell stock were placed into 250 ml flasks each containing 50 ml preculture medium. The flasks were incubated at 30 °C and 150 rpm for 24 h.

3.4. Flask experiments

All flasks experiments have been conducted in 250ml flasks filled with 50 ml medium. In this thesis, flask experiments have been carried out in order to evaluate different experimental conditions at the same time. The fermentation conditions were defined according to the experimental phase and they are described more in detail in the following paragraphs.

In general, each flask experiment was prepared according to the following procedure:

- Medium and carbon source preparation: media components solution was prepared by weighing each compound excluding the carbon source. The solution of carbon source (sugars or biomasses) was prepared separately in order to avoid the Maillard reaction (during the sterilization) which could adversely affect the performance of the fermentation;

- Sterilization: the medium and carbon source prepared were sterilized separately in autoclave at 120 °C for 21 minutes. After sterilization, medium and carbon sources were aliquoted in each flasks up to the desired medium and sugar concentration, according to the experimental design, and the final volume of 50 ml;

- Inoculum: under sterile conditions the flasks were inoculated with an aliquot of preinoculum, prepared according to section 3.3.2, in order to have a final biomass concentration of 0.06 g/L. Each experiment started when the flask was inoculated;

- Fermentation: the flasks were incubated at controlled agitation and temperature, in accordance with the experimental scheme. Periodically an aliquot of the medium was sampled in order to analyze the medium composition (analyte and biomass).

3.5. Bioreactor experiments

Batch and fed-batch experiments have been carried out in BIOSTAT B-Twin bioreactor (Sartorius AG, Germany), shown in Figure 3.1, equipped with pH, temperature, foam and dissolved oxygen probes.

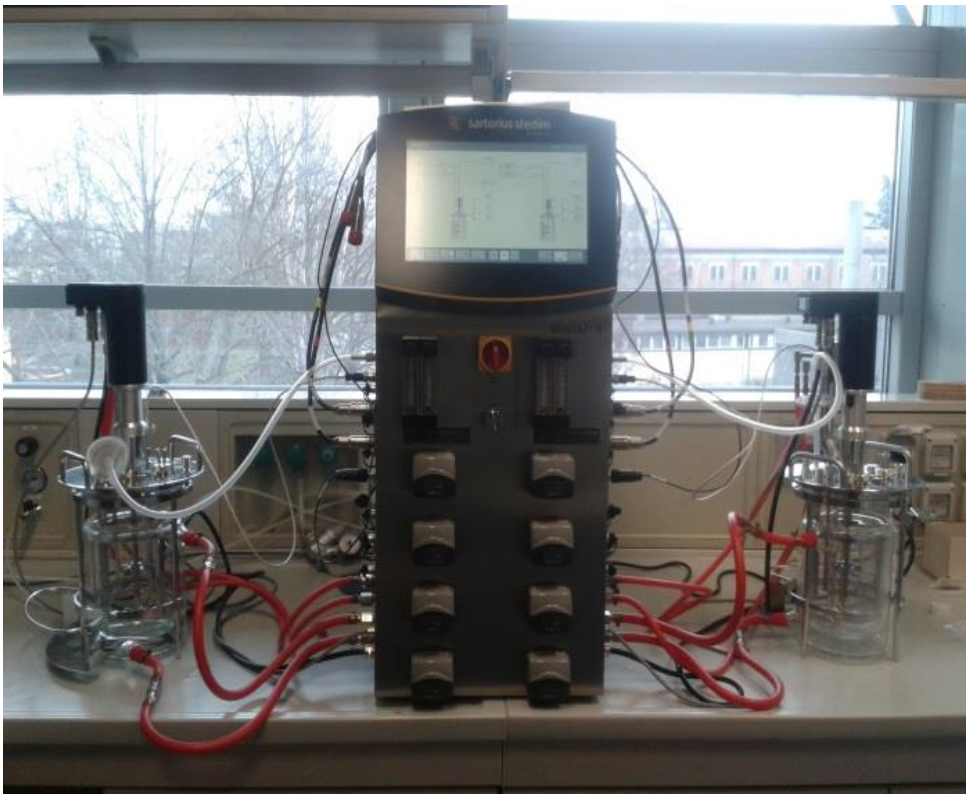


Figure 3.1 Bioreactors employed in this work: BIOSTAT B-twin bioreactor (Sartorius AG, Germany)

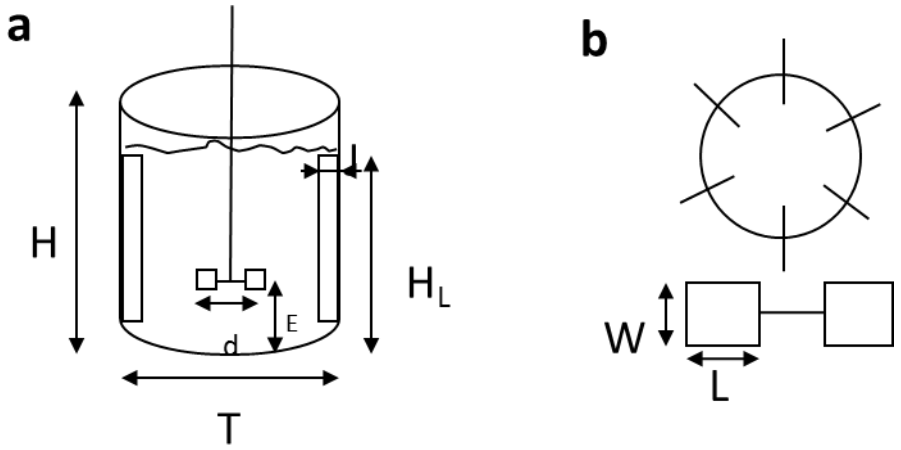


Figure 3.2 Bioreactor characteristic dimensions: vessel (a) and Rushton turbine (b). Internal vessel diameter T, impeller diameter d, total vessel height H, turbine height from the vessel bottom E, baffles width J, impeller blade height W, impeller blade width L

All the bioreactor experiments started from 1 L fermentation medium. The bioreactor employed (Figure 3.1) is characterized by the dimensions in Figure 3.2 and Table 3.6 230 mm total high (H_T), 81 mm liquid high (H_L), 130 mm internal diameter (T) and a section S = 0.0133 m^2. Four baffles of 1 cm depth are arranged on the walls of the bioreactor The agitation was transmitted to the liquid by a single six-blade Rushton turbine located 35 mm from the vessel bottom (E), and characterized by 50 mm diameter (D), each blade is 10 mm high (W) and 15 mm wide (L). Air was introduced through a perforated pipe shaped ring (4.5 cm diameter) located under the turbine. Table 3.7 shows the comparison between the dimension of the fermenter used and the dimension in a typical configuration of a bioreactor ("standard" in McCabe, Smith, & Hariott, 1985).

Table 3.6 Bioreactor dimensions

Acronym	Value	Unit
T	0.13	m
d	0.05	m
V	0.003	m3
A	0.013	m2
H_T	0.226	m
E	0.035	m
J	0.007	m
W	0.001	m
L	0.0015	m
V_L	1	L

Table 3.7 Bioreactor geometry for this work and for a standard mechanically agitated vessel.

	D/T	HL/T	J/T	E/T	W/D	L/D	Reference
This work	0.38	0.63	0.08	0.27	0.20	0.30	This work
Typical configuration	0.33	1	0.08	0.33	0.20	0.25	(McCabe et al., 1985)

Fermentation conditions were defined according to the experimental scheme described more in detail in Results paragraphs. In general, each bioreactor experiment was prepared according to the following procedure:

- Medium and carbon source preparation: media components solution was prepared by weighing each components excluding the carbon source. The solution of carbon source (sugars or biomasses) was prepared separately in order to avoid the Maillard reaction (during the sterilization) which could adversely affect the performance of the fermentation;

- Sterilization: bioreactor, filled with the required medium, and carbon source were separately sterilized in an autoclave at 120 °C for 21 minutes. After the sterilization, under sterile conditions, carbon sources was aliquoted in bioreactor up to the desired sugar concentration and the final volume of 1000 ml;

- Inoculum: under sterile conditions, the bioreactor was inoculated with an aliquot of preinoculum, prepared according to section 3.3.2, in order to have a final biomass concentration of 0.06 g/L. Each experiment started when the bioreactor was inoculated;

- Fermentation: in accordance with the experimental scheme, temperature, pH, stirring speed and agitation were set up to the bioreactor. Periodically an aliquot of fermentation broth was sampled in order to analyze the medium composition (analytes and biomass).

3.6. Analytical procedures

Analytical procedures are necessary to evaluate the composition of the fermentation broth and the parameters that can influence or control a fermentation process. This chapter aims to describe the analytical techniques applied in this thesis and the measurement procedures.

3.6.1. Biomass determination

During the fermentation, cell growth has been measured as optical density (OD). The measurement takes into account the turbidity of the fermentation broth. The measurement has been carried out at 600 nm wave length with a spectrophotometer (Prixma, Fulltech

Instruments). Before each measurement, the culture was diluted with the culture medium so that the absorbance was between 0.1 and 0.8. The culture medium was used as references.

Optical density was converted to cell dry weight (CDW) multiplying the OD for the proportionality factor 0.382 obtained from the OD vs CDW (g/L) calibration curve. The calibration curve was obtained from the slope between optical density and cell dry weight of samples, at different optical density, collected during a dedicated fermentation. The samples collected were centrifuged (8000 rpm for 10 min) and the cells washed in order to remove the residual analytes. The cell dry weight was obtained after drying cells overnight at 105 °C. Figure X shows the calibration curve.

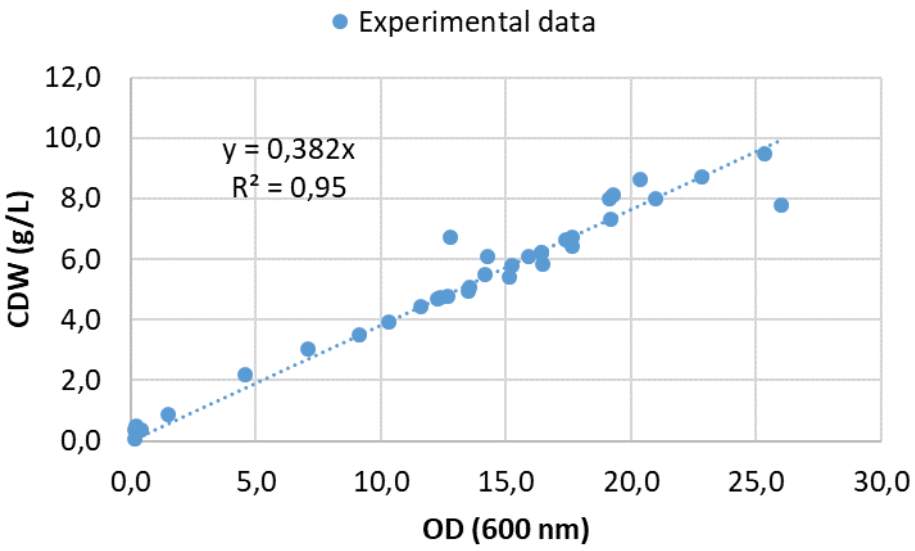


Figure 3.3 Optical Density vs Cell dry weight correlation

3.6.2. High-performance liquid chromatography (HPLC)

The qualitative and quantitative analysis of the compounds involved in the fermentation could be measured in many ways. The analytical technique applied in this thesis is the High-Performance Liquid Chromatography (HPLC). It consists of a chromatographic process in which the various constituents of a solution are separated according to physical or chemical technique between a stationary phase and a mobile phase. Depending on the relative bond strength between the compounds, to be separated, and the mobile and stationary phase, each

compound elute from the column at different retention times. Each compound can be qualitative recognized depending on the retention time. While the concentration of the components could be evaluated from a calibration curve between the concentration of the compounds and the area subtended by the peak that identifies the compound.

In this work, the analysis of the substrates, 2,3-BDO and the main by-products (ethanol, acetate, succinate, lactic acid, acetoin) in the fermentation broth were performed with a system Agilent 1260 Infinity Isocratic LC System, coupled with refractive index detector (RID). The column used was an Agilent Hi-Plex H, characterized by 300 mm length, 7.7 mm internal diameter and 8 pm pores diameter. The analytes were quantified as follow: mobile phase 5 mM H_2SO_4; flow rate 0.6 mL/min; injection volume of 0.5 µL; column temperature of 65°C.

Before the analysis, the samples of culture broth were centrifuged at 10000 rpm for 10 minutes to remove the cells and the supernatant was 0.4 µm filtered in order to remove particulate and suspended solids which could damage the column stationary phase. Then the liquid was diluted with ultrapure water in order to approximately keep the concentration of the analytes inside the calibration range.

3.6.3. Total proteins

In the present paragraph, the procedure for the analysis of the proteins content inside the fermentation medium is described. This method is based on the Bradford protein assay (Bradford, 1976), which is a simple procedure for determining the concentration of the protein in solution. It involves the addition of an acidic dye (Coomassie Brilliant Blue G-250) to the protein solution and subsequent measurement at 595 nm with a spectrophotometer. Comparison to a standard curve provides a relative measurement of protein concentration.

The calibration curve was done using the complex media (whom the composition is described in Table 3.4 as standard, in order to have as reference proteins as much as similar to the proteins usually inside the fermentation broth, excluding the biomass. Dilution of the complex media were prepared by aliquoting decreasing volumes of complex medium (3.20, 1.5, 0.5, 0.2, 0.15, 0.10, 0.05 ml), adding the same amount of reagent (0.4 ml) to each solution and bringing each solution to the final volume of 4 ml with the addition of water. Finally, the optical density of each of the prepared samples was measured, at 595 nm wavelength, by a spectrophotometer. The chart below shows the calibration graph (Figure 3.4).

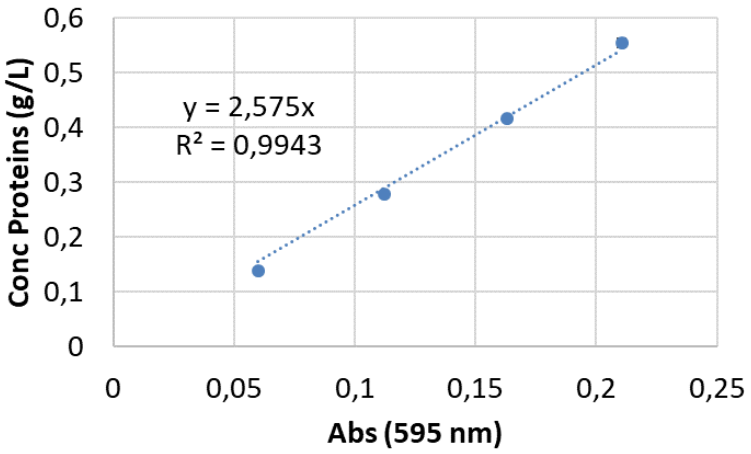


Figure 3.4 Proteins calibration curve

3.6.4. $k_L a$ and OTR determination: experimental and mathematical methods

In this chapter, methods for the determination of the oxygen transfer rate coefficient ($k_L a$) and oxygen transfer rate (OTR) used in this work are described.

Paragraph 1.1.5.5 of the Introduction describe that oxygen transfer in the production of 2,3-butanediol is the parameter that most influence the fermentation performances. In particular, 2,3-butanediol is produced preferentially in microaerobic condition, which is characterized by a concentration of oxygen in solution close to zero below the low detection limit of the oxygen probe. Thus, the measurement or estimation of $k_L a$ and OTR are crucial in order to evaluate the fermentation process. Thus, numerous methods have been developed to determine the oxygen transfer rate in bioreactor. In this work, different methods for the measurement or estimation of $k_L a$ or OTR have been used.

3.6.4.1. $k_L a$ determination

$k_L a$ could be determined in dfferent ways, the adoption of more than one determination technique allowed the validation by comparing of the data obtained. In this thesis, the $k_L a$ was experimentally and empirically quantified applying a physical method based on the measurement of the oxygen concentration variation in the fermentation broth (Dynamic

method) and applying empirical correlation obtained from literature. Both the methodologies are described below.

3.6.4.1.1. Dynamic method

Different dynamic and steady-state methods can be used to experimentally determine $k_L a$ (Pinelli et al., 2010). In this study, $k_L a$ was measured in the actual fermentation medium, before each fermentation test, for each condition studied in the 2,3-BDO production experiments in bioreactor, using a variation of the conventional dynamic method. These measurements were made with a Hamilton Visiferm electrode (Hamilton, Bonaduz, Switzerland), characterized by a 0.008 mg_{O2}/L quantification limit and a 10 s response time. This method is based on the measurement of the transient dissolved oxygen concentration in the medium in absence of biomass (no oxygen consumption) while applying a step change between two different conditions (from zero oxygen in the liquid to air saturation or vice versa). For all experimental conditions, $k_L a$ was measured by stripping oxygen from the fermentation medium through nitrogen sparging, then air was fed at the flow rate of the studied agitation/aeration condition. The step change from air saturated liquid to oxygen absence by N_2 sparging at the same airflow rate (Q_G) was also performed in some cases. As very similar $k_L a$ values were obtained with the two approaches, the two estimates were averaged. The experimental $k_L a$ evaluation method is based on the O_2 mass balance in the liquid written for a perfectly mixed system in semi-batch mode with no oxygen consumption:

$$\frac{dC_L}{dt} = k_L a \cdot \left(\frac{C_G}{m} - C_L\right) \quad (7)$$

Integrating equation 7 between two different times, the following equation is obtained:

$$ln\frac{(C_{G2}/m)-C_{L2}}{(C_{G1}/m)-C_{L1}} = -k_L a \cdot (t_2 - t_1) \quad (8)$$

Therefore, $k_L a$ can be assessed as the slope of a graph of the logarithm of the concentrations versus time (Garcia-Ochoa and Gomez, 2009; Scargiali et al., 2010). However, if the input gas is not a single component but a mixture of oxygen and nitrogen, characterized by different solubilities and diffusivities, the slope obtained may differ from the actual $k_L a$. The influence of this aspect on the estimated slope can be analytically predicted (Scargiali et al., 2010). On the other hand, if the mass transfer is not too high ($k_L a$ < 0.15 1/s), the deviation between the real

and experimental $k_L a$ is generally < 2% (Scargiali et al., 2010). A more severe limitation in all dynamic methods is the probe time lag or electrode response time, which influences the instantaneous slope of the curve. This effect can be neglected only if the probe time lag is one order of magnitude lower than the mass transfer characteristic time ($1/k_L a$). This constrain is easily satisfied in case of relatively low $k_L a$ values, such as those in this work where fully aerobic conditions must be avoided. The response time of the oxygen probe is generally specified by the probe producer and can also be experimentally determined. In our case the response time was 10 s, corresponding to a $k_L a$ threshold value of 0.01 1/s for complete negligibility of the probe response time.

3.6.4.1.2. Empirical correlations

As for the theoretical evaluation of $k_L a$, numerous correlations were proposed in the literature. The most common one, valid for a stirred tank reactor, relates $k_L a$ to the mechanical power dissipated in the aeration conditions (P_g) and to the inlet gas flow rate expressed in terms of superficial velocity (v_s), defined as ratio of the gas flow rate to the tank transversal section:

$$k_L a = K \cdot \left(\frac{P_g}{V}\right)^{\alpha} \cdot v_s^{\beta} \qquad (9)$$

where K, α, β are empirical coefficients that depend on liquid properties, bioreactor dimensions, and impeller type. The gassed power input P_g, in turn, can be estimated according to the following equation:

$$P_g = RPD \cdot N_p \cdot N^3 \cdot D^5 \cdot \rho_l \qquad (10)$$

where N_p is the impeller power number (about 5 for the six-blade Rushton turbine in turbulent regime (Re>10^4) used in this study), D the impeller diameter, ρ_l the liquid density and RPD the relative power demand. The latter can be calculated as $RPD = 0.18 \cdot Fr^{-0.25} \cdot Fl^{-0.2}$, where $Fr = N^2 \cdot D/g$ is the Froude number and $Fl = Q_G/(ND^3)$ is the gas flow number. Therefore, once the tank and impeller geometry are fixed, $k_L a$ is an increasing function of the operational conditions N and Q_G ($k_L a = const \cdot N^{2.7\alpha} \cdot Q_G^{\beta-0.2\alpha}$). The $k_L a$ values in the experimental conditions tested in this work were predicted using two sets of K, α and β values reported in the literature for a standard bioreactor for a liquid with coalescent behavior (Table 3.8). Density and viscosity were approximated to those of pure water at 30°C. The geometric ratios typical of a standard bioreactor are reported in Table 3.7.

Table 3.8 Constants for the empirical correlations used to estimate $k_L a$ equation 9

ID	K	α	β	Reference
1	0.026	0.40	0.50	(Van't Riet, 1979)
2	0.0037	0.59	0.35	(Scargiali et al., 2010)

3.6.4.1.3. Oxygen transfer rate

In this work, the oxygen transfer rate has been calculated in two ways. Indeed, equations 5 and 6 therefore represent two alternatives for evaluating the OTR for each tested experimental condition. Equation 6 is obtained from the oxygen mass balance in the gas phase, thus, OTR can be calculated from the difference in oxygen concentration between the inlet gas (C_G^{in}) and the outlet gas (C_G^{out}) (bioreactor headspace in a perfectly mixed system). While equation 5 is obtained from the oxygen mass balance in the liquid phase. In this case, OTR is derived from the product between the concentration of oxygen in the liquid phase at the saturation (C_L^*) and the oxygen transfer coefficient ($k_L a$). C_L^* can be calculated from the henry's low as product between , m_{O2} the gas/liquid partition coefficient for oxygen and C_G^{out} the gas phase concentration. In this work, the analysis of the gas headspace composition was carried out by using a MicroGC 3000 Agilent Technologies coupled with a TCD detector (injector temperature 90 °C; column temperature 60 °C; sampling time 20 s; injection time 50 ms; column pressure 25 psi; run time 44 s; carrier gas, N2).

3.7. Mathematical methods

In this paragraph, mathematical methods and calculation used for the process optimization and determination of the main fermentation performance parameters are described.

3.7.1. Design of experiment

Design of experiments (DoE) is a procedure of planning and analysis of experiments, using mathematical and statistical approaches, with the aim of obtaining information about the process studied. The application of a statistical approach and the analysis of the results allow

reducing the number of experiments leading to the optimization of the process (Montgomery, 2001). Such a technique could be applied to different field from the research to the industry.

Such a methodology allows to test more than one factor at a time as usual is done. The DoE shows how variables are interconnected each overs, outputting a mathematical model useful to predict the behavior of the response inside considered range and optimize the responses (Mandenius and Brundin, 2008).

Given its ability to predict the behavior of a process, its application depends on the specific objective of a study and it requires a deep knowledge of the process. The DoE consists of different steps which go from the selection of the object to the obtainment of the results you need, Figure 3.5 outlines the steps of the process.

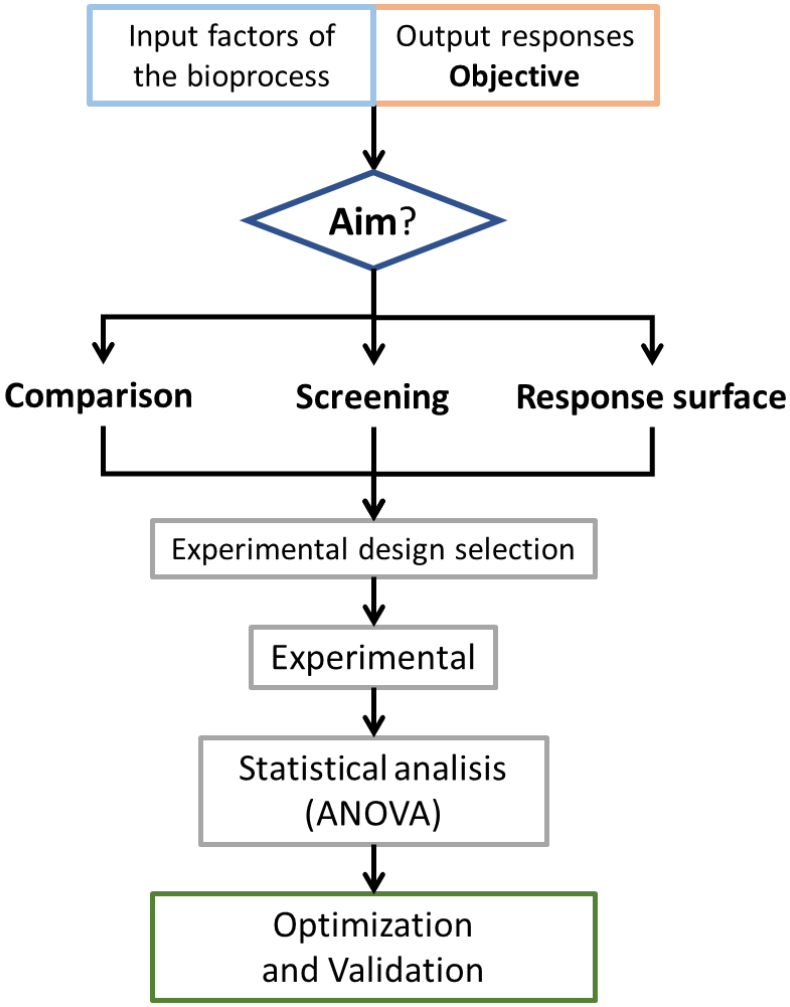


Figure 3.5 Schematic representation of the DoE stages.

The stages represented in Figure 3.5 could be summarized as follow:

1- Aim: First of all the aim of the application of the DoE has to be clear. Usually, DoE can be applied for different reasons:

   a. Screaming: evaluation of the input variables which more affects the responses. Often there are many possible input variables, some of which may be critical and others which may have little or no effect on a response. It may be desirable to reduce the number of factors;

   b. Comparison: choosing between alternatives (which input variable is the most effective on the responses);

   c. Response surface: optimization often is an ambiguous word indeed it could contain different meanings. In this case, the optimization consists in the building of a surface of the responses (a polynomial function of the input variables) which tells which combination of variables are able to: maximize or minimize the process, Making a Process Robust, hit a target.

   For this reason, a deep knowledge of the process is mandatory, since it could help to identify the factors that could affect the responses.

2- Variables selection: Once identified the objective of the experimentation the identification of the responses (output variables) and the process variables (input variables), which could influence the responses, is needed.

3- Planning: in this stage, the experimental design is selected. The choice of an experimental design depends on the objectives of the experiment and the number of factors to be investigated. In Table 3.9 are summarized the main design that could be applied depending on the number of factors and objectives.

Table 3.9 summary of the possible experimental design (modified from (Croarkin et al., 2012)

Input factors	Comparative	Screening	Response Surface
1	1-factor completely randomized design	-	-
2 - 4	Randomized block design	Full or factorial	Central Composite or Box-Behnken
5 or more	Randomized block design	Fractional Factorial or Plackett-Burmann	-

4- Experimentation: this step consists in the conduction of the experiments suggested by the experimental design and elaborate the data obtained in order to obtain the responses;

5- Analysis: once the planned experiments are done, the data obtained (responses) have to be analyzed graphically and statistically. Graphically, the distribution of the responses versus factor like histogram or plot, could help in the identification of the variables effect. Sometimes the right graphs or plots of the data lead to obvious answers. Furthermore, a statistical analysis such as the analysis of variance (ANOVA) for the identification of the p-values, residuals and the lack of fit could help in the identification of the less significant parameters and simplify the model;

6- Validation: this last step has the aim to validate the results obtained and to answer the questions of the experimental objectives. Further experiments are done in order to confirm, evaluate, optimize, etc. the data obtained.

In this work, the software for the DoE used is called MODDE 10.1 (Umetrics, Umeå, Sweden). The same software was used for the analysis of variance (ANOVA) and the optimization of the variables.

DoE is extensively applied in different field of fermentation processes (Mandenius and Brundin, 2008). The design of experiment has been already applied to the production of 2,3-butanediol. However, usually it is applied to the optimization of culture media (Dai et al., 2015; Li et al.,

2013; Yang et al., 2012) and only few works about fermentation conditions such as T and pH (Lee et al., 2013; Xin et al., 2016).

3.7.1.1. Central Composite Design

In the present thesis, a Central Composite Design (CCD) has been applied. CCD has the aim of the response surface building, and it is used when 2, 3 or 4 input factors occur.

The CCD is a very efficient design to describe second-order models and consists of three types of experimental points Factorial, axial and central as shown in Figure 3.6.

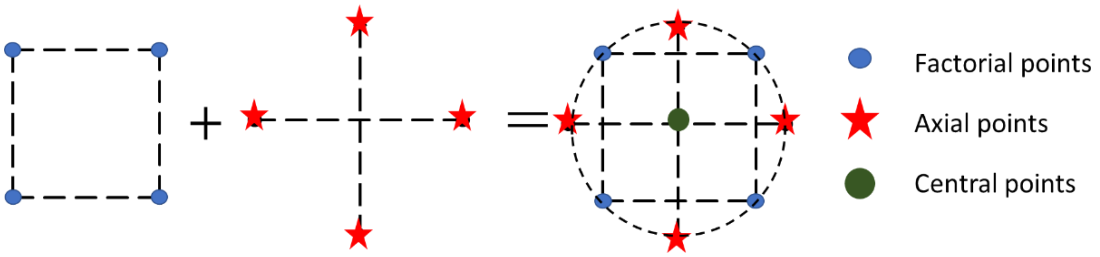


Figure 3.6 Central Composite Design for a two factors system

Two parameters of the experimental design must be specified: the distance α of the axial tests, and the number of replicas in the central points. The choice of α in the CCD is dictated primarily by the region of interest. If α is equal to 1, the axial points are arranged in an intermediate position between the factorial values at the top of the drawing virtually corresponding to the center point of the faces of a cube; in the center you have the central points and at the top the points of fact). Central points usually have replicates so as to estimate the experimental error and make the model more robust.

3.7.2. Calculation

This paragraph describes the main calculation necessary in order to evaluate the performances of the fermentation process.

Seven response variables were used to characterize the fermentation performance: 2,3-BDO yield (Y_{BDO}), 2,3-BDO productivity (P_{BDO}), acetoin yield (Y_{ACE}), glycerol yield (Y_{GLY}) ethanol yield (Y_{EtOH}) and selectivity in 2,3-BDO (S_{BDO}) expressed as the ratio between the mass of 2,3-BDO

produced and those of all the products from glucose (with the exception of biomass). They were calculated, as follows:

$$Y_{BDO} = \frac{m_{BD}}{m_{GL\_in}} \quad (11)$$

$$P_{BDO} = \frac{C_{BD}}{t} \quad (12)$$

where *t* indicates the sum of the growth and 2,3-BDO production phases

$$Y_{ACE} = \frac{m_{AC}}{m_{GL\_in}} \quad (13)$$

$$Y_{GLY} = \frac{m_{GLY}}{m_{GL\_in}} \quad (14)$$

$$Y_{EtOH} = \frac{m_{EtOH}}{m_{GL\_in}} \quad (15)$$

$$S_{BD} = \frac{m_{BD}}{m_{BD}+m_{AC}+m_{GLY}+m_{EtOH}} = \frac{Y_{BD}}{Y_{BD}+Y_{AC}+Y_{GLY}+Y_{EtOH}} \quad (16)$$

where m_{BD} (g) and CBD are the mass and the concertation of BD, m_{AC} (g) is the mass of acetoin, m_{GLY} (g) is the mass of glycerol and m_{EtOH} (g) is the mass of ethanol at the complete conversion of glucose and m_{GL_in} (g) is the mass of glucose initially present in the fermenter.

4. Results and Discussion

4.1. Microorganism selection and media evaluation: preliminary study

The first stage for the development of a pure culture fermentative bioprocess for the production of chemicals is the selection of a microorganism, able to produce the target molecule and applicable at industrial scale, and the selection of the operating conditions (culture medium, pH, Temperature).

4.1.1. Microorganism selection

2,3-butanediol as extensively described in chapter 1.1.3 is preferentially produced by pathogenic microorganism, these features make that these bacteria are hardly industrially applicable. In this thesis, the microorganism selection has been carried out, by consulting the available scientific literature, according to the following features:

i) wild-type microorganism (not genetically modified);

ii) ability to produce 2,3-butanediol from carbon sources commonly occurring in biomasses of plant origin;

iii) production performances comparable to that of the main pathogenic bacteria in terms of max concentration, productivity, and yield;

iv) the microorganism must be considered safe thus it must belong to the class 1 of risk that means that is unlikely to cause human disease;

v) occurring in one of the microbial culture collection.

The microorganism chosen is the *Bacillus licheniformis* ATCC-9789 which was bought from the American type culture collection. These bacteria belong to the class 1 of risk and it is generally recognized as safe (GRAS).

4.1.2. Medium selection

The second stage of a bioprocess upstream is the selection of an appropriate medium for the fermentation. This stage is mandatory in order to supply to the microorganism all the elements and substances necessary for the growth and the production. For this purpose, in this work, it has been decided to test the growth and production of 2,3-BDO with the microorganism, selected in the previous paragraph, on three medium which were selected from the literature. In particular, three papers, in which a microorganism of the same species selected for this work was used, were selected. The papers selected are described here below:

Jurchescu et al., 2013: the production of 2,3-butanediol is studied with the nonpathogenic bacteria *Bacillus licheniformis* DSM 8785 using glucose as substrate. In this article the effect of the initial concentration of glucose in flasks experiment was studied leading to the production of 72.5 g/L of 2,3-BDO from 180 g/L of glucose, the production of 144 g/L of 2,3_BDO was reached in fed-batch experiments in the bioreactor. Furthermore, the production of 2,3-BDO with the microorganism in immobilized form was assessed. The experiments were performed in 250 ml flasks with 50 ml medium whom the composition is shown in Table 3.2 at 30°C, 100 rpm and initial pH 6.6;

Perego et al., 2003: in the article of Perego et al the effect of different carbon sources (glucose, sucrose and cornstarch hydrolysate) and different operating conditions (temperature, inoculum size, and starting substrate concentration) have been studied using a microorganism of the specie *Bacillus licheniformis*, resulting in a high performance with the cornstarch hydrolysate.

Nilegaonkar et al., 1992b: the production of 2.3-butanediol from glucose by *Bacillus licheniformis* was studied in the article of Nilegaonkar. In this paper the effect of different operating parameters (temperature and pH) and initial glucose concentration was studied on the production of 2,3-BDO using a *Bacillus licheniformis*. In this case, the medium used (Table 3.4) is a complex medium composed of Beef extract and Peptone from soybean. the performances obtained are better in terms of yield of 2,3-BDO (0.47 g/g) than the other articles, however, the productivity (0.12 g/L/h) and the production of glycerol as a byproduct are undesirable. In this paper, the experiments were conducted in flasks at 37°C statically thus without agitation and pH 6 not controlled.

In this paragraph are described the results of the fermentations conducted in flasks with different media composition and conditions according to the experimental scheme described in Table 4.1, in which are reported the experimental conditions adopted.

Table 4.1 Selection of the culture medium: experimental scheme. In the second column are reported the name of the tables of materials and methods in which the medium used are described.

Test	Medium composition	Culture conditions	References
M1	Table 3.2	Initial pH 6.6 T 30 °C 100 rpm	(Jurchescu et al., 2013)
M2	Table 3.3	Initial pH 6.0 T 30 °C 100 rpm	(Perego et al., 2003)
M3	Table 3.4	Initial pH 6.0 T 37 °C Static	(Nilegaonkar et al., 1992b)

Figure 4.1 shows the evolution of the main fermentation parameters (concentration of biomass, glucose, 2,3-butanediol and the main by-products) in the experiments for the selection of the culture medium. It is evident that the microorganism is not able to growth end produce 2,3-BDO in the experiment M2 Figure 4.1b(Perego et al., 2003), probably this behavior is due to the absence of protein-rich substrates such as tryptone, peptone or beef extract. Indeed, such complete absence of growth it is not due to the growth conditions, which are similar to the conditions used in the experiment in Figure 4.1c. In Figure 4.1a (Jurchescu et al., 2013) the maximum concentration of 2,3-BDO (6.7 g/L) was reached after 23 hours when the complete consumption of glucose occurs, which correspond to a yield of 0.26 g/g of glucose. The concentration of 9 g/L of 2,3-BDO is reached in experiment M3 (Figure 4.1c) corresponding to a yield of 0.40 g/g of glucose, such a production was reached when the media and conditions of the article of Nilegaonkar et al., 1992 were applied. However, such a 2,3-butanediol concentration is reached only after 56 fermentation hours, and the glucose is not completely consumed, this suggests that extending the fermentation or increasing the fermentation rate to reach the complete substrate consumption it could be possible increase the 2,3-BDO

concentration and consequently the yield and productivity. The medium used in the fermentation M3 seems to be the most promising, not only because of the higher concentration of product obtained but also for its greater selectivity, indicating that a larger fraction of the substrate is converted to the desired product and not in by-products or biomass.

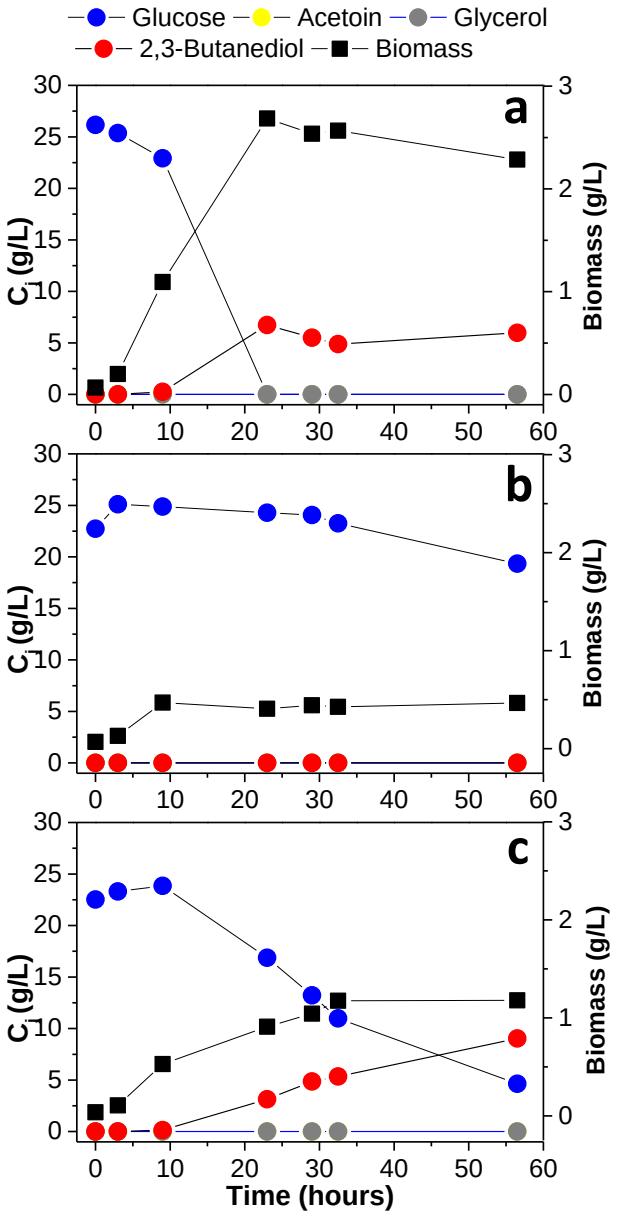


Figure 4.1 Evolution of the concentration of substrate, 2,3-butanediol, byproducts, and Biomass in the experiments for the selection of the medium. Experiments: (a) M1 (Jurchescu et al., 2013), (b) M2 (Perego et al., 2003), (c) M3 (Nilegaonkar et al., 1992b)

Another fermentation has been conducted in order to verify whether the fermentation time could be reduced using the same medium of the fermentation M3 and the conditions of the fermentation in M1 and M2. In particular, the main obstacle was probably the different mixing conditions. Indeed, fermentation M3 was conducted in absence of agitation unlike to the others. In Figure 4.2 are shown the concentration evolution of the biomass, substrate, 2,3-BDO and byproducts.

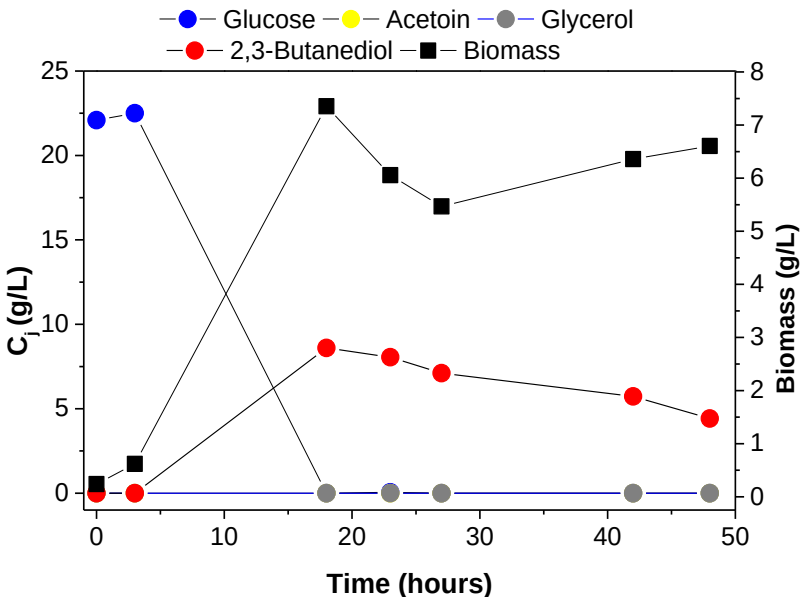


Figure 4.2 Evolution of the concentration of substrate, 2,3-butanediol, byproducts and Biomass obtained with the medium of the work of Nilegaonkar et al., 1992 at the conditions of T 30°C and 100 rpm in the flask.

Application of different growth conditions allowed to obtain a concentration of 2,3-BDO and yields comparable to those obtained previously with the same cultivation medium (experiment M3) as well as complete substrate consumption, but in a much shorter time (18 hours) and therefore with a significant increase in process productivity.

In conclusion, given the higher concentration of 2,3-BDO and higher fermentation performances, the complex medium shown in Table 3.4 has proven to be the best medium at the fermentation conditions of 30°C and 100 rpm for a process in the flask. This condition will be applied for the next flask experiments. The best performances obtained in this experiment could be due to the application of e medium reach aminoacids, vitamins and growth factors of

which the medium used is rich, furthermore, the evaluation of oxygen transfer condition more suitable could improve more the fermentation performances.

4.2. Conversion of sugars from agroindustrial biomasses in 2,3-butandiol

In the present chapter, the possibility of producing 2,3-BDO from sugars occurring in lignocellulosic biomass or waste from the food industry was assayed. For this purpose, the ability of the microorganism *Bacillus licheniformis* ATCC-9789 to convert different hexose and pentose monosaccharides, typically occurring in enzymatic hydrolysates of second-generation lignocellulosic biomass was assayed. Moreover, two agro-industrial wastes were assayed, namely cheese whey, i.e., the dairy industry waste rich in the disaccharide lactose, and molasses, the waste of the sugar production process rich in the disaccharide sucrose.

4.2.1. Conversion of monosaccharides in 2,3-butanediol

First, 2 hexoses (glucose and mannose) and 2 pentoses (xylose and arabinose) were tested individually. Furthermore, the strain was grown in absence of sugar, in order to verify microbial growth and 2,3-BDO production only in presence of the proteins and amino acids of the culture medium. The experimental scheme is shown in (Beef Extract and Peptone).

Table 4.2 Flask experiments with monosaccharides: experimental scheme (test name and monosaccharide assayed).

Test	Monosaccharides
S1	Glucose
S2	Mannose
S3	Xylose
S4	Arabinose
S5	No sugar

Both hexoses (glucose and mannose) were converted to 2,3-BDO. In particular, as shown in Figure 4.3, the fermentation profiles of the two hexoses were comparable in terms of biomass, glucose consumption, 2,3-BDO production, and byproducts accumulation, as well as in terms of substrate conversion, product concentration and yield.

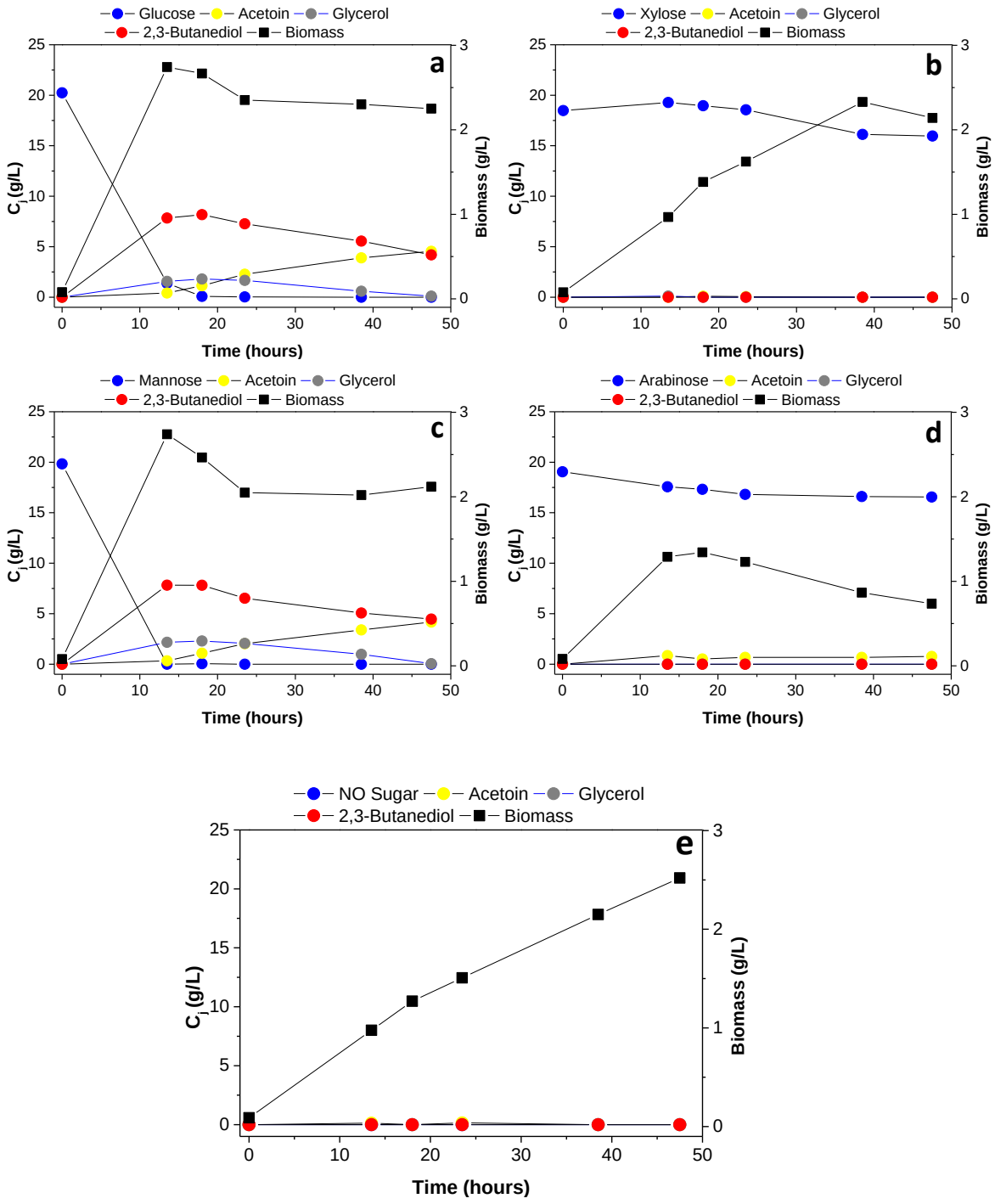


Figure 4.3 Microbial growth (Biomass) and concentration of analytes (substrate, product and by-products) over time, during the fermentation of the monosaccharides: glucose S1 (a) mannose S2 (b) xylose S3 (c) arabinose S4 (d) and without sugar S5 (e)

After 13.5 hours fermentation, the microorganism reaches the maximum growth (optical densities of 7.1±0.5 and 7.1±0.6 on glucose and mannose, respectively). The maximum concentration of 2,3-BDO is reached after 18 hours both from glucose (8.2 ± 0.1 g/L, yield equal to 40.4 ± 1.0 g/100g of glucose) and mannose (7.8±0.2 g/L, yield of 39.4±1.8 g/100g). Acetoin and acetic acid are produced after the complete consumption of the monosaccharide in concomitance with the degradation of 2,3-BDO.

The results also indicate clearly that the strain does not produce 2,3-BDO from pentoses, which are little consumed during 48 hours of fermentation, as shown in Figure 4.3c and d. In the presence of pentoses, the growth of the strain is markedly slowed down, leading to the achievement of a biomass of about 2.3 g/L after 38 hours of fermentation for xylose and about 1.3 after 18 hours for arabinose. The microorganism grows slowly even when grown only on the proteins and amino acids of Beef extract and Peptone without any sugars; in particular, after 48 hours the highest biomass of 2.5 g/L is reached. This growth is in line with that observed in the presence of the pentoses, indicating that the strain grows more slowly on the amino acidic of the media and that the production of 2,3-BDO occurs from sugars, in particular, hexoses. Microorganisms of *Klebsiella* species are able to metabolize a broader spectrum of sugars (Yang et al., 2011) than the *Bacillus licheniformis* strain used in this work. However, the yield of 2,3-BDO obtained from glucose by *Klebsiella spp*. Usually ranges from 43 to 50 g 2,3-BDO/100 g glucose, which is comparable with that obtained in this work with *B.licheniformis* ATCC 9789. Higher 2,3-BDO titers, up to 150 g/L, have been also reported for *Klebsiella pneumoniae* from glucose under fed-batch conditions (Ma et al., 2009), suggesting that similar 2,3-BDO concentrations might be obtained after process optimization with *B. licheniformis* ATCC 9789.

4.2.2. Conversion of biowastes in 2,3-butanediol

Afterwards, cheese whey and molasses were tested individually as substrate. In addition, the pure disaccharides occurring in the wastes were tested, in order to verify the possible presence of inhibitory effects on the process by other substances of the waste. Furthermore, the two monosaccharides that constitute the disaccharides were assayed singularly as a substrate, in order to verify the ability of the strain to convert both and the possibility of using the enzymatic hydrolysate of the disaccharide, if the latter is not efficiently used or hydrolyzed by the microorganism. Table 4.3 summarize the experiments carried out.

Table 4.3 Flask experiments with biowastes and the di and monosaccharides occurring in them: experimental scheme (test name and sugars or biowastes assayed).

Test	CW	Test	MOL
CW1	Cheese whey	MOL1	Molasses
CW2	Lactose	MOL2	Sucrose
CW3	Galactose	MOL3	Fructose
CW4	Glucose	MOL4	Glucose

When cheese whey was used as a substrate, the initial concentration of lactose in the fermentation broth was about 10 g/L (instead of 20 g/L), because the content of lactose is about 50% w/w. The organism slightly consumes the lactose present in the cheese whey but it does not ferment it to 2,3-BDO. Likewise, pure lactose and pure galactose are not converted in 2,3-BDO by the strain (Figure 4.4). This evidence indicates that cheese whey is not an agro-industrial waste suitable for the production of 2,3-BDO with this microorganism. Furthermore, when cheese whey, lactose, and galactose are used as a substrate the microorganism grows slowly on the amino acidic fraction of the medium as described above. Lactose is apparently not converted to 2,3-BDO also by *Klebsiella oxytoca* (Champluvier et al., 1989), while can be fermented to 2,3-BDO by *Klebsiella pneumoniae* with yields comparable to those obtained from glucose (46 g2,3-BDO/100g of lactose) (Lee and Maddox, 1984).

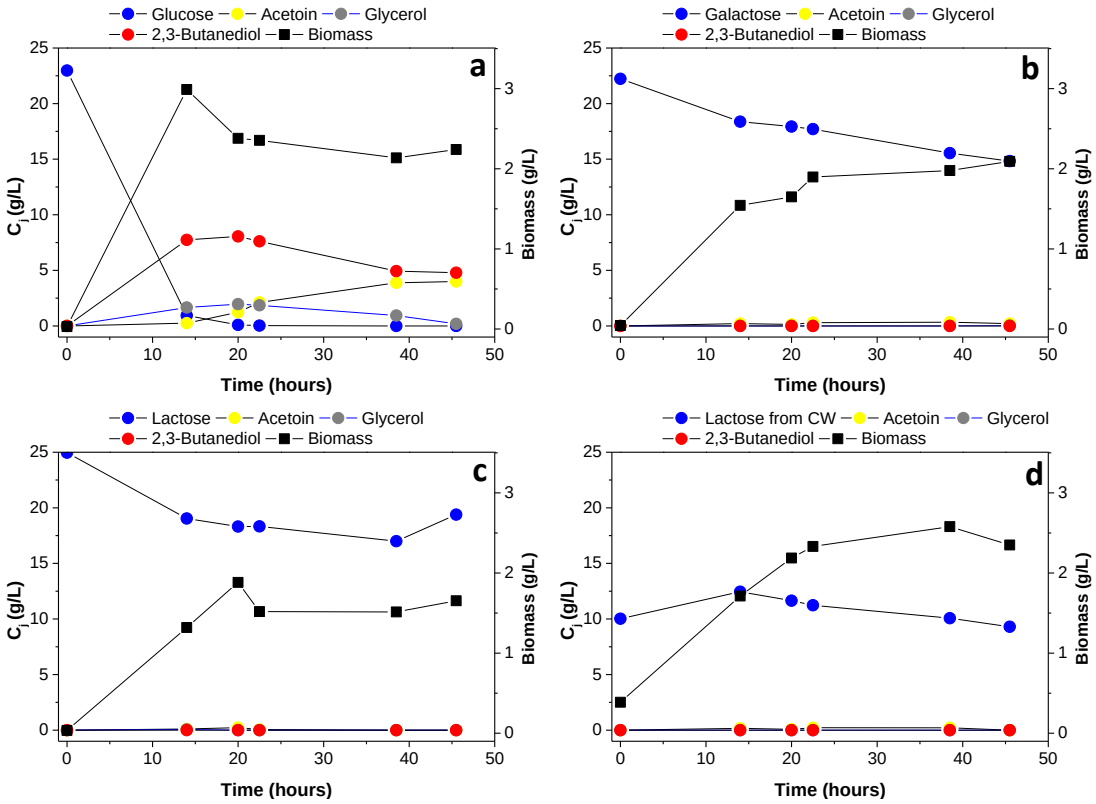


Figure 4.4 Microbial growth (biomass) and concentration of analytes (substrate, product and by-products) over time, during the fermentation of glucose CW4 (a), galactose CW3 (b), lactose CW2 (c), cheese whey (lactose) CW1 (d).

Figure 4.5 shows the Biomass and the concentration of the substrate, product, and by-products versus fermentation time when sucrose occurring in molasses, pure sucrose and the monosaccharides occurring in sucrose (glucose and fructose) are used as a substrate. As regards glucose and fructose, the biomass reaches the maximum of 2.6 and 2.5 g/L, respectively, after 14.5 hours. The maximum 2,3-BDO concentration and yield are reached after almost 19 hours for both monosaccharides, with a yield of 42 g/g and 30 g/g respectively. When pure sucrose is used, the same 2,3-BDO concentration is reached with a yield of 0.36 g/g. The lower yield obtained with sucrose with respect to glucose probably is not due to a partial hydrolysis of the disaccharide in the two monosaccharides, but to the lower conversion efficiency of fructose, which represents 50% by weight of sucrose.

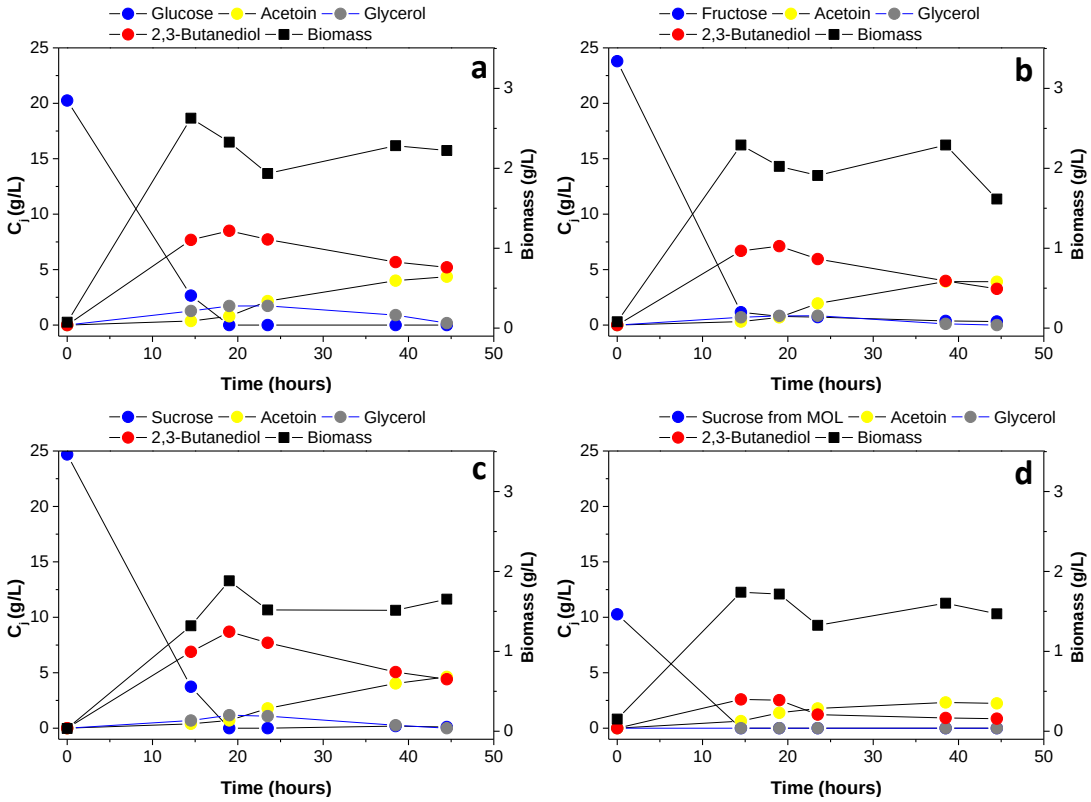


Figure 4.5 Microbial growth (Biomass) and concentration of analytes (substrates, product and by-products) over time, during the fermentation of glucose MOL4 (a), fructose MOL3 (b), sucrose MOL2 (c), molasses (sucrose) MOL1 (d).

As regards molasses, the initial concentration of sucrose in the fermentation is about 10 g/L (instead of 20 g/L), since the content of sucrose in molasses is about 50% by weight. Sucrose is completely consumed after 14.5 hours, although complete sucrose consumption might have been reached earlier. The corresponding maximum 2,3-BDO concentration of 2.6 ± 0.3 g/L and yield (0.27 g/g sucrose) are recorded after that time (14.5 h). This yield is significantly lower than that obtained with pure sucrose and lower than that reported from cane molasses for *Klebsiella oxytoca* (42 g 2,3-BDO/100 g; (Afschar et al., 1991). It should be recognized, however, that the initial concentration of sucrose in this fermentation was about the half of that used for pure sucrose, and that sucrose may have been completely consumed before the first sampling carried out after 14.5 hours of incubation. Thus, it is possible to speculate that a higher concentration and yield of 2,3-BDO from molasses could be obtained during the first 14.5 hours of fermentation, at the moment of complete sucrose consumption. Similarly, a higher concentration of the product and a higher yield could be obtained after 14-18 hours of

fermentation using a double concentration of molasses, corresponding to an initial concentration of sucrose of 20 g/L. The data obtained indicate that it is, therefore, possible to produce 2,3-BDO from molasses, and suggest that concentrations and yields comparable to those obtained from pure sucrose might be obtained after process optimization.

In conclusion, the possibility of producing 2,3-BDO with the strain *Bacillus licheniformis* ATCC 9789 from sugars occurring in lignocellulosic biomasses or biowaste from the food industry has been evaluated. The results show that the microorganism is not able to use and produce 2,3-BDO from pentoses (xylose and arabinose) and the hexoses galactose, from the disaccharide lactose and the cheese whey. Contrariwise, it grows and produces 2,3-BDO starting from hexoses monosaccharides such as glucose, mannose, fructose, from the disaccharide sucrose, and from molasses. Molasses could be considered a good substrate for the following stages of process optimization.

4.3. Effect of the oxygen mass transfer rate on the production of 2,3-butanediol

The production of 2,3-butanediol, as shown in the introduction (Paragraph 0), is highly influenced by the oxygen supply conditions. In particular when microaerobic conditions occur, high 2,3-butanediol fermentation performances take place. Thus, a fine tuning of the oxygen mass transfer rate (OTR) is crucial in order to maintain microaerobic conditions and maximize BD yield and productivity. The optimization of the aeration conditions is therefore critical in order to scale-up the process. In particular, aerobic fermentation are typically scaled-up by maintaining a constant volumetric mass transfer coefficient ($k_L a$), whereas for microaerobic fermentations the most effective scale-up criterion is constant OTR (Garcia-Ochoa and Gomez, 2009). The goal of the work described in the present paragraph is to perform an optimization of the 2,3-BDO production process by identifying the OTR range that maximizes 2,3-BDO yield and productivity using glucose as carbon sources. OTR control can be attained by varying the $k_L a$, which is in turn is determined by two operating parameters: agitation and aeration in the fermenter. If a conventional mechanically agitated vessel is used, agitation is regulated by the impeller rotational speed (N) and aeration by the air flow rate (Q_G). In this work, fermentations experiments have been carried out at different combinations of N and Q_G values, in order to

increase the robustness of the process optimization, and this in turn means different values of OTR. For this scope, a design of experiments (DoE) approach is particularly suitable and useful.

In order to assess the optimal conditions of agitation and aeration, it was decided to apply the following general approach:

1) visual analysis to evaluate the N and QG operating range to guarantee correct fluid dynamic conditions and complete gas dispersion,

2) application of the Central Composite Design approach (CCD) to define the set of experiments (N and Q_G values) required to identify the optimal (N, Q_G) combination;

3) conduction of the fermentations proposed by the CCD and determination of the oxygen transfer rate of each of the conditions assayed by means of the dynamic method, empirical estimation or exhaust gas analysis;

4) identification of the optimal operational conditions:

    a. application of the response surface methodology approach to the results of the fermentation carried out according to the Central Composite Circumscribed Design scheme;

    b. empirical approach based on the interpolation of the trend of performance parameters versus OTR.

Fluid dynamic in a bioreactor must be reserved in order to keep a complete mixing and thus a complete dispersion of the bubble in the bioreactor in order to avoid undesired bubble flow such as both agitator flooding (too high Q_G and too low N) and surface aeration by formation of a pronounced central vortex (too high N). This is the reason why the impeller rotational speed was confined between 250 and 550 rpm, whereas the gas flow rate could vary between 0.1 and 0.5 L/min. Table 4.4 summarize the conditions described above.

Table 4.4 ranges of N and Q_G (input variables of the DoE) used in this work for the optimization of the oxygen supply conditions.

	Symbol	Units	Low limit	High limit
Impeller rotational speed	N	rpm	250	500
Gas flow rate	Q_G	vvm	0,1	0,5

The application of a Design of experiment, as described in material and methods (paragraph 3.7.1) requires the definition of input and response parameters. In this case, the aim is the identification of the optimal oxygen transfer conditions able to maximize the process performances. Thus, the input variables are the Gas flow rate and the impeller rotational speed in the range described in Table 4.4. Furthermore, the response variables applied are the fermentation performances described in material and methods (paragraph 3.7.2) named 2,3-BDO Yield (Y_{BDO}), 2,3-BDO Productivity (P_{BDO}) 2,3-BDO Selectivity (S_{BDO}), Acetoin Yield (Y_{ACE}) and Glycerol Yield (Y_{GLY}). The experimental design applied is a central composite design as described in (paragraph 3.7.1.1), which includes 4 factor points (vertices), 1 central point and 4 axial points. Since the DoE is a statistical software, the center point has been repeated 3 times. The experimental scheme (Figure 4.6) and the list of tests performed (Table 4.5) are shown below.

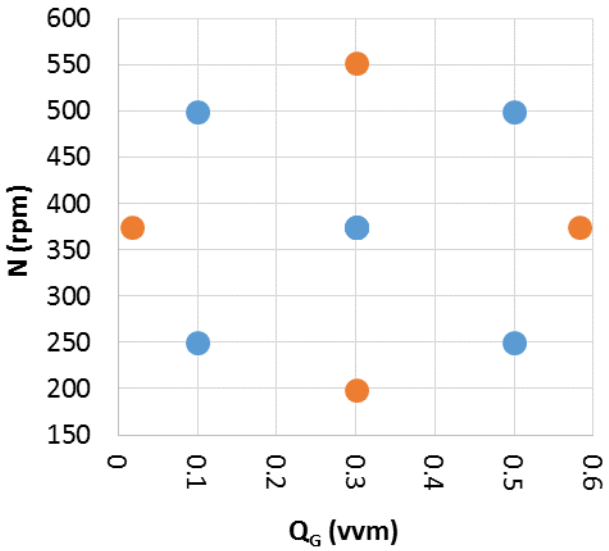


Figure 4.6 Experimental scheme of the tests proposed according to the design CCD of the DoE

Table 4.5 List of tests performed. *Central point carried out in triplicate

Test	N	QG
	Rpm	Vvm
O1	250	0.1
O2	198	0.3
O3	250	0.5
O4	375	0.05
O5*	375	0.3
O6	375	0.58
O7	500	0.1
O8	500	0.5
O9	552	0.3

4.3.1. Fermentations

All the fermentation listed in Table 4.5 have been carried out as shown in Figure 4.7. As described in M&M were conducted at 30°C changing aeration and agitation according to the experimental scheme (Table 4.5). At the moment of the inoculum, the concentration of oxygen in the bioreactor is the maximum and it's equal to the oxygen solubility as shown in Figure 4.7 which means aerobic conditions. After a time lap of few hours (from 4 to 15 h depending on the operating condition), as the biomass grew, the metabolic oxygen consumption (namely the OUR) overwhelmed the oxygen mass transfer (namely the OTR, not controlled), the dissolved oxygen decreased and microaerobic conditions were reached. In these conditions, glucose is rapidly converted into pyruvic acid and, then, into either acetoin and 2,3-butandiol or glycerin and ethanol.

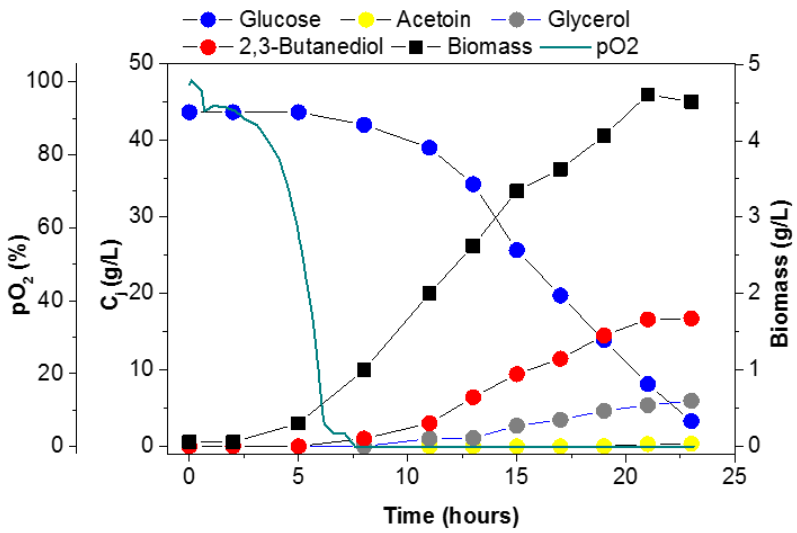


Figure 4.7 Example of a fermentation. Time evolution of the main concentrations in the batch. Experiment O4: Q_G=0.05L/min, N=375rpm.

The results of the 11 fermentations designed through the CCCD approach are shown in Table 4.6 (central point repeated 3 times, average values reported in Table 4.6. In the fifth column of Table 4.6 are shown the conditions reached during the fermentation. All the fermentation, except the experiment O9, have been conducted in microaerobic conditions and consequently were proved to be suitable for 2,3-BDO production. Indeed, with exception of experiment O9, the 2,3-BDO yield varies between 0.37 and 0.45 g/g with productivities between 0.62 and 0.98 g/L/h confirming that microaerobic conditions are mandatory to obtain high performances from the process. The only experimental test that gave very poor results was the experiment O9 (N=552 rpm, QG=0.31 L/min), corresponding to more extreme test toward aerobic conditions: a very low 2,3-BDO concentration (2 g/L) and a high level of acetoin (11 g/L) at the end of the experiment were observed. Indeed, this experiment was almost fully aerobic with an oxygen concentration in the liquid phase always higher than 2 mg/L. Moreover, Table 4.6 shows the values of $k_L a$ and OTR for each fermentation. OTR was calculated applying, both, equation 5 from the liquid mass balance and equation 6 oxygen mass balance in the gas phase. However, preliminary calculations indicated that the first approach was affected by a lower relative error than the second one. The *OTR* evaluated for each experimental condition with the first approach was therefore utilized in this work. Hence, $k_L a$, calculated by mean of the dynamic method (Material & Methods 3.6.4.1.1) and OTR, calculated from the liquid mass balance(Material & Methods 3.6.4.1.3) are shown in Table 4.6.

Table 4.6 Fermentation results of the CCD tests at the complete glucose conversion *average of experiments in triplicate. micro = microaerobic condition, aerobic = aerobic condition.

Experimental scheme					Oxygen transfer rate coefficient		Oxygen transfer rate		Concentrations				
ID test	Test type	N	QG	Condition	kLa dynamic method		OTR		2,3-BDO	Acetoin	Glycerol	Ethanol	Biomass
					mean	dev.std	mean	dev.std	conc.	conc.	conc.	conc.	conc.
		rpm	L/min		s-1	s-1	mmol/L/h	mmol/L/h	g/L	g/L	g/L	g/L	g/L
O1	CCCD	250	0,10	micro	0,0018	0,0006	1.4	0.5	17,6	0,0	9,4	0,1	4,5
O2	CCCD	198	0,31	micro	0,0024	0,0002	1.9	0.2	15,6	0,0	9,5	1,0	3,1
O3	CCCD	250	0,51	micro	0,0036	0,0007	2.8	0.5	17,0	0,1	7,6	0,8	4,2
O4	CCCD	375	0,05	micro	0,0038	0,0004	2.8	0.3	19,7	0,2	6,8	1,2	5,1
O5*	CCCD	375	0,31	micro	0,0061	0,0007	4.7	0.5	18,0	0,1	5,8	0,4	4,8
O6	CCCD	375	0,59	micro	0,0090	0,0009	7.1	0.7	17,1	0,8	3,9	0,7	5,1
O7	CCCD	500	0,10	micro	0,0130	0,0011	7.6	0.8	18,4	1,7	0,3	0,0	6,6
O8	CCCD	500	0,51	micro	0,0225	0,0024	16.6	1.8	16,3	2,4	0,1	0,0	6,3
O9	CCCD	552	0,31	Aerobic	0,0235	0,0016	17.9	1.2	2,0	11,0	0,0	0,0	5,2
10	Optimization	460	0,10	micro	0,0096	0,0008	6	0.7	19,7	2,1	1,2	0,0	6,4
11	Validation	430	0,24	micro	0,0073	0,0013	5.7	1.1	16,8	1,4	0,4	0,0	6,0
12	Validation	375	0,14	micro	0,0057	0,0001	4.4	0.1	17,5	0,0	5,5	0,7	4,9
13	Validation	405	0,51	micro	0,0109	0,0007	8.3	0.5	19,3	3,8	0,0	0,0	6,1
14	Validation	425	0,43	micro	0,0107	0,0015	8	1.1	19,0	2,7	0,0	0,0	4,3

4.3.2. Identification of the optimal operational condition

In the present chapter, the fermentation performances are compared applying two different approaches. The results showed in Table 4.6 are elaborated applying the response surface methodology in order to identify an optimal combination of N and Q_G values and an empirical method that correlates the performances with the effective scale-up parameter OTR.

4.3.2.1. Response surface methodology approach

The results obtained from fermentations of the central composite design (CCD) were elaborated with the software of design of experiment so as to obtain functions of the input variables (N and Q_G) able of predicting the evolution of the response parameters (Y_{BDO}, P_{BDO}, S_{BDO}, Y_{ACE} and Y_{GLY}), within the ranges considered. The fermentation O9, as previously described is characterized by completely aerobic conditions leading to poor concentration of 2,3-BDO and consequently low performances. An initial data fitting, carried out including the performances of such fermentation, led to models not statistically significant and not enough adequate to describe the experimental values. Thus, for this reason, the results of this test (O9) were excluded from the DoE procedure. Below the functions (equations 18, 19, 20, 21, and 22) and the response surfaces (Figure 4.8) concerning the response performances used for the DoE are reported.

$$\text{2,3-BDO Yield} = 0.102+0.227*Q_G+0.002*N-0.271*Q_G^2-2.160E-6*N^2 - 4.0E-4*Q_G*N \quad (17)$$

$$\text{Productivity} = -0.450+0.952*Q_G+0.005*N-0.243*Q_G^2-4.90E-6*N^2 -0.002*Q_G*N \quad (18)$$

$$\text{Selectivity} = 0.528+0.163*Q_G+1.950e-5*N+0.100*Q_G^2+1.434e-6*N^2 -0.0005*Q_G*N \quad (19)$$

$$\text{Acetoin Yield} = 0.100-0.147*Q_G-0.001*N+0.153*Q_G^2+9.988e-7*N^2 +0.0002*Q_G*N \quad (20)$$

$$\text{Glycerol Yield} = 0.204-0.0002*Q_G+0.0006*N-0.337*Q_G^2-1.916e-6*N^2 +0.0003*Q_G*N \quad (21)$$

The goodness of the model function with respect to the experimental is shown by the coefficient of determination (R^2) in our case, the R^2 of the models is higher than 0.9 for each of the responses that means the models are capable of fitting the experimental data. The high value of the adjusted determination coefficient (R^2 Adj.) also indicated an high significance of the model. Furthermore, according to the ANOVA, the models could be considered significant

because the p-value of the model F-value are less than 0.05 and the p-value of the lack of fit is higher than 0.05.

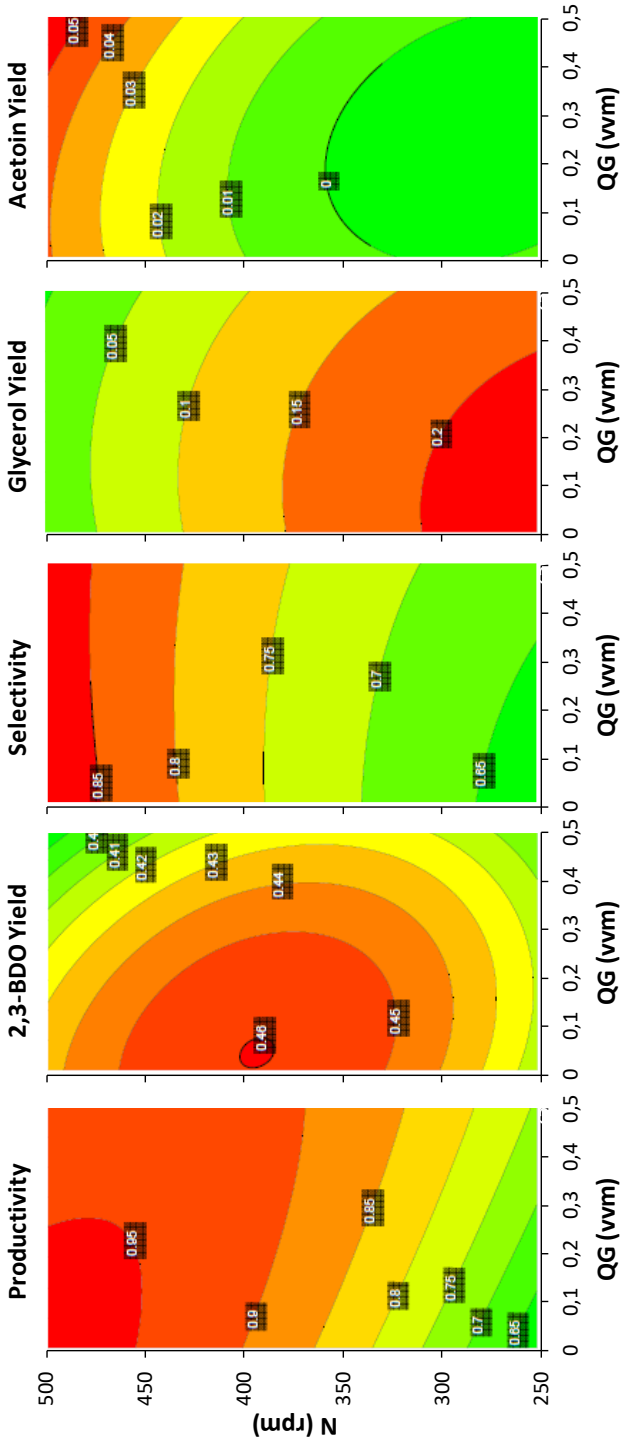


Figure 4.8 Contour plot of the responce variables, Product and by products Yields, 2,3-BDO Productivity and Selectivity

Inside the range of aeration and agitation considered BDO Yield is higly influenced by the agitation, low yields at 250 rpm and at high rpm and lpm (500rpm-0,5vvm) are obtained (Figure 4.8). Productivity seems to be more influenced by the agitation than aeration indeed productivities higher than 0.9 g/L/h can be obtained at rotational speed higher than 400 rpm. The byproducts Acetoin and Glycerol, are mostly produced at opposite conditions as showed in Figure 4.8. Glycerol is preferentially produced at low N and low QG, conditions closed to the anaerobiosis, conversely, Acetein is preferentially produced in conditions closed to aerobiosis. Such behaviour is confirmed by the experiment O9 (Table 4.6), conducted in compleately aerobic conditions, this aspect will be further explored in the next chapter. Selectivity is the performances parameters who correlates fermentation products and by-products, we have defined it as the relative product distribution at the end of the fermentation, indeed, it is defined as the ratio between 2,3-BDO and the sum of all the fermentation products (2,3-BDO Acetoin, Glycerol and Ethanol) (Equation 16). Practically, it combines in one parameter the Glycerol Yield and Acetoin Yield. It is high at a high value of rpm and low at a low value. This is due to a low concentration of acetoin at high rpm than the concentration of glycerol at low rpm this is evident from Table 4.6 in which in microaerobic conditions (excluding experiment O9) the highest concentration of glycerol obtained is equal to 9.5 g/L and the highest concentration of acetoin is 2,4 g/L.

As described in paragraph 3.7.1 the last step of the DoE is the validation and optimization of the process studied. The aim of the model validation is to verify the model predictions conducting experiments at values inside the model ranges but excluding the values used for the model fitting. In the present work, we decided to validate the models conducting three experiments at Q_G and N randomly chosen inside the considered interval. The results of such fermentation are showed in the bottom part of the Table 4.6 (Validation) experiments O11, O12, O13 and O14.

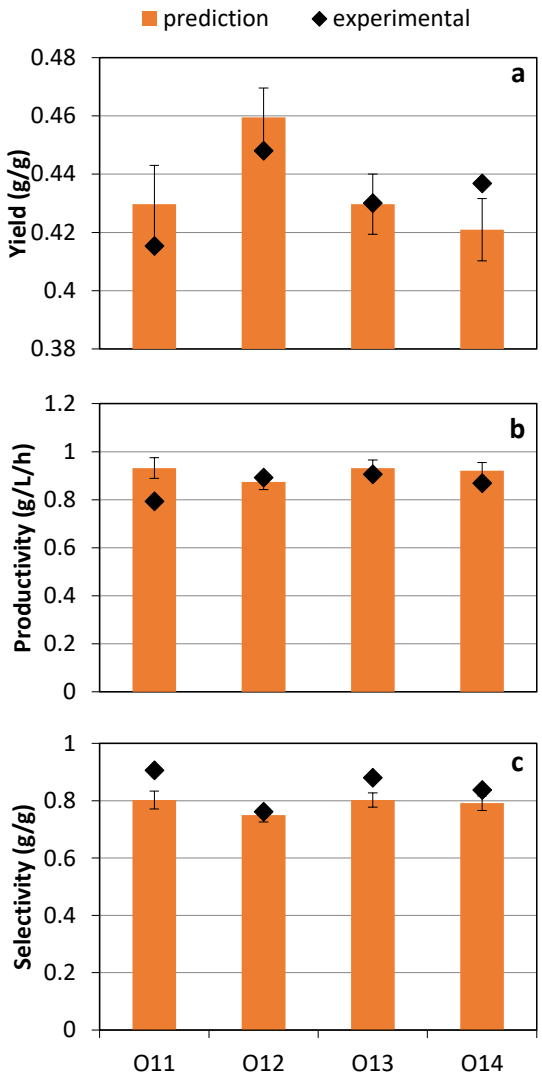


Figure 4.9 Performances of the validation experiments: a) Yield, b) Productivity and c) Selectivity. Orange bars are the model prediction and black points are the experimental data.

In Figure 4.9 the performances of the validation fermentations are compared to the DoE prediction. Almost all the experimental values are well predicted by the software, except for the selectivity and the productivity of the fermentation at 430 rpm and 0.1 vvm probably due to the production of 1,4 g/L of acetoin at the end of the fermentation. Anyway, in general the models are able to predict the process performances at different combination of N and Q_G.

The last stage of the design of experiments is the evaluation of the conditions that allow the process optimization. In this case, the optimum is the condition of agitation and aeration in which Yield Productivity and Selectivity are maximized and acetoin and glycerol Yields are minimized. According to the software optimization a set of solutions were proposed. One of the

proposed solution was characterized by a rotational speed (N) of 460 rpm and a gas flow rate (QG) of 0.1 vvm. In Figure 4.10 the evolution of the analytes are reported.

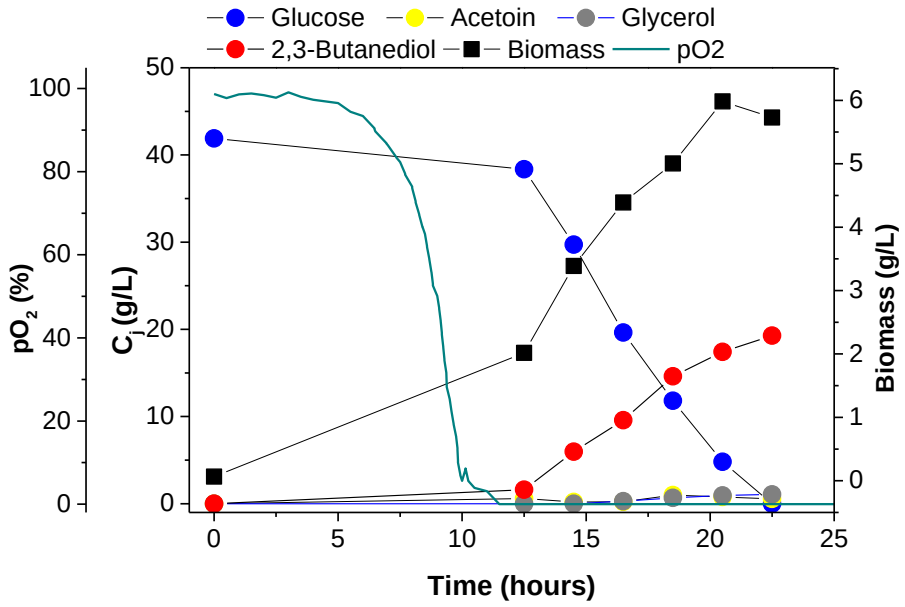


Figure 4.10 Microbial growth (biomass) and concentration of analytes (substrate, product and by-products) over time, during the fermentation at N = 460 rpm and Q_G = 0.1 vvm test O10

The product concentration and the fermentation performances are shown in Table 4.6 test O10. This fermentation lead to the production of 19.7 g/L of 2,3-BDO and 2.1 g/L of acetoin after 22 hours which corresponds to a yield of 0.47 g/g, selectivity f 0.84 g/L and 0.94 g/L/h such a results are well predicted by the DoE (Productivity (g/L/h) 0.96±0.04, Yield (g/g) 0.45±0.01 and Selectivity (g/g) 0.84±0.32).

4.3.2.2. Empirical process optimization

In this paragraph, the fermentation performances of all the fermentations carried out (CCD, Validation and optimization tests), showed in Table 4.6 have been compared with the OTR, which is actually the real process control parameter.

In this work, the OTR has been calculated from the mass balance in the liquid (equation 5). According to the equation, being OTR function of the kLa it is necessary to have a check on the

soundness of its values. For this reason, for each of the fermentations in Table 4.6, the kLa values were measured with the dynamic method (according to the procedure described in M&M, 3.6.4.1.1) and also estimated using the empirical correlations (equation 9), using each of the two constant sets reported in Table 3.8. The experimental $k_L a$ and the calculated ones are shown in a parity plot in Figure 4.11; two dotted lines corresponding to a ±30% variation from the diagonal corresponding to a reasonable estimation error (Pinelli et al., 2010) are shown as well. An acceptable agreement was observed in every case at low $k_L a$ values, in particular with parameters obtained from the work of Scargiali (Scargiali et al., 2010). The only data that have a deviation larger than 30% are those at $k_L a$ > 0.01 s-1 and the experimentally determined values are substantially higher than the predicted ones. This behavior was ascribed to an unwanted surface aeration obtained in that conditions which caused by a not perfect correspondence of the bioreactor used in this work and the standard bioreactor configuration used for the empirical correlations. In particular, the H/T ratio is less than 1 in our experiments and this causes surface aeration at high impeller rotational speed. The experimental $k_L a$ values were therefore considered acceptably reliable, and they were used to calculate for each test a $k_L a$ -based OTR (equation 5) value and ultimately to identify the optimal OTR range for BD production by *Bacillus licheniformis* ATCC9789.

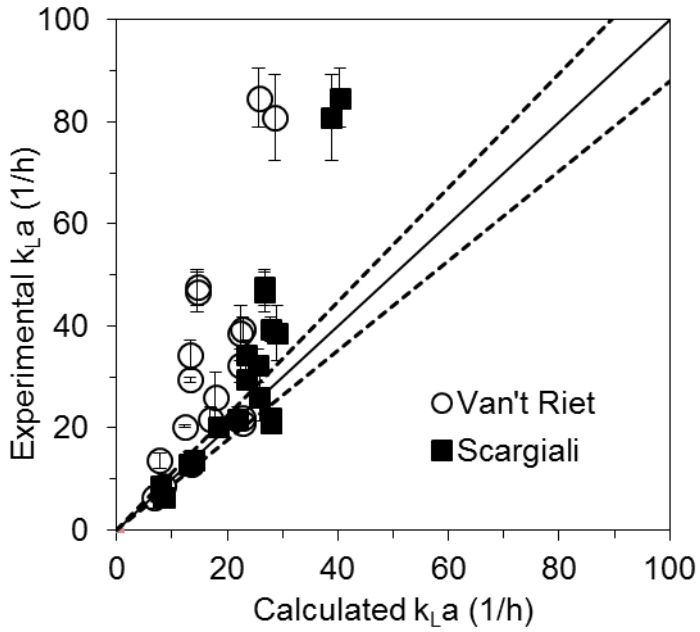


Figure 4.11 Comparison between the experimentally determined $k_L a$ values and those calculated according to Eq. 9, using the parameter values reported by Scargiali et al., 2010 and Van't Riet, 1979.

The plots of the 5 selected performance parameters (Y_{BDO}, P_{BDO}, S_{BDO}, Y_{ACE} and Y_{GLY}), versus *OTR* are represented in Figure 4.12 with full symbols for the CCD tests (O1 to O9) and empty symbols for the validation tests (O10 to O14).

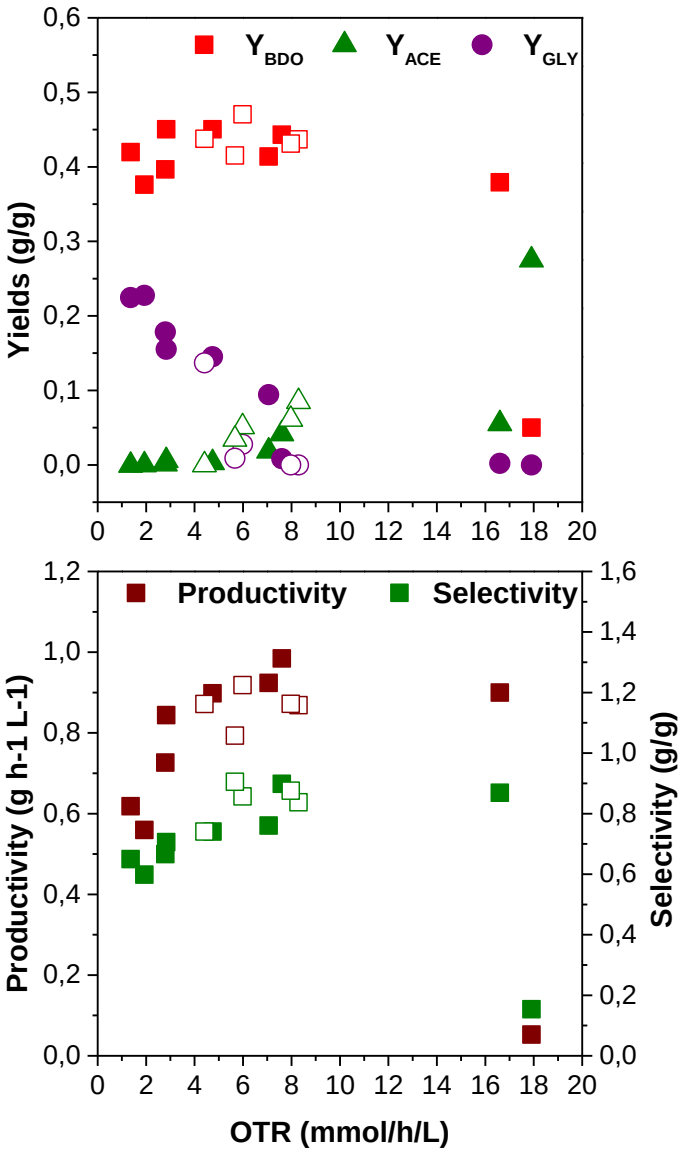


Figure 4.12 Oxygen supply experiments: performance indicators versus OTR. Full symbols refer to the CCD tests (O1 to O9), whereas empty symbols refer to the validation tests (O10 to O14).

With the exception of test O9, that reached fully aerobic conditions during the 2,3-BDO production phase (Table 4.6), in all the other tests the plots of glucose, 2,3-BDO, cells and – where present – acetoin concentration versus time resulted linear and not exponential. This finding indicates that the biological activity was controlled by the oxygen mass transfer rate,

which – according to equation 5 - remained constant during each 2,3-BDO production phase. This represents an indication that micro-aerobic conditions were actually achieved. In agreement with this observation, these tests resulted in satisfactory values of both 2,3-BDO productivity (0.56-0.98 g/h/L) and 2,3-BDO/glucose yield (0.38-0.47 g/g), corresponding to 75-94% of the theoretical maximum yield (0.50 g/g), i.e. the yield that could be obtained if 2,3-BDO was the only product and no biomass formation from glucose occurred. In line with the rather high 2,3-BDO yields, rather low yields of acetoin and glycerol production and negligible ethanol concentrations were found in these tests. Conversely, the aerobic test (O9) resulted in very poor performances (Y_{BDO} = 0.05 g/g, 2,3-BDO productivity = 0.05 g/h/L).

The 2,3-BDO yield (Figure 4.12a) presents a slight increase in the low-OTR range (from 1.4 to 3 mmol/h/L), followed by a roughly constant yield (0.44 ± 0.02 g/g) in the 3-8 mmol/h/L *OTR* range. A gap of experimental data is then found from 8 to 16 mmol/h/L, whereas the Y_{BD} obtained at an *OTR* of 16.6 mmol/h/L, equal to 0.38 g/g, resulted lower than the optimal values obtained in the 3-8 mmol/h/L range. An OTR equal to about 17 mmol/h/L determined the shift to aerobic conditions, leading at 17.9 mmol/h/L to an extremely poor Y_{BDO} (0.02 g/g). Under this condition, the most abundant product is acetoin, as O_2 is used instead of acetoin as the main final electron acceptor. 3-8 mmol/h/L was thus identified as the OTR range that safely leads to high BD yields. Within this OTR range, the glycerol yield gradually decreased and reached zero in correspondence with an OTR equal to 8 mmol/h/L, whereas the acetoin yield slowly increased with increasing OTR, with a maximum value of 0.04 g/g.

In terms of 2,3-BDO productivity (Figure 4.12b), an about linear increase up to an OTR equal to 5 mmol/L/h – corresponding to the *OTR* interval where the 2,3-BDO production process was completely controlled by the oxygen transfer rate - was followed by a roughly constant value (0.94 ± 0.03 mmol/h/L) in the 6-17 mmol/h/L OTR range. At an OTR equal to 17 mmol/h/L the shift to aerobic conditions determined a drastic decrease in 2,3-BDO productivity and production rate. 6-17 mmol/h/L was thus identified as the OTR range leading to optimal values of BD productivity.

In the perspective to identify an optimal *OTR* range for 2,3-BDO production by *Bacillus licheniformis* ATCC9789, the optimal ranges identified for the single performance indexes on the basis of the data reported in Figure 4.12 are 3-8 mmol/h/L for the 2,3-BDO yield, and 6-17 mmol/h/L for the 2,3-BDO productivity. Only a comprehensive economical evaluation could lead

to the identification of the optimal *OTR* value, i.e. of the best compromise between the conditions that favor either yield or production rate / productivity. As the economic analysis of the process is beyond the scope of this work, the conclusion that can be drawn from the above-illustrated analysis is that the optimal *OTR* value lies in the region of overlap between the ranges that optimize the BD yield and production / productivity, i.e. in the 6-8 mmol/h/L range. The drastic drop in BD production performances observed in correspondence with a 17 mmol/h/L OTR indicates that a careful *OTR* control is crucial in order to optimize the BD production process by *Bacillus licheniformis* ATCC9789, and that the *OTR* set point should be sufficiently lower than the borderline value of 17 mmol/h/L.

In conclusion, the effect of the oxygen supply for the production of 2,3-butanediol from glucose with the microorganism *Bacillus licheniformis* lead to a yield near to the maximum theoretical value. This result could be achieved by optimizing the oxygen mass transfer so as to maintain microaerobic condition suitable to produce the BD and avoid the accumulation of the oxidized precursor acetoin. A range of the values of the OTR (6-8 mmol/h/L) suitable for the best performance was determined and the corresponding range of the volumetric mass transfer coefficient was estimated (between 0.008÷0.011 1/s).

A Design of Experiment approached was successfully tested to define a minimum set of experiments that allowed the optimization of the process. The best performance was (experiment O10): final C_{BD}= 19/L with a Y_{BDO} = 0.47 g_{BD}/g_{GL}, productivity = 0.98 g h^{-1} L^{-1}, selectivity = 0.92 $g_{BD}/g_{products}$.

The BD production performances obtained in this work are comparable or higher than those reported by other studies conducted with *Bacillus licheniformis* wild types at the same temperature (30°C) and with similar media, under batch conditions. For example, Nilegaonkar et al. (Nilegaonkar et al., 1992a) obtained in shake flask experiments a similar yield (0.47 gBD/gGL) but a significantly lower productivity (0.12 g/h/L). Jurchescu et al. (Jurchescu et al., 2013) report similar performances for both yield (0.42 gBD/gGL) and productivity (0.86 g/h/L), but they were able to increase the latter up to 1.14 g/h/L thanks to the switch from batch to fed-batch process. In other studies conducted with the same strain (Li et al., 2013) (Ge et al., 2016), the increase in temperature from 30°C to 50°C determined the attainment of higher BD productivities (2.2-2.8 g/h/L) whereas the BD yields resulted comparable to those obtained in this work at 30 °C.

The 2,3-BDO yields obtained in this work are comparable with those achieved with other BD producing wild type strains belonging to the risk group 2. For example, Anvari and Motlagh (Anvari and Safari Motlagh, 2011) reached a yield of 0.44 g/g using *Klebsiella oxytoca* and Hazeena et al. (Hazeena et al., 2016) report a 0.48 g/g yield with *Enterobacter cloacae*. No productivities were reported in these latter studies. Higher productivities are reported in the literature for genetically modified strains. For example, in a glucose-fed batch process (Wang et al., 2012) reached a 0.37 g/g yield and a 2.64 g/h/L productivity by knocking out the *ldh* gene of *B. licheniformis* BL1.

4.4. Study of the fed-batch operation mode

The application of a fed-batch strategy was studied in order to maximize the production of 2,3-dutanediol. Preliminary experiments were carried out in order to verify the effect of the initial glucose concentration and the complex medium concentration, which are essential for the production of high yield of 2,3-BDO. Finally, the experiments described in this paragraph have taken into account the oxygen supply condition studied in the previous chapter (paragraph 4.3).

4.4.1. Effect of the substrate concentration

As part of the fermentation process optimization, the effect of the initial glucose concentration on the 2,3-BDO production was evaluated by carrying out fermentations at increasing initial glucose concentrations according to the experimental scheme shown in Table 4.7. The fermentation medium and conditions were identical to those evaluated previously (Beef extract 10 g/L, peptone 10 g/L and NaCl 5 g/L 30°C and pH 6). Moreover, these experiments included the oxygen transfer conditions which were optimized before . .

Table 4.7 Experimental scheme of the tests at different initial glucose concentration

Test	Initial glucose concentration (g/L)
G1	20
G2	40
G3	60
G4	80
G5	100

Figure 4.13 compares the evolution of the concentration of biomass, glucose and 2,3-butanediol

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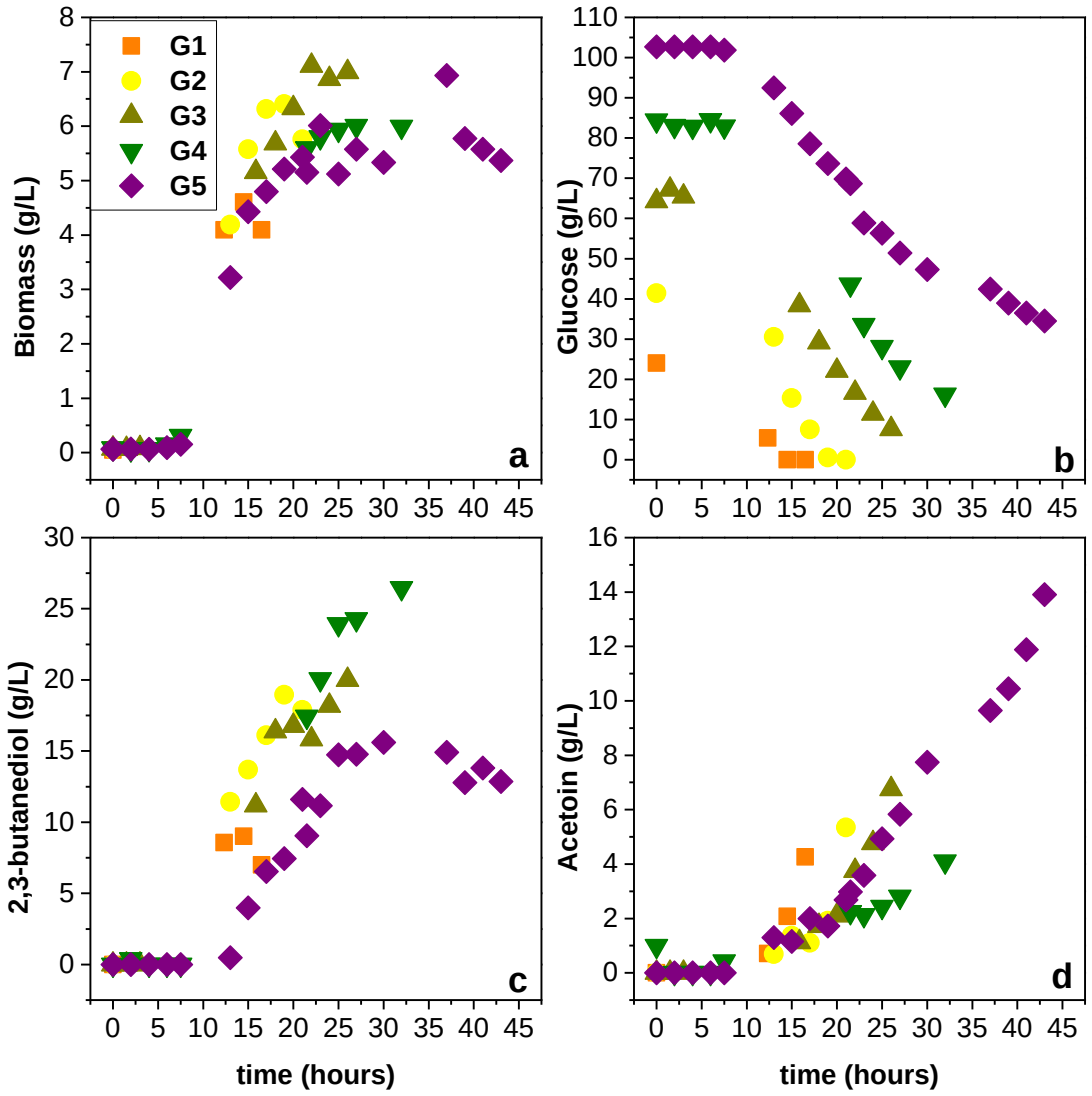

Figure 4.13 Evolution of biomass (a), glucose concentration (b), 2,3-butandiol (c) and acetoin (d) concentration in the fermentations at increasing initial glucose concentration. All the experiments were carried out at the optimal oxygen transfer conditions.

Figure 4.13a demonstrates the comparison of biomass evolution in the fermentation broth at different initial glucose concentrations. Apparently, the experiments with 20, 40 60 80 g/L of initial glucose reached the same final biomass concentration between 6 and 7 g/L, whereas, experiment G5 reached the max concentration of 5 g/L. Although only little information is available on the initial growth rate, fermentation G5 seems to be the most affected by the glucose concentration. Furthermore, experiments G3, G4 and G5 indicate that the microbial growth stops after 25 hours, even though glucose is still present in the fermentation broth. This behavior could be linked to the complex media on which the microorganism is able to growth, as demonstrated in the flask experiments in chapter 4.2.1.

An additional element of comparison is the substrate consumption (Figure 4.13b). The rate of glucose consumption is apparently the same in fermentations G1, G2, G3, G4 while it seems to be lower in the fermentation with 100 g/L of glucose. In particular,in experiment G5 the initial substrate consumption rate seems to be lower compared to experiments G1, G2, G3 and G4. Furthermore, in experiment G5, the consumption rate seemed to slow down between 20 and 25 hours , this is likely to be connected to the growth rate decrees and could be linked with the complex media.

Moreover, 2,3-butanediol production is affected by the initial glucose concentration too. Indeed, when observing Figure 4.13 c, 2,3-BDO evolution in experiment G5 seems to be affected mostly by the initial glucose concentration than in experiments G1, G2, G3 and G4. The highest 2,3-BDO concentrations of 19, 20, 26 and 15 g/L were respectively produced in experiments G2, G3, G4 and G5. The maximum concentration of 2,3-BDO is apparently proportional to the initial glucose concentration until the fermentation with 80 g/L of initial glucose and it has a drop in experiment G5. Such a behavior would suggest the occurring of inhibition due to the substrate. The production of acetoin, of which evolution is shown in Figure 4.13 d, is higher in experiment G5 in which a concentration of 13 g/L was reached after 43 hours.

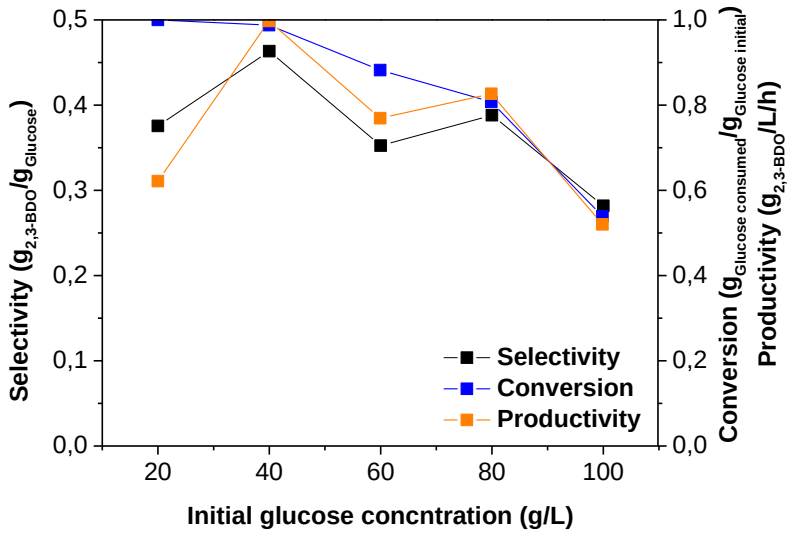

Figure 4.14 Fermentation performances Selectivity and Conversion in the fermentations at different initial glucose concentration

Since glucose is not completely converted in all the fermentations, a comparison of the fermentations in terms of selectivity, productivity, and glucose conversion was made when the highest concentration of 2,3-BDOwas reached . Figure 4.14 shows the trend of selectivity productivity and glucose conversion in the fermentations. Glucose is completely consumed when the initial glucose concentration is 20 and 40 g/L whereas glucose concentrations of 60 and 80 g/L showed a conversion decrease as it dropped to 0.55 g/g in the fermentation with 100 g/L of the initial glucose. The same behavior can be observed regarding the selectivity which is in the range 0.35 and 0.45 g/g in fermentations G1, G2, G3 and G4, while it dropped to 0.28 g/g in the fermentation at 100 g/L. As for the productivity, when the initial concentration of glucose was 20 g/L, the productivity was around 0.62 g/L/h, this is probably due to the low concentration of 2,3-BDO that was reached. Instead, when the concentration of glucose was between 40 and 80 g/L, the productivity was in the range 0.76 - 0.94 g/L/h. As observed for selectivity and conversion, productivity dropped in the experiment at 100 g/L (0.52 g/L/h).

In conclusion, the initial concentration of glucose of 100 g/L seems to be inhibitory for the production of 2,3-BDO from glucose with the microorganism *Bacillus licheniformis.* Therefore, it would be better to avoid the concentration of 100 g/L of glucose during the development of a fed-batch process in order to avoid inhibitory effects. Moreover, it is better to keep a concentration of glucose between 40 and 80 in order to obtain the best fermentation

performances. Since that, the complex media has shown to affect the growth and the production of 2,3-butanediol, further experiments have been carried out in order to evaluate its effect.

4.4.2. Effect of the complex media concentration

The present paragraph presents the study of the effect of the concentration of the complex media on the production of 2,3-BDO. The selection of the medium and the literature review presented in paragraph 4.1.2 have both led to choose a medium mainly composed by Beef extract and Peptone from soy bean (Table 3.4). This medium is composed by proteins, aminoacids, vitamins and growth factors which can be assumed to promote the growth and the production of 2,3-BDO. In the previous chapter a depletion of the microbial growth rate in the fermentations with 60, 80 and 100 g/L of initial glucose was noted when glucose was still present in the fermentation broth. This behavior could be due to the absence of a growth nutrient that is inside the complex media.

The study of the effect of the complex media concentration on the process is also useful for the optimization of the fermentation process in fed batch mode. Indeed, in order to obtain high 2,3-BDO concentrations and productivity, glucose shouldn't be considered the sole feeding substrates.

The experiments were set in as shown in Table 4.8 where the initial concentration of glucose was constant, but the concentration of complex media was changed. The glucose concentration chosen for these experiments was 80 g/L which corresponded to the highest concentration at which inhibitory effects do not occur.

Table 4.8 Experimental scheme of the experiments at different concentration of complex medium

Test	Beef extract g/L	Peptone g/L	Total g/L	Glucose g/L
M1	5	5	10	80
M2	10	10	20	80
M3	20	20	40	80
M4	40	40	80	80

The evolution of the main anilities and the concentration of the dissolved oxygen is demonstrated in Figure 4.15. A key parameter for the production of 2,3-BDO is the concentration of oxygen in solution. In fact, 2,3-BDO is produced when the dissolved oxygen concentration is equal to zero, a particular condition called microaerobiosis. Figure 4.15d shows the evolution of dissolved oxygen. The time necessary to reach the microaerobiosis is proportional to the concentration of complex media: increasing the concentration of complex media increase the time necessary to reach the microaerobiosis. Microaerobiosis requires 32 hours when 80 g/L of complex media are present. While 15, 7.5 and 7.5 hours are respectively necessary in cases of 40, 20 and 10 g/L of complex media,. Since the production of 2,3-BDO is chained to the microaerobic conditions, its production starts when such conditions are reached as shown in Figure 4.15a. Thus, in order to avoid a long lag phase, it is better to initially start a fermentation with a concentration of complex media of 10 or 20 g/L and then feed contemporary glucose and complex media to the fermenter.

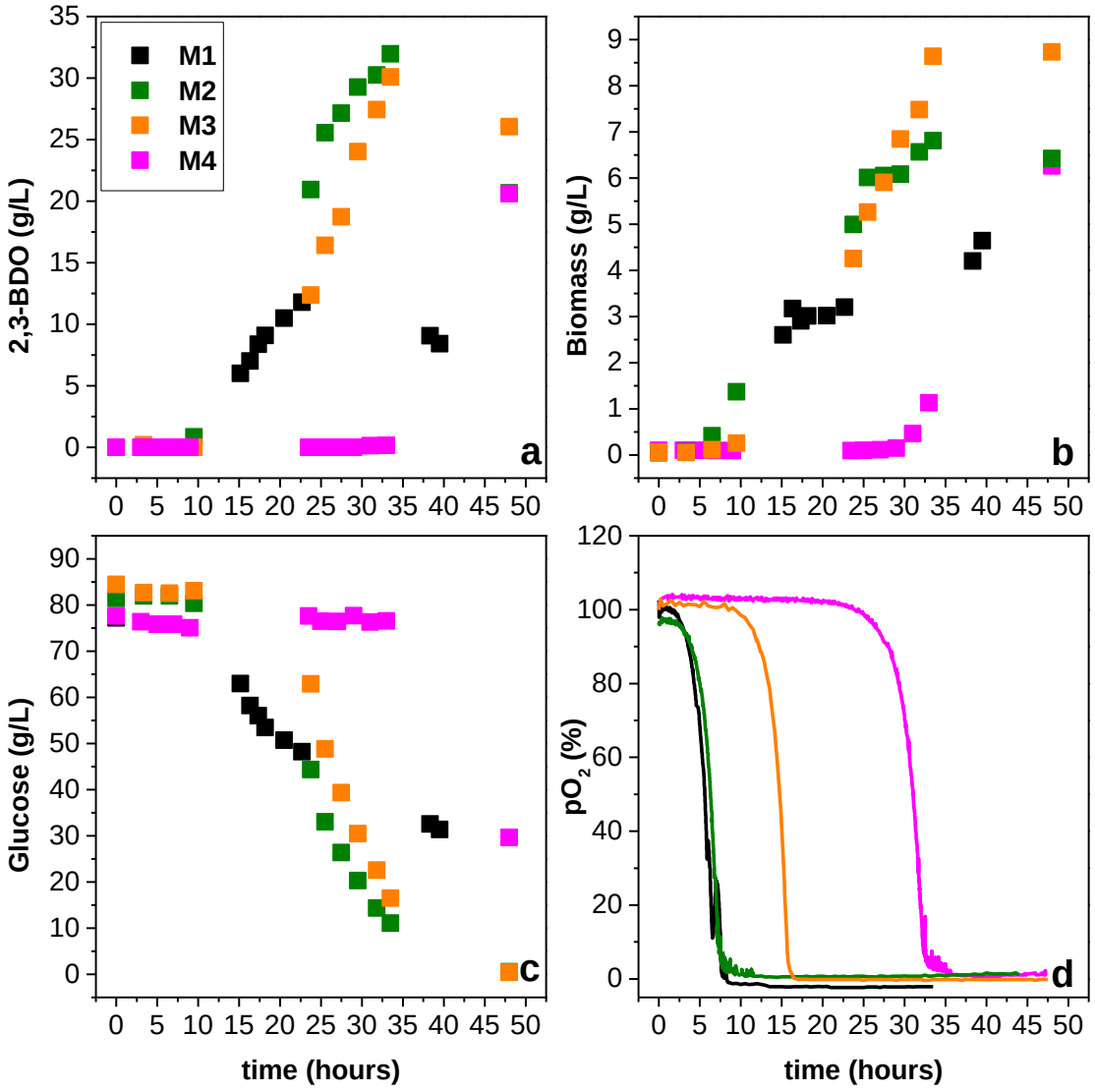

Figure 4.15 Evolution of 2,3-butandiol (a), biomass (b), glucose concentration (c) and oxygen partial pressure in the liquid (d) in the fermentations at increasing initial glucose concentration.

Furthermore, observing the biomass evolution (Figure 4.15b), the highest concentration of biomass production was proportional to the concentration of the complex media, 4.6, 6.8 and 8.7 g/L of biomass were produced in experiments M1, M2 and M3 respectively. Since the fermentation was very long, it was not possible to measure the maximum biomass concentration in experiment M4. In Figure 4.15c and a, the glucose consumption and the 2,3-BDO production are shown. Experiment M1 with 10 g/L concentration of complex media seemed to consume glucose and produce 2,3-BDO slower than the others. While fermentations M2 and

M3 respectively may have had the same rate of glucose consumption,, it should be noted that experiment M2 reached the microaerobiosis a few hours before experiment M3 did, thus a comparison between the experiments after the microaerobiosis was deemed more appropriate. In particular, an attempt has been done to calculate the fermentation performances (glucose conversion, 2,3 BDO Yield, and selectivity) 15 hours subsequent to the start of the microaerobiosis due to the lack of experimental points in some experiments.

Table 4.9 represents the fermentation performances when compared in terms of complex media concentration and ratio glucose complex media. The ideal ratio glucose/complex media will be applied in the fed batch fermentation process.

The results demonstrated in Table 4.9 were calulated approximately 14-15 hours the microaerobiosis began, this was necessary due to the lack of data in some experiments.

Table 4.9 Fermentation performances (Conversion, Selectivity and Yields) in the experiments at different concentration of complex media, calculated after 15 hours from the beginning of the microaerobiosis.

		M1	**M2**	**M3**	**M4**
Glucose	g/L	80	80	80	80
Complex media	g/L	10	20	40	80
Glucose/Complex media Ratio	g/g	8	4	2	1
Conversion	g/g	0,38	0,59	0,64	0,62
Yield	g/g	0,15	0,31	0,28	0,27

The performances dropped when the concentration of complex media was 10 g/L (experiment M1), whereas when 20, 40 and 80 g/L of complex medium were initially present high conversion was reached. Being the fermentation performances comparable in such experiments (M2, M3 and M4), giving the fact that the fermentation preformances were comparabile in these expriments, it can be argued that a concentration of 80g/L complex medium is too high for the fermentation process.. Thus, being the concentrations of 80 g/L glucose and 20 and 40 g/L complex medium not inhibitory for the fermentation process, a ratio glucose complex medium between 2 and 4 should be fed to the bioreactor in a fed-batch process.

The previous two chapters discussed the study of the effect of the substrates, glucose and complex media. Such information is useful for the development of a fed batch process. Indeed, in order to avoid glucose inhibition effects it is necessary to keep the concentration of glucose between 20 and 80 g/L. Moreover, the complex medium, which is the main growth substrate, should not be higher than 20 g/L at the beginning of the fermentation in order to avoid effect of long lag phase. In addition, it should be fed to the bioreactor during the fermentation at a ratio glucose complex media (g/g), between 2 and 4.

4.4.3. Optimization of the process in fed batch mode

The development of a fed batch process was studied in order to obtain a high concentration of 2,3-BDO and increase the productivity of the process. 2,3-BDO fermentation process is influenced by different variables. In fact, the previous chapters investigated on the effect of the substrates concentration which lead to an inhibitory effect according to thier low or high concentration.. Furthermore, the effect of the oxygen transfer rate has to be taken into account.

In this chapter, the possibility of producing 2,3-BDO at high concentration through a fed batch process was studied having taken into consideration the results of the effect of the substrates (glucose and complex media) and focused on the effect of the oxygen transfer rate.

As extensively studied above, oxygen is the key parameter in the production of 2,3-BDO for achieving high yields, selectivity, and productivity. In particular, the key parameter for the scale-up of this process is the oxygen transfer rate which in turn depends and could be controlled by the oxygen transport coefficient (k_La). In chapter 4.3, an aeration agitation combination (460rpm – 0,1vvm) and a range of OTR equal to 6-8 mmol/h/L corresponding to a k_La range of 0.008-0.011 s-1, within which the best performance falls, was identified in batch fermentations. Such parameters can be used to carry out the optimization of the process in different operation mode as well as for the scale-up of the process. In this chapter, the possibility of keeping k_La constant was studied.

Indeed, during a fed-batch fermentation, feeding a solution leads to the increase of fermentation broth volume. With the oxygen transfer rate coefficient proportional to the fermentation broth volume, according to equation 9, and at a constant stirring rate and aeration rate during the fermentation, the increasing of the volume leads to a decreasing in the k_La coefficient. Thus, in this chapter, the strategy applied to keep the k_La constant was to increase

the stirring rate while keeping constant the air flow rate. In particular, it was decided to keep the k_La in the optimal range of 0.008-0.011 s-1 as previously determined in the oxygen supply test (Chapter 4.3).

The stirring rate has been calculated starting from the empirical k_La calculation (correlation 9 materials and methods) at the condition of stirring rate and aeration optimized in chapter 4.3.2.1. It was decided to apply the empirical coefficients derived from Scargiali et al. 2010 (Scargiali et al., 2010) (m = 0.0037, α = 0.59 and β = 0.35) since these coefficients were the ones that allowed to have the smallest gap between the dynamic measure of k_La and the empirical calculation. Table 4.10 demonstrated the data concerning agitation, aeration and the relatives k_La determinated experimentally or calculated from the empirical equation.

Table 4.10 Comparison between dynamic and empirical *kLa* under fermentation conditions optimized at N = 460 rpm and QG = 0.1 vvm (test O10)

N (rpm)	Qg (L/min)	kLa dinamico (s-1)	kLa empirico (s-1)
460	0.1	0.0096	0.0065

The empirical k_La value indicated in the last column of Table 4.10 is the k_La value used to derive the values of agitation (N) at the variation of the fermentation volume (V).

An iterative calculation was conducted to calculate the gassed power to the change in the fermentation volum from the equation 9 and subsequently to calculate the number of turns (N) from equation 10 to be set to the change of the fermentation volume that varies with the feed rate.

The different fed batch fermentations and experimental scheme that were carried out in this chapter are summarized in Table 4.11 . Mainly the effect of the oxygen transfer conditions was studied by attempting to compare a fermentation at constant agitation and aeration with fermentations at constant k_La. Furthermore, the effect of different feeding rate of a concentrated glucose complex media (ratio 2-4) solution was evaluated in order to maintain the concentration of glucose between 20 and 80 g/L.

Table 4.11 Experimental scheme for the fed batch fermentations.

Test	Oxygen transfer condition	Feeding (Concentration of the feed solution)	Feeding rate
FB1	Constant stirring end aeration	Glucose 630 g/L Complex 300 g/L	0.108 ml/min (a) 0.124 ml/min (b)
FB2	Constant kLa	Glucose 567 g/L Complex 280 g/L	0.126 ml/min (c) 0.155 ml/min (d)
FB3	Constant kLa	Glucose 545 g/L Complex 273 g/L	0.122 ml/min (e) 0.6 ml/min (f)
FB4	Constant kLa	Glucose 545 g/L Complex 273 g/L	Da 0.12 a 0.3 ml/min

In Figure 4.16 the fermentation FB1 (constant aeration and agitation) is shown, where the dotted lines indicate the beginning, change and end of the feeding. The glucose and complex media solution feeding started8 hours after the beginning of the fermentation. 2,3-BDO starts to be produced when the microaerobic conditions are reached. At the same time, the concentration of glucose in the fermentation broth slowly decrease. After 30 hours from the beginning of the fermentation, feeding rate was increased to 0.124 ml/min in order to keep the concentration of glucose around 40 g/L. After 56 hours, the concertation of 2,3-BDO was 86 g/L and a concentration of 15 g/L of biomass was produced. Thus, the feeding was stopped in order to consume all the glucose. It should be noted that after 76.6 hours of fermentation, cell growth reaches 15.1 g / L of cell biomass and the microorganism produced 100 g / L of 2,3-BD. At the end of the fermentation, the yield was 0.43 g/g and the productivity 1.3 g/L/h. The byproducts which were accumulated to the greater extent was glycerol at a concentration of 14.8 g / L.

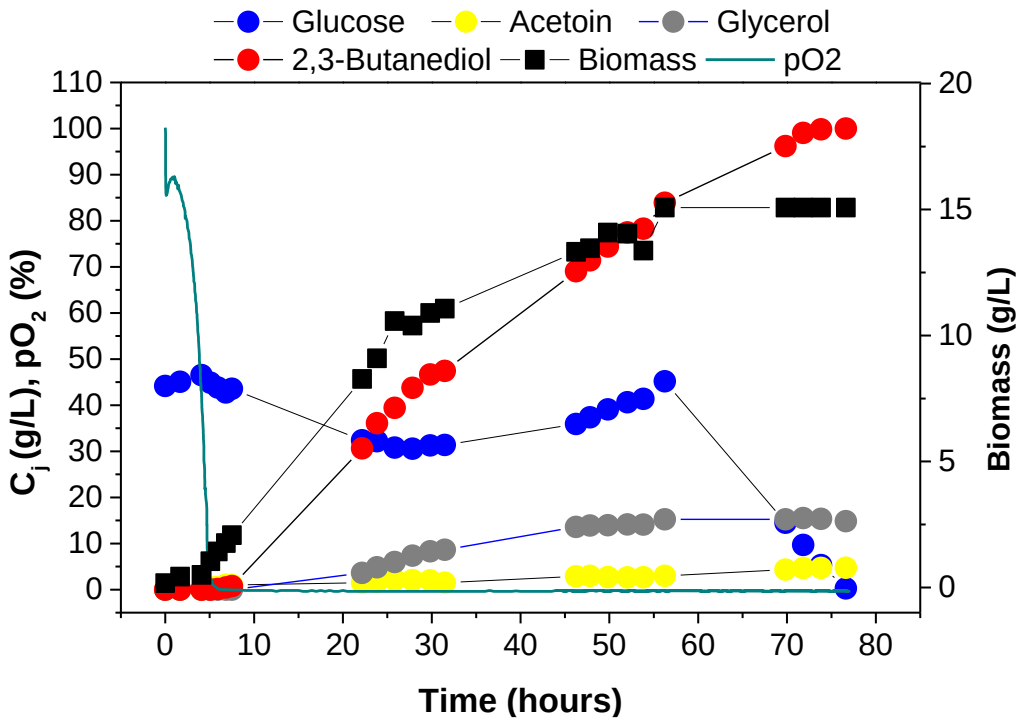

Figure 4.16 Microbial growth (biomass) and concentration of analytes (substrate, product and by-products) over time, during the Fedbatch fermentation FB1 constant agitation and aeration. The dot lines indicate the beginning, change of rate and end of the feeding. Two feeding rate has been applied: first dot line 0.108 ml/min, second dot line 0.124 ml/min. Feeding was stopped after 56 hours of fermentation (red dot line).

Figure 4.17 shows fermentation FB2. This fermentation was the first attempt at constant k_La. Feeding started after 9 hours, at a rate of 0.126 ml/min, when microaerobic conditions were reached. In this case, while the substrate was feeded, the glucose concentration decreased and after 30 hours it reached the concentration of 10 g/L, i.e., lower than the limit of 20 g/L. Thus, with the aim of counteracting the glucose decrease, it was decided to increase the feeding rate to 0.155 ml/min, however after 50.7 hours the glucose was finished. After 50.7 hours 18.5 g / L of biomass were produced with a concentration of 2.3-BD equal to 87.1 g/L, with a yield of 0.388 gBD/gGLU and a productivity of 1.7 g/L/h. The byproducts accumulated were glycerol and acetoin at the concentration of 10 g/L.

The effect of the constant k_La seemed to be present on the production and consumption rate, and therefore the productivity was higher than the previous experiment. The aim of the next experiment was to increase the feeding rate in order to control the glucose consumption.

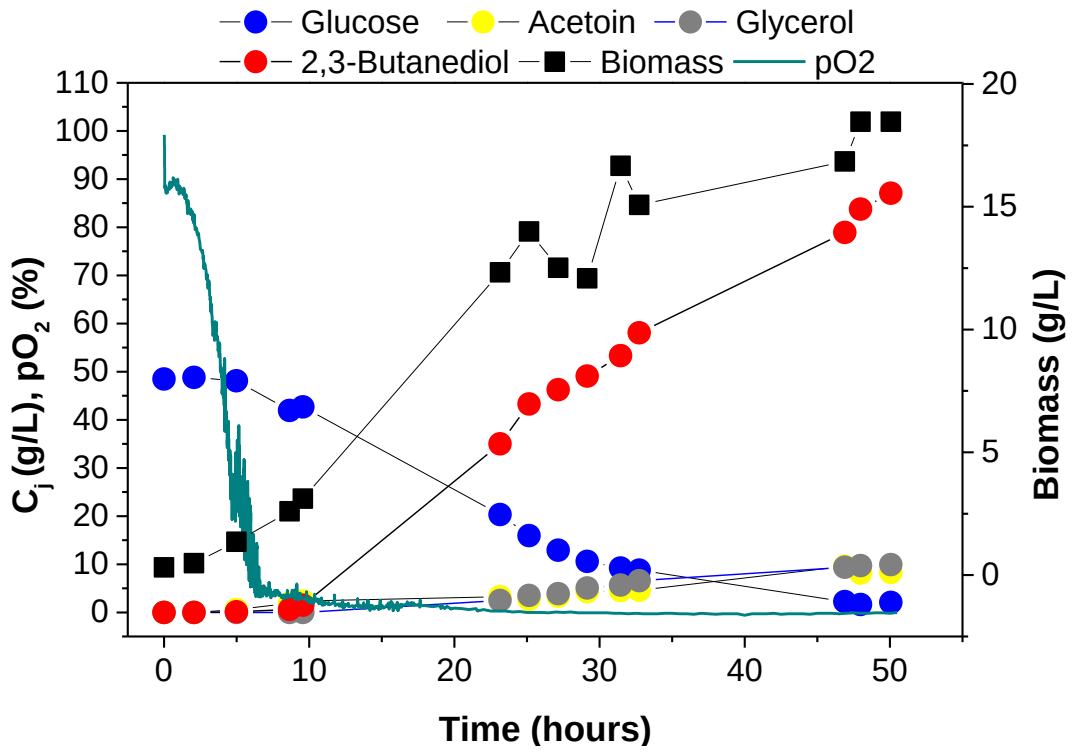

Figure 4.17 Microbial growth (biomass) and concentration of analytes (substrate, product and by-products) over time, during the Fedbatch fermentation FB2 constant agitation and aeration. The dot lines indicate the beginning, change of rate and end h of the feeding. Two feeding rate has been applied: first dot line 0.124 ml/min, second dot line 0.155 ml/min. Feeding is stopped after 52 hours (red dot line).

Figure 4.18 shows fermentation FB3. The production of 2,3-BDO started 6 hours after the beginning of the fermentation, when the microaerobic conditions were reached. The feeding was switched on 9 hours after the beginning of the fermentation, with a flow of 0.122 ml/min. However, this flow was not sufficient to allow to keep the glucose concentration constant. Thus after 28 hours its concentration was 8.5 g/L, and the flow was increased to 0.6 ml/min in order to increase the glucose concentration in the broth. In this case the flow rate was higher than the glucose consumption rate, hence the glucose concentration in the broth reached the concentration of 78 g/L after 35 hours and the concentration of 2,3-BDO remained constant between 28.5 and 35 hours. Considering that the concentration of glucose after 35 hours was close to the inhibitory concentration, the feeding was stopped. At the end of the fermentation,56 hours after it began, the concentration of 2,3-BDO was 101.3 g/L corresponding

to a yield of 0.447 g/g and a productivity of 1.8 g/L/h. However, glucose remained present in the broth at the concentration of 23 g/L which meant that the glucose conversion was 0.9 g/g, thus indicating that10 g/L of 2,3-BDO more could be produced. When comparing the performances of fermentations FB2 and FB3 it is possible to observe that the 2,3-BDO concentration and productivity were higher. This behavior can indicate how controlling glucose concentration high fermentation performances can be obtained.

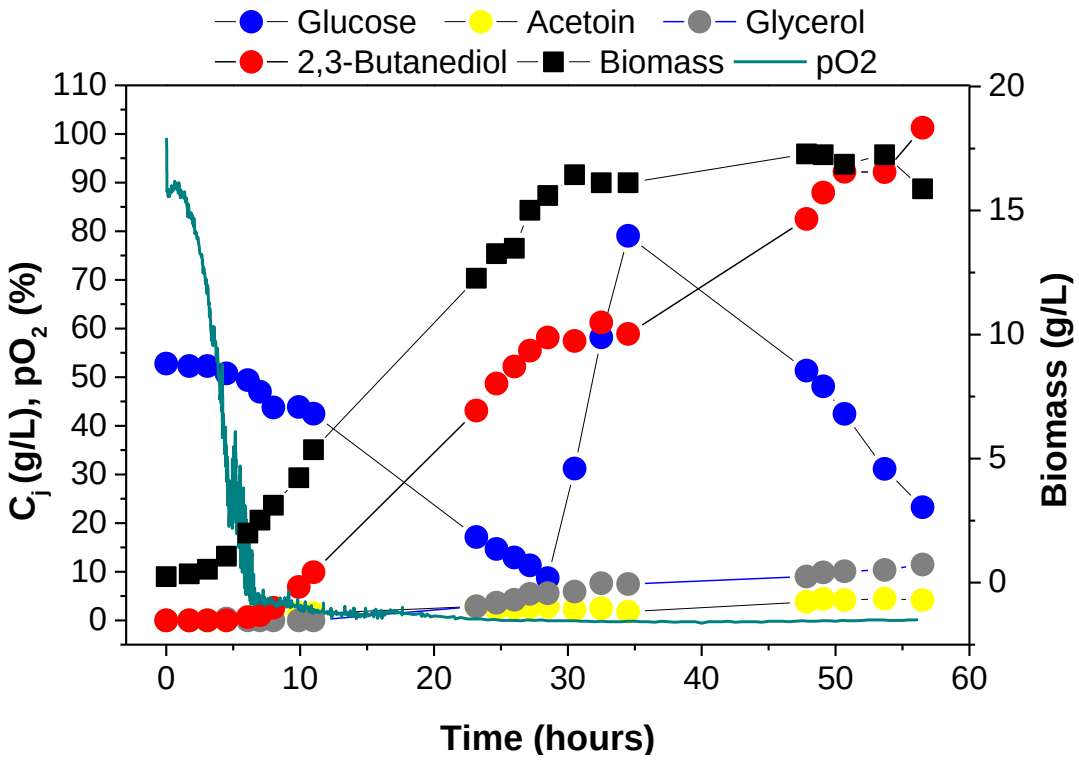

Figure 4.18 Microbial growth (biomass) and concentration of analytes (substrate, product and by-products) over time, during the Fedbatch fermentation FB3 constant agitation and aeration. The dot lines indicate the beginning, the change of rate and end of the feeding. Two feeding rate has been applied: first dot line 0.122 ml/min, second dot line 0.6 ml/min. Feeding is stopped after 36 hours (red dot line).

In fermentation FB4 a more accurate glucose concentration control was applied. In particular, considering fermentations FB2 and FB3, 4 different feeding flow rates were applied. By plotting the feeding flow rate vs glucose concentration consumption rate it is possible to calculate the

feeding flow necessary to keep its concentration constant(glucose consumption rate equal to zero).

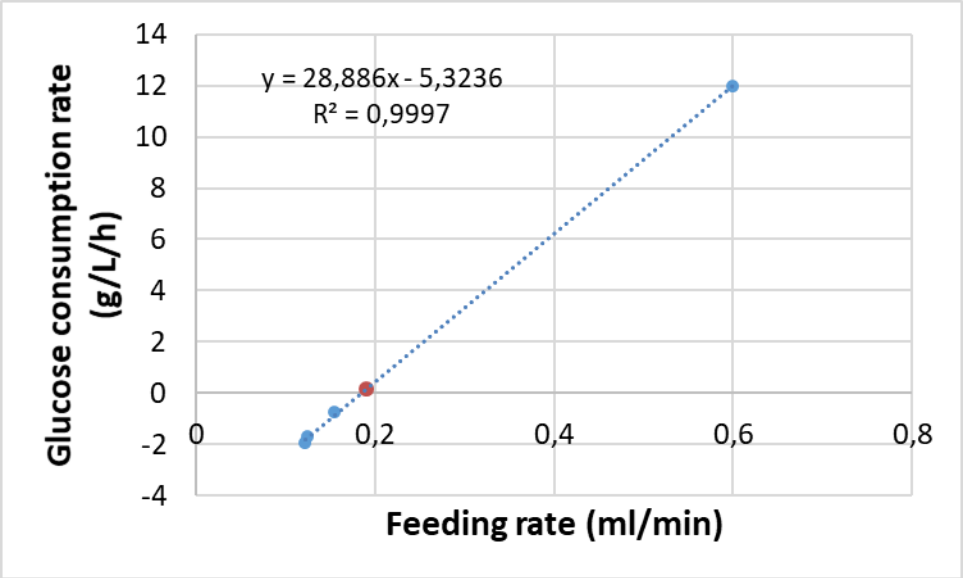

Figure 4.19 Feeding rate vs glucose consumption rate in the fermentation FB2 e FB3. The blue points represent the feeding rate applied in the fermentations FB2 and FB3, while the red point is the predicted feeding rate.

In Figure 4.19 the dot line represents the intercept of the glucose consumption rate/feeding rate from which is possible to calculate the feeding rate necessary to keep the broth's glucose concentration constant. A calculation of experimental data shows that this value should be around 0.18 ml/h. , However, it must be stated that the feeding rate should be calculated from the mass balance of glucose during the fermentation.

Following this reasoning, in fermentation FB4 a solution was fed at almost the same concentration of fermentations FB2 and FB3, at a growing feeding rate calculated experimentally from the previous experiments, in a range between 0.122 and 0.4 ml/min.

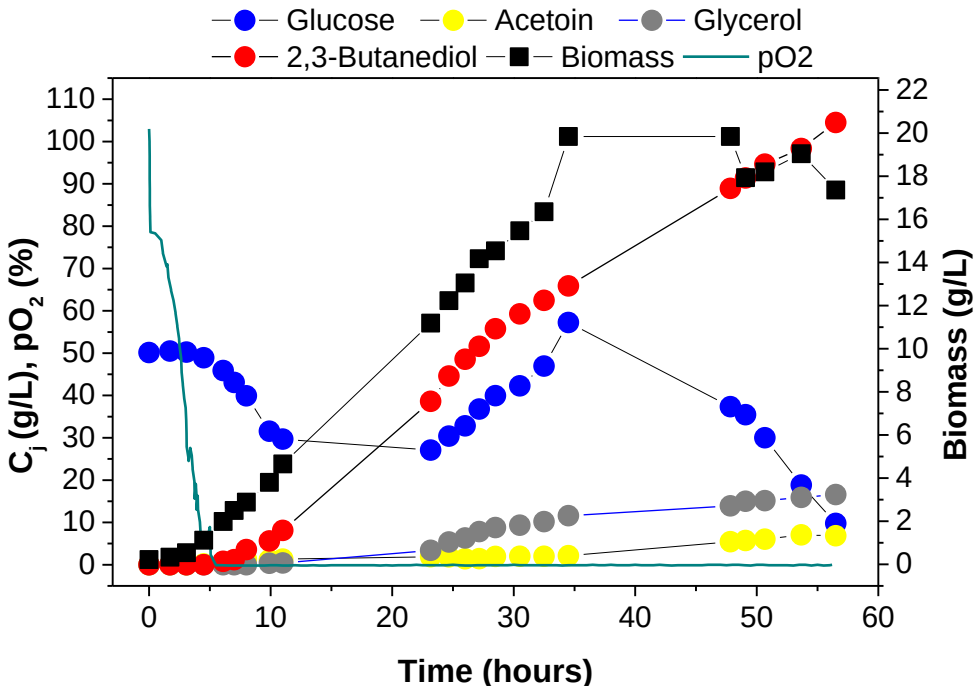

Figure 4.20 Microbial growth (biomass) and concentration of analytes (substrate, product and by-products) over time, during the Fedbatch fermentation FB4 constant agitation and aeration. The dot lines indicate the beginning, the change of rate and end of the feeding. The feeding rate started after 11 hours from the beginning with a rate between 0.124 ml/min and 0.4 ml/min. Feeding was stopped after 40 hours of fermentation (red dot line).

In Figure 4.20 the fermentation FB4 is demonstrated. The feeding began after 8 hours, at a feeding rate wich gradually increased from 0.122 to 0.4 ml/min. As shown before the production of 2,3-BDO starts when microaerobic conditions are reached. Glucose initially decreases until it reaches the concentration of 30 g/L after 10 hours. Its concentrationprobably decreases even more during the night between the 10 and 22.5 hours of fermentation, than it increases due to the increase of the feeding rate. After 35 hours the feeding was stopped in order to consume all the glucose. At the end of the fermentation (56 hours) 104.5 g/L of 2,3-BDO were produced, corresponding to a yield of 0.48 g/g and a productivity of 1.7 g/L/h. The glucose was not completely converted as 9.7 g/L glucose was still present in the fermentation broth (conversion 0.96 g/g). In addition, glycerol was the main byproducts with a concentration of 16.6 g/L and acetoin 6.8 g/L.

In order to better understand the effect of oxygen transfer on the 2,3-BDO production process, Table 4.12 **Comparison between fedbach fermentations and batch fermentation** shows data

on product concentrations and fermentation performances for all the fermentations in which the concentration of 2,3-BDO is the maximum. This data was compared with the optimized batch fermentation in order to understand the fed batch performance increase compared to a batch.

Table 4.12 Comparison between fedbach fermentations and batch fermentation, *batch experiment is referred to the experiment at 460 rpm e 0.1 vvm

		FB1	**FB2**	**FB3**	**FB4**	**Batch***
Time	hours	76,63	50,07	56,50	56,50	22
residual Glucose	g/L	0,24	2,07	23,26	9.8	0,00
Glycerol	g/L	14,84	9,91	11,45	16.6	1,2
Acetoin	g/L	4,74	8,31	4,15	6.8	2,11
2,3-BDO	g/L	99,99	87,08	101,27	104.5	19,7
Biomass	g/L	15,08	18,46	15,87	17.5	6,4
Conversion	g/g	1,00	0,99	0,90	0.96	1,00
Yield	g/g	0,48	0,50	0,45	0.48	0,47
Productivity	g/L/h	1,30	1,74	1,80	1.8	0,92

Regarding the fermentation at constant k_La, the concentration of biomass is greater probably due to increased oxygen availability. The fermentation yield is similar in all the four fermentations, while the effect of the fermentation mode is more prominent on the productivity. While apparently, the biggest effect is on the productivity, indeed, it has an overall productivity increase of 1,3 g/L/h in the experiment FB1 at 1,74, 1,80 and 1,80 g/L/h respectively in FB2, FB3 and FB4. In comparison with the batch fermentation, fed batch fermentations are comparable in terms of yield. In fact, batch yield was 0.47 g/g, which is comparable with that obtained in fed batch fermentations, while there is a remarkable increase in concentration of 2,3-BDO product by reaching up to a concentration of 104.5 g/L and an increase in global productivity doubling from 0.92 to 1.80 g/L/h.

Furthermore, to better understand the effect of the two operating modes (constant agitation and aeration or constant *kLa*), a comparison of the performance of the fermentations in

generative terms (g/h) was made rather than considering the absolute speeds (g/L/h) that are affected by the increase of volume of medium due to feeding.

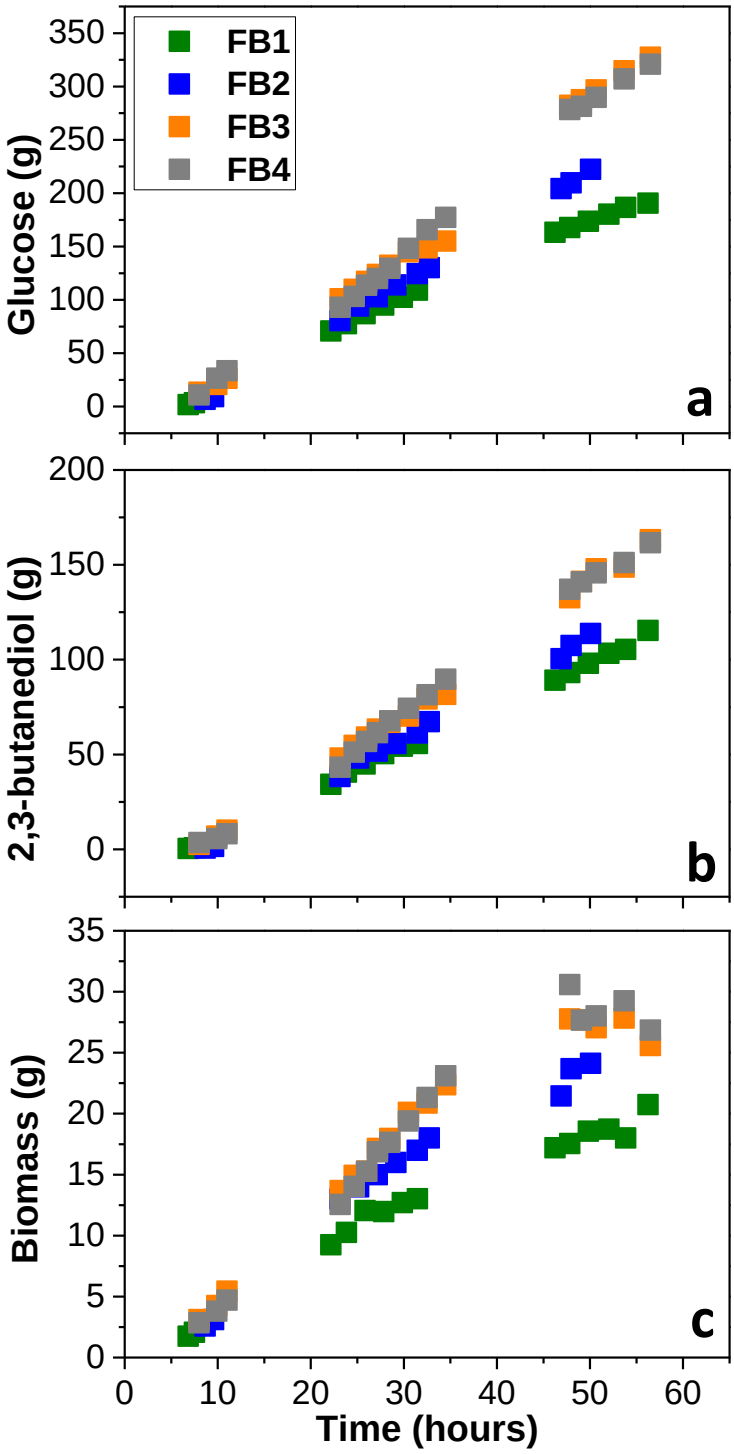

Figure 4.21 Comparison of the fedbatch fermentations in mass a) glucose, b) 2,3-butanediol and c) biomass. Experiments: Green FB1, Blu FB2, Grey FB3 and Orange FB4

Figure 4.21 shows the trend of glucose consumption (4.21a), 2,3-butanediol production (4.21b) and biomass production (4.21c) in terms of mass in the four fedbatch experiments. The green line in the pictures corresponds to the fermentation FB1 which was conducted at constant aeration and agitation. It is possible to observe that its slope is lower than experiments FB2, FB3 and FB4, a behavior achieved thanks to different aeration strategies. In fact, the increase in volume affect the oxygen transfer rate and consequently the fermentation performances, and therefore fermentations at constant k_La have a higher slope.

4.5. Production of 2,3-butanediol with alternative organic nitrogen source and carbon source

In the present paragraph, the replacement of the organic nitrogen source used for the optimization of the 2,3-butandediol fermentation process is evaluated. Furthermore, the replacement of the carbon source used for the optimization of the process with molasses, which was the biowaste with the best performances for the production of 2,3-butanediol (chapter 4.2), has been assayed.

4.5.1. Flask experiments

The aim was to verify the possibility to substitute completely the complex medium (Beef extract and Peptone) and the Carbon source (glucose), for this reason, three medium were compared:

- Complex medium (COM): is the medium used for the process optimization, described in Table 3.4 of materials and methods;
- Mineral salt medium (MSM): medium completely avoid of organic nitrogen whom composition is described inTable 3.5 of materials and methods;
- chicken meat and bone meal (CMBM): this is the medium originating from the rendering of animal byproducts, whose composition is described in materials and methods. The concentration used of CMBM for the experimentation was equal to 30 g/L, so as to maintain the total protein concentration of the batch tests previously conducted (based on the proteins calibration, chapter 3.6.3).

Each medium has been tested on two substrates (glucose GLU and molasses MOL). The substrates were tested at two distinct initial sugars concentrations (20 and 40 g / L). Table 4.13 shows the experimental scheme of the flask experiments with different medium and substrate.

Table 4.13 Flask tests with different medium and substrate: experimental scheme

Test	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
Substrate	GLU	GLU	MEL	MEL	GLU	GLU	MEL	MEL	GLU	GLU	MEL	MEL
Conc subs	20	40	20	40	20	40	20	40	20	40	20	40
Media	MSM	MSM	MSM	MSM	COM	COM	COM	COM	CMBM	CMBM	CMBM	CMBM

Figure 4.22 shows the comparison of yields and productivity obtained in the batch flask experiments. It can be seen that the tests with worse results, in terms of yield and productivity, are those in which the MSM is used (experiments M1, M2, M3 and M4), confirming the fact that organic nitrogen sources positively influences the production of 2,3-BDO.

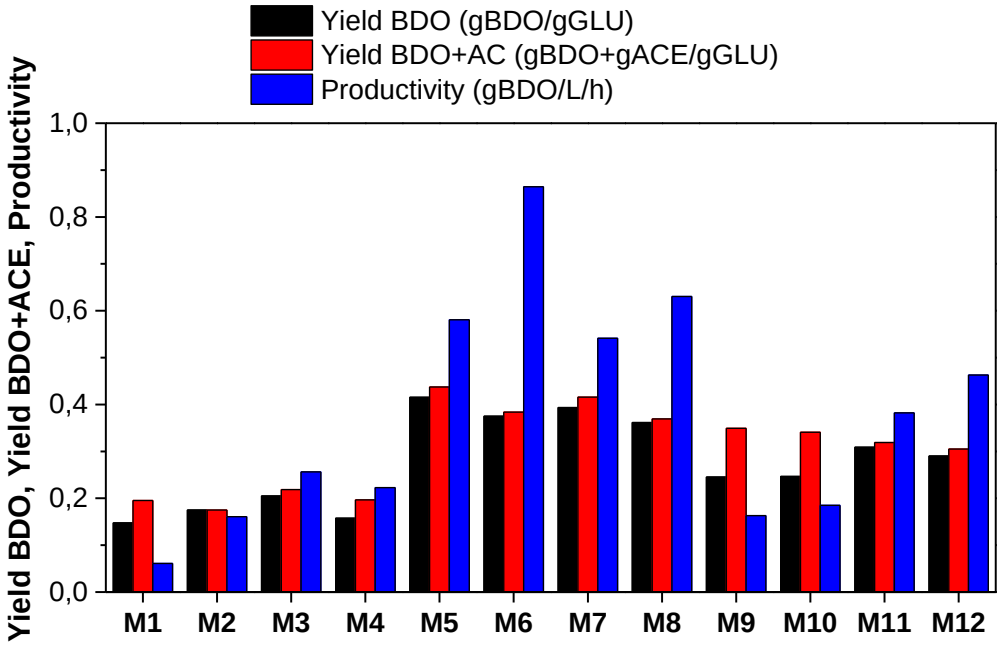

Figure 4.22 Comparison of yields and productivity obtained from the batch flasks experiments at different medium and substrate.

The best performance was obtained, as expected, with the complex medium (M5, M6, M7 and M8) which is the one used for the whole research period.

Regarding the medium coming from animal residues, performances are still lower compared to the complex medium, especially the yields are slightly lower than those obtained with the complex medium. Indeed, looking at Figure 4.22, the yield of acetoin + butanediol (red bars), in the experiments with the CMBM (M9, M10, M11 and M12) are around 30%, that although can be considered a good result considering that to have only 2,3-BDO it would be enough to modify the aeration conditions which is difficult to control in flask. On the other hand, unexpectedly, the results of the tests with molasses and flour (M11 and M12) have better performances than the respective ones with flour and glucose (M9 and M10), although the molasses is a less valuable substrate. This behaviour can be attributed to the components contained in the molasses that does not occur in glucose. However, meat and bone meal could be considered a good substitute to the standard commercial sources of organic nitrogen. A more accurate control of the aeration conditions with this new culture medium could improve the fermentations performances.

4.5.2. Bioreactor experiments

The tests with the CMBM medium and molasses as substrates were repeated in bioreactor to investigate further the possibility of using the meat and bone meal in place of the complex media. In particular, experiments at different OTR have been carried out. The range of OTR considered was close to the one evaluated in the paragraph 4.3.2.2 for the complex medium and glucose. Table 4.14 shows the experimental scheme of the tests carried on. These tests were conducted with a concentration of chicken meat and bone meal and molasses equal to the flask experiment M12 so as to have a concentration of 30 g/L of CMBM (equal to the content of proteins in the experiments with the standard medium) and a concentration of molasses of 74 g/L, so as to maintain the total sugars concentration equal to 40 g/L.

Table 4.14 Bioreactor tests at different medium and substrate: experimental scheme

Test	Type of medium	Agitation rpm	Aeration Vvm
CM1	Molasses + CMBM	405	0.5
CM2	Molasses + CMBM	460	0.1
CM3	Molasses + CMBM	500	0.1

The *OTR* and the 2,3-BDO production performance parameters obtained in these tests are compared in Table 4.15 with the corresponding average values obtained in the standard medium tests with *OTR* values in the optimal range (6-8 mmol/h/L).

Table 4.15 Oxygen transfer rate and 2,3-BDO production performances obtained in the byproduct tests, and corresponding average values obtained in the standard medium tests with *OTR* values in the 6-8 mmol/h/L range

Test	k_La 1/s	OTR (mmol/h/L)	Conversion (gSUG/gSUG)	Y_{BD} (gBD/gSUG)	Y_{BD+AC} (gBD+AC/gSUG)	P_{BD} (g/h/L)
CM1	0.0058	4.3	0.84	0.22	0.26	0.54 ± 0.05
CM2	0.0082	6.1	0.87	0.25	0.27	0.42 ± 0.04
CM3	0.0132	9.8	0.81	0.20	0.26	0.43 ± 0.04
Standard medium tests in the optimal OTR range[b]		6 - 8	1	0.44[b]	0.44[b]	0.91b

[b] Average value obtained in the standard medium tests characterized by an *OTR* in the 6-8 mmol/h/L range.
SUG sum of the sugars

All the three experiments have not reached the complete sugar conversion at the end of the experiments as shown in column 4 Table 4.15 (conversion 0.85 g/g) thus probably, when glucose is completely converted, the performances could be little higher than that showed in Table 4.15. However, comparing the results with that obtained with the standard medium, in all the

experiments, the 2,3-BDO yield varied in the 0.20-0.25 g/g range (45-55% decrease in comparison with the optimal tests), the productivity was equal to 0.42-0.54 g/h/L range (50% decrease) furthermore in column 6 of Table 4.15 the yield BD+AC is shown, these values are slightly higher than the BD yield only, which means that the aeration considered in these experiments could be optimized for the new medium. In conclusion, the lower performances obtained in such tests could be ascribed for sure to the different types of carbohydrates and proteins utilized, but also to the lower availability of oxygen. Anyway in these tests, for the first time, meat and bone meal are used as culture medium instead of commercial products (Beef extract, yeast extract, peptone from soybean, …).

5. Conclusions

The research work developed during the present PhD thesis on the optimization of a fermentative process for the production of 2,3-butanediol, has led to the following conclusions:

Microorganism preselection and evaluation of the growth and production conditions:

- the microorganism *Bacillus licheniformis* ATCC9789 has been identified and acquired. According to a bibliographic research, this microorganism is not pathogenic and it is able to produce 2,3-BDO with good performances;
- a medium rich in organic nitrogen sources (Beef extract an Peptone from soybean) in which the microorganism is able to grow and produce 2,3-BDO, has been selected.

Evaluation of the possibility of producing 2,3-BDO from different sugars and biowastes:

- hexoses and the disaccharide sucrose are used as carbon and energy sources and fermented into 2,3-BDO, while pentoses cannot be metabolized by the microorganism considered;
- Cheese whey could be potentially partially converted in 2,3-BDO only after enzymatic hydrolysis of lactose in glucose and galactose;
- Molasses represent an interesting feedstock for the production of 2,3-BDO with the selected strain.

Optimization of the oxygen supply conditions:

- DoE application for the prediction of the optimal agitation speed and aeration was successfully applied to the microaerobic production of 2,3-BDO, leading to the identification of an agitation aeration combination able to maximize 2,3-BDO productivity and yield close to the theoretical one (0.5 g/g), and minimize the byproducts concentration as well;
- The best fermentation performances can be obtained thanks to the application of an empirical approach which allowed for the identification of a range of OTR values that can be used for scale-up purposes. =

Study of the fed batch operation mode:

- the effect of the concentration of the carbon source (Glucose) and the organic nitrogen sources allowed for the identification of the inhibitory concentrations to the process;

- *OTR*, the parameter that governs the process, is controlled by the k_La. Fed-batch experiments, conducted keeping constant the k_La, allowed the production of 104 g/L of 2,3-BDO with high productivity and a yield close to the theoretical one;

Replacement of the complex media:

- the replacement of expensive organic nitrogen and carbon sources with meat and bone meal (byproduct of the meat production industry never tested before for 2,3-BDO production), and molasses respectively, has led to lower performances than those obtained with glucose and the complex medium. However, the results obtained could be considered more promising since they were achieved from biowastes.

In conclusion, the biotechnological production of 2,3-BDO at high concentration, yield and productivity, was obtained with a non-pathogenic microorganism, under optimized oxygen supply conditions, and in fed-batch mode. Furthermore, the possibility of producing 2,3-BDO starting from biowastes, resulting from agroindustrial processes, was evaluated. Future works should be focused on the optimization of the fermentation performances from biowastes and on the economic analysis of the process.

6. International exchange research period: Complutense University of Madrid

The activities and the results discussed in this section were obtained as part of the period of study at the Department of Chemical Engineering of the Universidad Complutense de Madrid (Spain) under the supervisor of the professor Felix Garcia-Ochoa. The research group guided by professor Garcia-Ochoa has an expertise in bioreactor oxygen transfer and kinetic modelling of biochemical reactions. For this reason, the opportunity to take part in said research group has had a fruitful impact as it was relevant and complementary to my Ph.D. project.

The activities undertake at the research group concerned the development of a kinetic model able to describe the 2,3-butanediol production process from glucose with the microorganism *Bacillus licheniformis* ATCC 9789 at different Oxygen transfer rate conditions. In fact, this activity is complementary with the experimentation carried out in Chapter 4.3.

6.1. Introduction

Mathematical modeling of bioprocesses is a useful tool in the field of industrial biotechnology. Due to the complexity of the microbial metabolism, in which a large number of reactions and regulatory mechanisms are involved, there is a growing interest for the development of mathematical models able to understand, predict and optimize properties and behaviors of biological systems. Essentially, the purpose of a kinetic model is to describe those aspects of the cell that are involved in the biotechnological process of interest (Almquist et al., 2014).

A kinetic model consists in the definition of equations that correlate rates and concentrations of products and reagents that when integrated into a material balance, allow the prediction of the substrates conversion and products performance at the operating conditions (Gombert and Nielsen, n.d.).

The aim of the present chapter is to present the development of a kinetic model for the production of 2,3-butanediol from glucose by *Bacillus licheniformis* ATCC 9789 that can describe the evolution of biomass, glucose, 2,3-butandediol, acetoin and glycerol concentration at different oxygen uptake rates.

6.2. Methodology and mathematical methods

Basically, the purpose of a kinetic model is to describe the cellular behavior involved in the biotechnology process of interest. A kinetic model consists in the definition of equations that correlate the evolution rate and concentrations of all the compounds involved in the process under specific operating conditions. The development of a kinetic model can be divided into the following phases:

1) Kinetic model formulation: in this stage, the system of reactions which describes the considered bioprocess and the kinetic equations describing each reaction is hypothesized. The kinetic equations could come from phenomenological hypotheses or empirical descriptions. Starting from the reaction scheme and kinetic equations, the equation of production or consumption of each compound is build;

2) Model parameter estimation: this stage consists in the determination of the kinetic parameters and the stoichiometric coefficients. In this stage, the kinetic model will be adjusted to the experimental data in order to obtain the parameters of the hypothesized kinetic equations. In this work, the numerical integration of the system of equations of each compound has been carried out using the Euler implicit method, and NL2SOL (*non-linear least-squares solver*) for the minimization of the residual sum of squares.

3) Model validation: the parameters obtained from the previous point must be validated by means of physical criteria, and the application of statistical parameters. In this work, we have considered the following statistical parameters:

- Residual Sum of Squares (RSS). It refers to the difference between the experimental value and that predicted by the model for each response. The lower the value of this parameter, the better the adjustment of the model to the experimental data, therefore it is desirable to minimize its value.

$$\boldsymbol{RSS} = \sum_{i=1}^{n} (\boldsymbol{y}_{exp} - \boldsymbol{y}_{pred})^2 \qquad (22)$$

- Residual Mean Squared Error (RMSE): a measure of the difference between the predicted values of the variable and the observed ones. It takes into account the total amount of data (N) and the number of model parameters (P), as shown in equation 23

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(y_{exp}-y_{pred})^2}{N-P}} \quad (23)$$

- Variation explained (VE): this parameter explains the goodness of the model to predict trends.

$$VE\ (\%) = 100 \cdot \left(1 - \frac{\sum_{i=1}^{N}(y_{exp}-y_{calc})^2}{\sum_{i=1}^{N}(y_{exp}-\overline{y_{exp}})^2}\right) \quad (24)$$

- F-fisher: this parameter suggests the goodness of each model. The higher the value of the mentioned parameter, the better the fitting, and the model is more representative. F-fisher could be calculated as follow:

$$F-fisher = \left.\sum_{1=1}^{N}\left(\frac{y_{i,calc}}{M}\right)^2\right/\sum_{i=1}^{N}\frac{SSR}{N-M} \quad (25)$$

The model parameter estimation and validation has been carried out employing the software *Aspen Custom Modeler ®*.

6.1. Results

In order to formulate the kinetic model, it is first of all necessary to suggest a reaction scheme for the species involved in the bioprocess. As described in Figure 1.6, there is a huge number of species and reactions involved in the production of 2,3-butanediol from glucose by the microrganism *Bacillus licheniformis*. Due to the complexity of the biological system, it is necessary for the proposed reaction scheme to be simplified.

Thus, the first stage is the identification of the main species involved in the studied bioprocess. The present chapter has considered eight of the experiments carried out in chapter 4.3 and shown in Table 4.6. The experiments considered are the O1, O2, O3, O4, O5, O6, O7 and O8. The

results obtained in chapter 4.3, i.e., the production of 2,3-butanediol from glucose using *Bacillus licheniformis* ATCC-9789 at 30°C, pH 6 and microaerobic conditions (oxygen concentration in the broth equal to zero, oxygen transfer rate = oxygen uptake rate), have led mainly to the production of three compounds: 2,3-butanediol, acetoin and glycerol. Undoubtedly , the biomass as well as the substrates (glucose and the complex media) should be considered.

The proposal of a reactions scheme of a fermentative process should consider the pathway of the microorganism, and each reaction should be obtained from experimental or theoretical considerations. Figure 6.1 demonstrates three fermentations in order to better understand the microorganism's behavior and help to implement the reaction model.

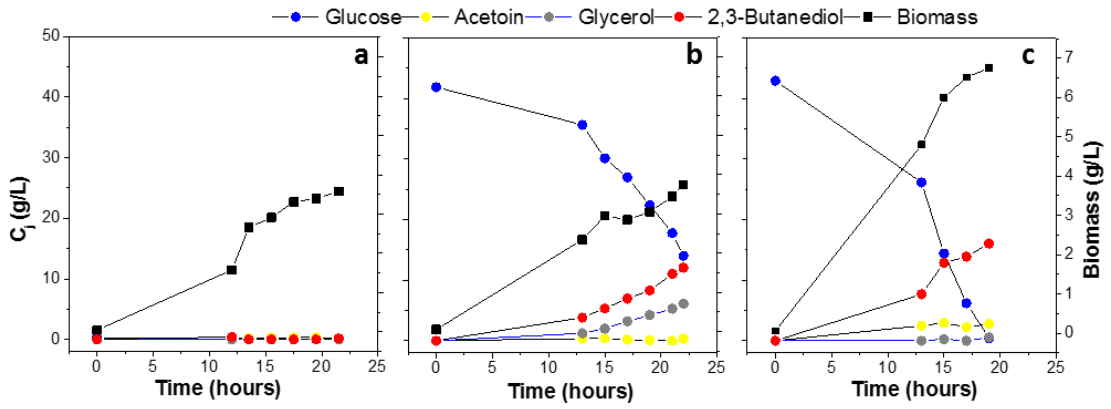

Figure 6.1 Evolution of the main anilities in three bioreactor fermentations: a) Fermentation carried out at 460 rpm and 0.1 vvm in a medium composed by complex media without glucose, b) fermentation O1 (Table 4.6 Fermentation results of the CCD tests at the complete glucose conversion *average of experiments in triplicatecarried out at 250 rpm, 0,1 vvm and c) fermentation O7 (Table 4.6 Fermentation results of the CCD tests at the complete glucose conversion *average of experiments in triplicatecarried out at 500rmp 0,1 vvm.

The graph in Figure 6.1a shows the evolution of the main anilities in a fermentation conducted without glucose, in the sole presence of the complex media. The complex media (described in Table 3.4 materials & methods) is mainly composed by beef extract and peptone which are rich in proteins, amino acids, vitamins and growth factor which is useful for the microbial growth and 2,3-butanediol production. In this experiment, only the biomass is produced, at the concentration of 3.7 g/L after 22 hours, whereas no production of 2,3-butanediol or by products has been detected. This fact means that the microorganism is able to grow on the complex medium without glucose and that it is able to use the organic nitrogen source as carbon and

energy sources. However, this consideration is not sufficient to argue that the microorganism does not use glucose, when present, for its growth. Indeed, observing Figure 6.1 b and c (experiments O1 and O7 Table 4.6 respectively) the concentration of biomass reached higher values than in the experiment of Figure 6.1 a (3,8 and 6,8 g/L in O1 and O7 respectively). Furthermore, in experiments O1 and O7, 2,3-BDO, acetoin and glycerol were produced as well as biomasses. Thus, the biomass can be produced thanks to both substrates: glucose and complex media. In order to better understand which substrate is mainly involved in the production of the biomass and the biomass yield from both the substrates, more focused experiments should be carried out . Furthermore, the carbon balance during the fermentation could help in the phenomenological description. However, in this chapter as a first approach to the process modeling of the yield biomass complex media has been approximated. Considering the fermentation in figure 9.1a in which biomass is produced from the organic nitrogen sources, it is possible to calculate the biomass yield assuming that all the substrate is consumed for the biomass production. Considering that the complex media is composed by 20 g/L of organic nitrogen (10 g/L beef extract and 10 g/L peptone), the biomass yield is 0.18 g of biomass per g of organic nitrogen.

From the considerations made thus far, the reaction that describe the production of biomass could be written as follow:

$$\upsilon_{X/S} \cdot S + \upsilon_{X/G} \cdot G \xrightarrow{r_1} X \qquad (26)$$

where, $\upsilon_{X/S}$ and $\upsilon_{X/G}$ are the biomass (X) production stoichiometric coefficients from the complex media (S) and glucose (G) respectively, and r_1 is the reaction rate for the production of biomass. Since that biomass is produced mainly from the complex media, according to the previous considerations, the $\upsilon_{X/S}$ coefficient has been fixed at 6 g/g (being $Y_{S/X}$ = 0.18 g/g).

Regarding the production of 2,3-butanediol and by-products, these are strictly chained to the metabolic pathway. In this case, in order to simplify the reaction model, some observations and simplifications have been taken into account. The metabolic products of the *Bacillus licheniformis* metabolism are several, however, observing Figure 6.1 b, c and Table 4.6 in chapter 0, the products involved at the conditions considered are glycerol, 2,3-butanediol, and acetoin. The pathway involved in the production of 2,3-butanediol starts from pyruvate and involves acetoin which is the precursor for the production of 2,3-butanediol. The reaction

between acetoin and 2,3-butanediol is an equilibrium between them, in particular, 2,3-butanediol is produced from acetoin. Such reaction is reversible, in fact, when the glucose is finished, 2,3-butanediol is reduced to acetoin. Furthermore,in aerobic or close to aerobic conditions, acetoin is produced , since the reaction from acetoin to 2,3-BDO is inhibited by the presence of oxygen (Xiao Jun Ji et al., 2011). This behavior is observable in Table 4.6 experiment O9 which was conducted under compleately aerobic conditions. In this case, in order to simplify the reaction scheme, 2,3-butanediol and acetoin have been considered as two separate products and the reactions that characterize them are shown below

$$\upsilon_{ACE/G} \cdot G \xrightarrow{r_2} ACE \tag{27}$$

$$\upsilon_{BDO/G} \cdot G \xrightarrow{r_3} 2{,}3 - BDO \tag{28}$$

where r_2 and r_3 are the reaction rate for the production of acetoin (ACE) and 2,3-BDO respectively. Glycerol is produced directly from glucose as a ramification of the glycolysis (Figure 1.6) and its reaction could be written as follow:

$$\upsilon_{GLY/G} \cdot G \xrightarrow{r_4} GLY \tag{29}$$

in which r_4 is the reaction rate for the production of glycerol (GLY). The kinetic equations proposed to describe the reaction speed of each of the reactions proposed in the kinetic model are the following:

$$r_1\left({}^{g_X}/_{L*h}\right) = \mu \cdot C_X \cdot \left(1 - \frac{C_X}{C_X^{max}}\right) \tag{30}$$

$$r_2\left({}^{g_A}/_{L*h}\right) = K_{GA} \cdot C_G \cdot C_X \tag{31}$$

$$r_3\left({}^{g_P}/_{L*h}\right) = K_{GP} \cdot C_G \cdot C_X \tag{32}$$

$$r_4\left({}^{g_{GLY}}/_{L*h}\right) = K_{GGLY} \cdot C_G \cdot C_X \tag{33}$$

The microbial growth rate has been modeled according to the logistic equation, where μ is the specific growth rate, C_X is the concentration of biomass and C_X^{max} maximum cell concentration. The kinetic equations r_2 , r_3 and r_4 have been described by equations 32, 33, 34. Where k_{GA}, k_{GP} and k_{GGLY} are the respective kinetic constants. The model in the form of production rates is expressed by the following equations:

$$R_X\left({}^{g_X}/_{L*h}\right) = {}^{dC_X}/_{dt} = r_1 \quad (34)$$

$$R_G\left({}^{g_G}/_{L*h}\right) = {}^{dC_G}/_{dt} = -\upsilon_{G/X} \cdot r_1 - \upsilon_{G/BDO} \cdot r_2 - \upsilon_{G/ACE} \cdot r_3 - \upsilon_{G/GLY} \cdot r_4 \quad (35)$$

$$R_S\left({}^{g_S}/_{L*h}\right) = {}^{dC_S}/_{dt} = -\upsilon_{S/X} \cdot r_1 \quad (36)$$

$$R_P\left({}^{g_P}/_{L*h}\right) = {}^{dC_P}/_{dt} = r_2 \quad (37)$$

$$R_A\left({}^{g_A}/_{L*h}\right) = {}^{dC_A}/_{dt} = r_3 \quad (38)$$

$$R_{GLY}\left({}^{g_{GLY}}/_{L*h}\right) = {}^{dC_{GLY}}/_{dt} = r_4 \quad (39)$$

Including the expressions that describe the reaction rates, the kinetic model that was going to be used for the description of the production of 2,3-BDO from glucose using the microorganism *Bacillus licheniformis* as biocatalyst at different oxygen supply, can be expressed by the following equations:

$${}^{dC_X}/_{dt}\left({}^{g_X}/_{L*h}\right) = \mu \cdot C_X \cdot \left(1 - \frac{C_X}{C_X^{max}}\right) \quad (40)$$

$${}^{dC_G}/_{dt}\left({}^{g_G}/_{L*h}\right) = -\upsilon_{\frac{G}{X}} \cdot \mu \cdot C_X \cdot \left(1 - \frac{C_X}{C_X^{max}}\right) - \upsilon_{\frac{G}{BDO}} \cdot K_{GA} \cdot C_G \cdot C_X - \upsilon_{G/ACE} \cdot r_3 - \upsilon_{G/GLY} \cdot r_4 \quad (41)$$

$${}^{dC_S}/_{dt}\left({}^{g_S}/_{L*h}\right) = -\upsilon_{S/X} \cdot \mu \cdot C_X \cdot \left(1 - \frac{C_X}{C_X^{max}}\right) \quad (42)$$

$${}^{dC_P}/_{dt}\left({}^{g_P}/_{L*h}\right) = K_{GA} \cdot C_G \cdot C_X \quad (43)$$

$${}^{dC_A}/_{dt}\left({}^{g_A}/_{L*h}\right) = K_{GP} \cdot C_G \cdot C_X \quad (44)$$

$${}^{dC_{GLY}}/_{dt}\left({}^{g_{GLY}}/_{L*h}\right) = K_{GGLY} \cdot C_G \cdot C_X \quad (45)$$

Once the kinetic model is established, the differential equations representing the production or consumption rate of the species involved in the metabolism (selected previously) are adjusted to experimental data in order to obtain the kinetic parameters of the hypothesized kinetic equations. In this work the experimental data are the batch experiments conducted at different oxygen supply whom results are shown in Table 4.6.

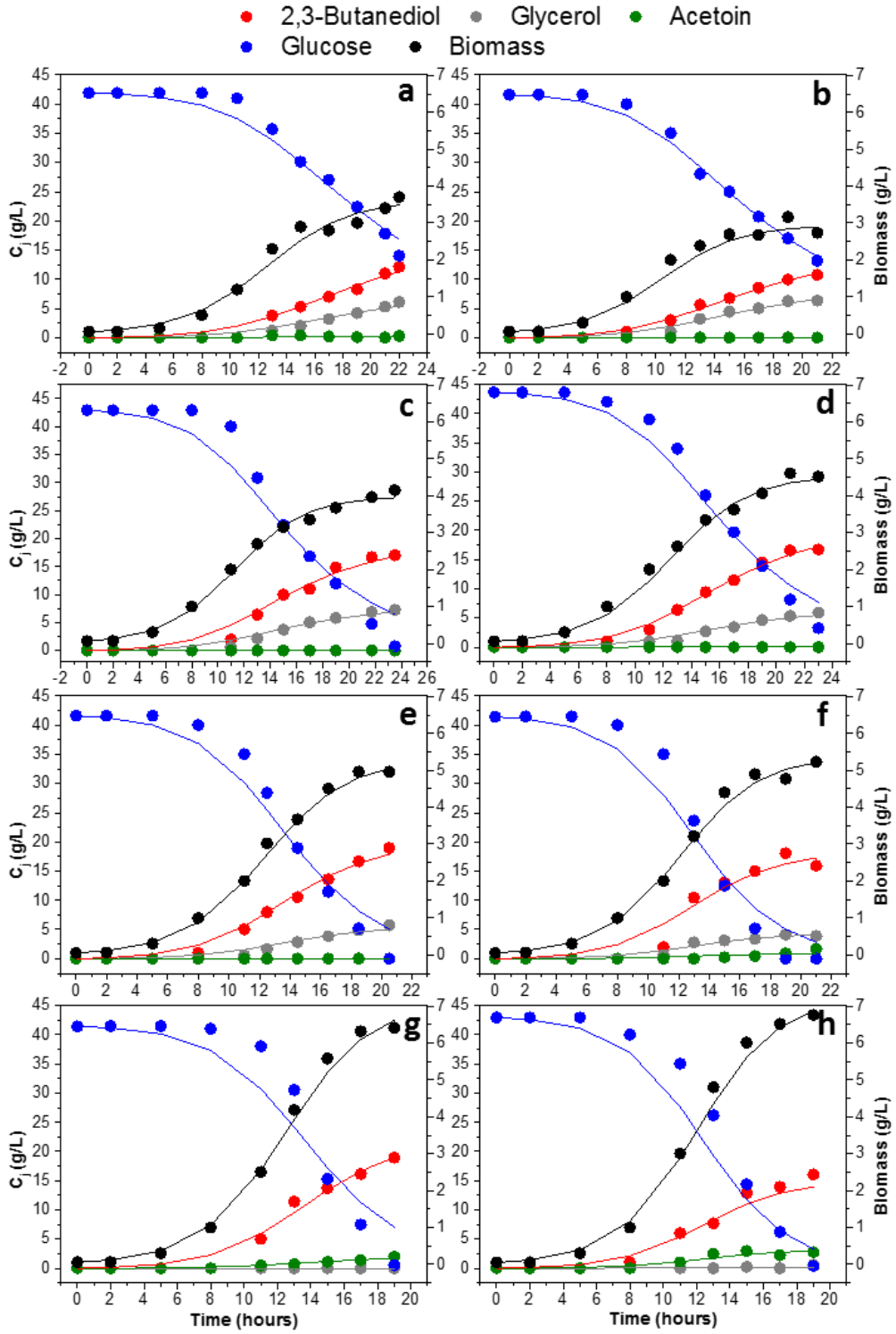

Figure 6.2 Experimental data (points) and kinetic model predictions (curves) for the 2,3-BDO production process by *B.licheniformis* using different oxygen supply conditions (see Figure 4.9). Data points: Glucose (blue), glycerol (grey), biomass (black), 2,3-BDO (red), Acetoin (green). Experiments: O1 (a), O2 (b), O3 (c), O4 (d), O5 (e), O6 (f), O7 (g), O8 (h),

In Figure 6.2 where the results of the fitting are shown, it is possible to note that in each fermentation the experimental data (points) is well predicted by the model fitting (lines). In fact, the goodness of the fitting and the statistical parameters are also shown in Figure 6.2.

The model parameter values obtained after the fitting of all the experiments considered are shown in Table 6.2 (statistical parameters) and Table 6.1 (kinetic parameters and coefficients).

As it can be seen in Table 6.2, the F-fisher values are high enough to denote the significance of the model. RMSE and SSR values were acceptable in all fittings, considering the high number of available experimental data. The values of VE are all closed to 100%, this indicates the goodness of the fittings and further reinforces the validity of the proposed kinetic model.

Table 6.1 Kinetic model parameter values obtained from the experimental data fitting for the different experiments.

Exp	OTR	μ	Xm	k_{GA}	k_{GLY}	k_{GP}	v_{GA}	v_{GGLY}	v_{GP}
	mmol/L/h	h-1	g	L/g/s	L/g/s	L/g/s	g/g	g/g	g/g
O1	1.4	0.34	3.63	0.0003	0.0057	0.0119	0.0000	1.2250	1.6197
O2	1.9	0.38	2.94	0.0000	0.0084	0.0140	0.3405	1.1676	1.8007
O3	2.8	0.37	3.98	0.0000	0.0074	0.0179	0.0000	1.4869	1.5539
O4	2.8	0.36	4.55	0.0002	0.0055	0.0173	0.0000	1.5582	1.5827
O5	4.7	0.37	5.29	0.0000	0.0066	0.0236	0.0000	0.8693	1.7930
O6	7.1	0.38	5.38	0.0013	0.0059	0.0249	0.0000	0.5257	2.1107
O7	7.6	0.39	7.12	0.0018	0.0000	0.0205	0.2181	0.0000	1.7892
O8	16.6	0.41	7.25	0.0038	0.0003	0.0176	2.2317	0.0000	2.3542

Table 6.2 Statistical parameters of the kinetic model proposed for the production of 2,3-BDO from glucose at different oxygen supply conditions.

Exp	OTR mmol/L/h	F-fisher	RMSE	SSR	VE (%)
O1	1.4	2172.9	0.85	39.73	97.22
O2	1.9	4923.6	0.51	13.18	99.02
O3	2.8	777.9	1.61	142.30	95.74
O4	2.8	1287.9	1.08	63.72	97.81
O5	4.7	677.9	1.33	88.31	97.00
O6	7.1	359.5	1.76	154.20	95.64
O7	7.6	286.6	2.00	180.00	93.34
O8	16.6	24.73	7.17	143.40	94.72

The kinetic parameters demonstrated in Table 6.2 refer to the hypostasized kinetic equation system. Observing the kinetic parameters of the logistic equation (equation 30) in Table 6.1 that characterize the microbial growth, the specific growth rate (μ) and the maximum biomass concentration (Xm) increase with the OTR, such a behavior indicates that the microbial growth is greater at higher oxygen supply.

Concerning the parameters related to the products and by products formation, and taking into consideration Table 6.1, this case as well represents a clear relation with the oxygen supply. The kga, glucose acetoin kinetic parameter (equation 31), increased at high OTR, contrariwise, the kggly, glucose glycerol kinetic parameter (equation 33), was higher at low OTR. The 2,3-BDO kinetic constant (kgp, equation 32) showed a different trend, whereas it has a maximum at a OTR equal to 0.009 s-1 instead. Similarly to the kinetic constants, the stoichiometric coefficients (ν) are characterized by the same trends.

The behavior showed in these paragraphs can be observed as well in paragraph 4.3 (Effect of the oxygen mass transfer rate on the production of 2,3-butanediol). In fact, the considerations that were made are strictly linked to the microbial metabolism in which different yields and productivities at different OTR have been observed.

6.2. Conclusions

The proposed model is able to simulate the experimental data obtained from eight of the experiments that were conducted at different OTR conditions. The results obtained confirm that the production of 2,3-BDO is influenced by the oxygen transfer rate. However, the application of a more detailed equation system involving both side reactions and a more accurate determination of the complex medium on the microbial growth end 2,3-BDO production should be carried out.

Furthermore, next activities should aim to the determine each of the kinetic and stoichiometric parameters, and empirical equations of the oxygen transfer rate. The determination of these equations could allow to predict the fermentation performances at different OTR in order to control the process.

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