University of Minho
School of Engineering

Ana Margarida Magalhães Ribeiro

Erythritol production from crude glycerol by *Yarrowia* species: strains comparison and oxygen influence

Thesis submitted in fulfilment of the requirements for Master's degree in Biotechnology

Work developed under the guidance of:
Doctor Isabel Maria Pires Belo

January 2021

ACKNOWLEDGMENTS

Ao terminar mais um etapa importante da minha vida não posso deixar de agradecer a todas aquelas pessoas que de forma direta ou indireta ajudaram-me a chegar a bom porto ao fim destes dois anos. Assim deixo aqui os meus mais sinceros agradecimentos:

À minha orientadora, Doutora Isabel Belo que me acolheu no seu grupo, que me guiou e ajudou sempre em todas as etapas da dissertação, por me ter transmitido todo o conhecimento e sabedoria, por todo o apoio, motivação, orientação e carinho que ajudaram na concretização desta dissertação e por ter acreditado no meu trabalho.

À Ana Sofia Pereira que foi a minha maior ajuda no laboratório, por toda a disponibilidade e carinho que demonstrou, por todo o apoio prestado em todas as etapas da realização da dissertação, pela paciência que teve comigo e por contribuir para o sucesso deste trabalho.

À Doutora Patrícia Ferreira, que mesmo não estando no laboratório foi incansável e ajudou-me e apoio-me sempre que eu precisava.

A todo o grupo do laboratório de Bioprocessos (Sílvia M., Joana, Patrícia, Rita, Marta, Margarida, Sílvia F., Bruna D., Bruna F., Daniel, Paulina, Marlene e José) pelo fantástico ambiente de trabalho que me proporcionaram e por me terem ajudado sempre que precisava ou tinha alguma questão. Um agradecimento especial à Joana, à Sílvia M. e à Patrícia por me terem acompanhado mais de perto nesta jornada.

Aos meus amigos Ju, Vitor, Gi e Bia por todo o apoio incondicional ao longo destes anos, por me compreenderem melhor do que ninguém, por acreditarem sempre em mim e por todos os bons momentos que me proporcionaram.

À minha família pelo amor e apoio incondicional ao longo destes anos e sempre, por toda a paciência que têm para me aturarem mas sobretudo por nunca deixarem que eu desistisse dos meus sonhos e por serem o meu maior pilar.

À minha avó que sempre sonhou com este dia, que sempre me motivou a ser melhor, que me criou e viu crescer e que estará para sempre no meu coração.

"Chutando a sílaba im da palavra impossível,

qualquer pessoa terá a certeza de que consegue chegar mais à frente."

Robert Stephenson Smyth Baden-Powell

Para a minha avó Lindinha,

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ABSTRACT

The global emerge of metabolic disorders caused by excessive consumption of food with refined sugars triggered a demand for healthier options, such as low-calorie sweeteners. Erythritol is a natural sweetener with nearly 70 % of sucrose sweetness, that has no calories, is non-glycemic and is non-cariogenic. Industrially, this polyol is produced *via* fermentation from glucose by osmophilic/osmotolerant fungi. The production of erythritol by yeasts from *Yarrowia* genus can be affected by several parameters, such as yeast strain, operational conditions and dissolved oxygen concentration (DOC) in the medium. The main goal of the present work was the study of erythritol production by different yeast strains of *Yarrowia* genus, from crude glycerol derived from biodiesel production.

The performance of three strains of *Y. lipolytica* and two of *Y. divulgata* was compared in flasks batch culture. Erythritol and mannitol production, as well as, cellular growth and glycerol consumption were monitored for 7 days. *Y. lipolytica* species proved to be the most suitable for the production of erythritol, under the tested conditions, being the strains *Y. lipolytica* W29 (ATCC 20460) and *Y. lipolytica* Ch 3/4 the ones that produced the highest concentration (34 and 25 $g \cdot L^{-1}$), leading to highest yield (38 % w/w) and productivity of erythritol (0.20 and 0.15 $g \cdot L^{-1} h^{-1}$).

In a stirred tank reactor (STR), the effect of different operational conditions on $k_L a$ values were analysed. The highest experimental $k_L a$ value (162 h^{-1}) was observed in the experiment with the conditions of 3 vvm of aeration rate and 900 rpm of stirring rate. These conditions were found to be optimal for erythritol production and they also prevented the exhaustion of DOC in the medium. The scale-up of the bioprocess, with the strain W29 and the strain Ch 3/4 allowed a 2.4-fold and a 3.3-fold increase on productivity, respectively, reducing the operation time from 168 hours to only 72 hours. The erythritol concentration produced (35 $g \cdot L^{-1}$), the erythritol yield (40%, w/w) and the erythritol productivity (0.5 $g \cdot L^{-1} \cdot h^{-1}$) were approximately equal for both strains.

A stepwise fed-batch experiment with the addition of glycerol pulses was also performed as alternative of using high initial glycerol concentration in batch mode experiments, that was not successful. The stepwise fed-batch strategy allowed to reach almost the double of erythritol concentration for both strains, without decreasing global productivity. The best results of erythritol concentration (64 $g \cdot L^{-1}$), yield (42 % w/w) and productivity (0.45 $g \cdot L^{-1} \cdot h^{-1}$) were obtained in the experiments with *Y. lipolytica* W29.

KEYWORDS: Crude glycerol; Erythritol; *Yarrowia divulgata; Yarrowia lipolytica*.

RESUMO

O aparecimento global de distúrbios metabólicos causados pelo consumo excessivo de alimentos com açúcares refinados, levou a uma crescente procura por opções mais saudáveis, como é o caso dos adoçantes com baixas calorias. O eritritol é um adoçante natural com cerca de 70 % da doçura da sacarose, que não possui calorias, é não-glicémico e é não-cariogénico. Industrialmente, este poliól é produzido via fermentação a partir de glucose por fungos osmofílicos/osmotolerantes. A produção de eritritol por leveduras do género *Yarrowia* pode ser afetada por diversos parâmetros tais como a estirpe da levedura, as condições de operação e a concentração de oxigénio dissolvido no meio. O principal objetivo deste trabalho foi o estudo da produção de eritritol, por diferentes estirpes de levedura do género *Yarrowia*, a partir de glicerol bruto derivado da produção do biodiesel.

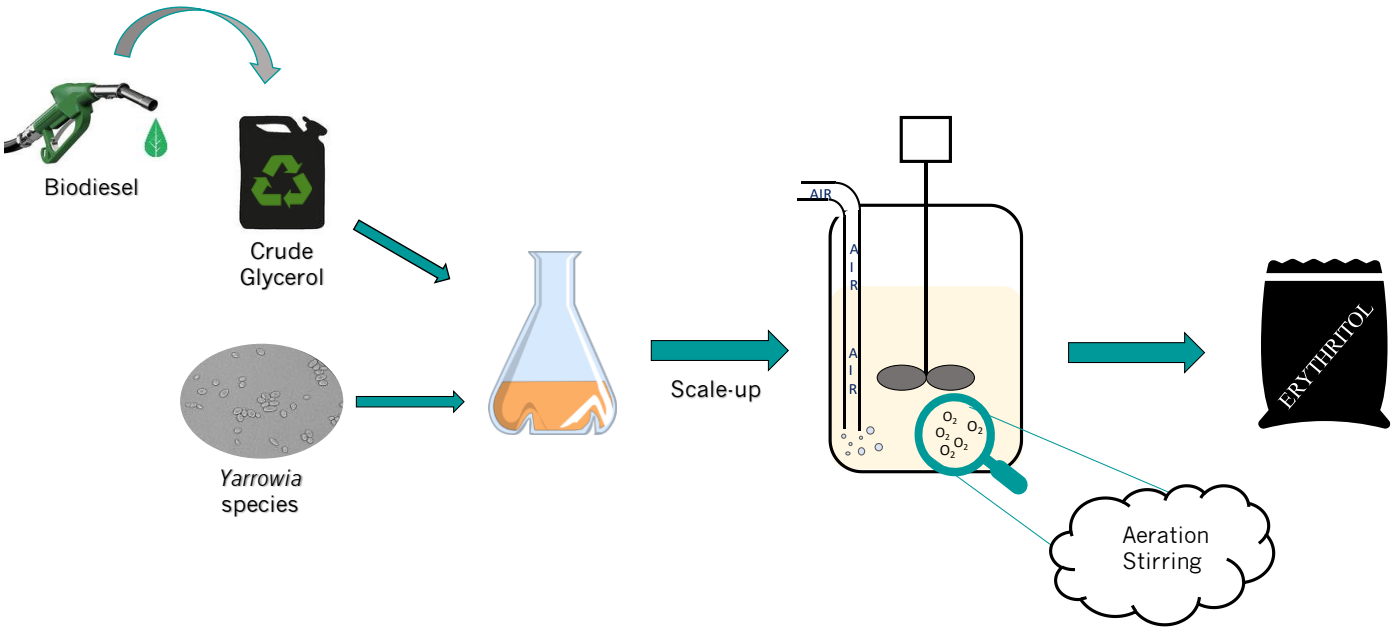
Em cultura descontínua em matraz foi comparado o desempenho de três estirpes de *Y. lipolytica* e de duas de *Y. divulgata*. A produção de eritritol e manitol, bem como o crescimento celular e o consumo de glicerol foram monitorizados durante 7 dias. A espécie *Y. lipolytica* foi a mais adequada para a produção de eritritol nas condições testadas, sendo as estirpes *Y. lipolytica* W29 (ATCC 20460) e *Y. lipolytica* Ch 3/4 aquelas que produziram uma maior concentração (34 e 25 g/L), rendimento (38 % p/p) e produtividade em eritritol (0,20 e 0,15 g/L/h).

Num reator do tipo tanque agitado foi analisado o efeito de diferentes condições de operação no valor experimental de k_La. O maior valor experimental de k_La (162 h^{-1}) foi observado no ensaio com as condições de taxa de arejamento de 3 vvm e taxa de agitação de 900 rpm. Estas condições foram consideradas as ideais para a produção de eritritol, prevenindo ainda o esgotamento da concentração de oxigénio dissolvido no meio. Com o aumento de escala do processo observou-se um aumento de cerca de 2,4 vezes para a estirpe W29 e de 3,3 vezes para a estirpe Ch 3/4, na produtividade, sendo o tempo de ensaio reduzido de 168 horas para apenas 72 horas. A concentração de eritritol produzida (35 g/L), o rendimento em eritritol (40 % p/p) e a produtividade em eritritol (0,5 g/L/h) foram aproximadamente iguais para ambas as estirpes.

Foram também realizados ensaios em modo semí-contínuo com a adição de um pulso de glicerol como alternativa ao uso de elevadas concentrações iniciais de glicerol bruto em ensaios em modo descontínuo, que não foi bem sucedido. A estratégia em modo semi-contínuo com pulso de glicerol permitiu que se atingisse quase o dobro da concentração de eritritol para ambas as estirpes, sem que se tenha observado uma diminuição da produtividade global. Os melhores resultados de concentração de eritritol (64 g/L), rendimento em eritritol (42 % p/p) e produtividade em eritritol (0,45 g/L/h) foram obtidos nos ensaios com a *Y. lipolytica* W29.

PALAVRAS-CHAVE: Eritritol; Glicerol Bruto; *Yarrowia divulgata; Yarrowia lipolytica*.

GRAPHICAL ABSTRACT



LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
C	
C:N	Carbon/Nitrogen ratio
CSL	Corn steep liquor
C	Dissolved oxygen concentration in the liquid phase
C*	Solubility of oxygen in the liquid phase
D	
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
DOC	Dissolved oxygen concentration
E	
E4PP	Erythrose 4-phosphate phosphatase
ER	Erythrose reductase
F	
FBA	Fructose-biphosphate aldolase
FDA	Food and Drug Administration
G	
GA3P	Glyceraldehyde-3-phosphate
GDH	Glycerol-3-phosphate dehydrogenase
GK	Glycerol kinase
GPI	Glucose-6-phosphate isomerase
GRAS	Generally recognized as safe
G6PDH	Glucose-6-phosphate dehydrogenase

H	
HK	Hexokinase
HPLC	High performance liquid chromatography
K	
kLa	Oxygen volumetric mass transfer coefficient
L	
LOD	Limit of detection
LOQ	Limit of quantification
N	
NADPH	Nicotinamide adenine dinucleotide phosphate
O	
OD	Optical density
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
P	
PEG	Polyethylene glycol
PFK	Phosphofructokinase
PGDH	Phosphogluconate dehydrogenase
PPP	Pentose phosphate pathway
Q	
Q_E	Erythritol productivity
R	
RNA	Ribonucleic acid
RPM	Revolutions per minute
RI	Refractive index

S	
STR	Stirred tank reactor
T	
TIM	Triosephosphate isomerase
TK	Transketolase
TAL	Transaldolase
V	
VVM	Volume of air *per* volume of medium *per* minute
Y	
$Y_{X/S}$	Biomass yield
$Y_{E/S}$	Erythritol yield
YPDA	Yeast extract, peptone, dextrose and agar medium
YPD	Yeast extract, peptone, glycerol medium
6	
6PGL	6-phosphonogluconolactonase

1. LITERATURE REVIEW

Erythritol is four-carbon sugar alcohol used by food industry as a natural nutritive sweetener. This polyol is mostly produced by fermentation from glucose, using osmophilic fungi, however its high retail price leads to a demand for production alternatives. Yeasts from the genus *Yarrowia*, namely *Yarrowia lipolytica*, are capable to produce erythritol from crude glycerol, a by-product of biodiesel industry. This production method can be a plausible and interesting alternative to the process currently used by the erythritol industry.

In this section, is emphasized erythritol properties and its production by yeasts of the genus *Yarrowia.* A brief overview on metabolic pathways involved in glycerol consumption and erythritol production is also presented, as well as, the effect that different culture conditions could have in erythritol production.

1.1. Sweeteners

Since the prehistoric times, the Human being developed a preference and desire for a sweet taste, using this to identify foods with high caloric and nutritional values [1]. The food sweetness is related with natural sugars that are presented, for example, in fruits, vegetables and honey [2]. Although natural sugar has been present since earliest times in human diet to provide energy, acting as the main source of carbon and energy for most of the cell types, nowadays, with the development of food industry there are an enormous diversity of products that contain high amounts of added sugars [3]. The refined sugar is obtained from sugar cane or high fructose corn syrup prepared from corn starch. This sugar added during the processing and preparation of the food, has commonly high energy/caloric value but a very low nutritional value [1,4]. The high consumption of food rich in refined sugar, observed in the last century, lead to an increase in metabolic disorders, such as overweight and obesity among children, teenagers and adults, cardiovascular disease, insulin resistance and diabetes [2,3,5]. A variety of epidemiological studies carried during the last years have demonstrated an association between energy imbalance (energy intake higher than energy expenditure) and the obesogenic role of sugar [6]. The increase of health problems has generated a growing demand, between the scientific and industrial community, for food that maintain the sweet taste, once sweetness induces feelings of happiness in the brain, but that have a lower energy/caloric value.

Sweeteners are natural or synthetic chemical compounds, which have a sweet taste that, therefore, determines their use as sweeting agents [4]. These compounds can be classified according to their origin (natural or synthetic agents) or their intrinsic properties like technological function (semisynthetic fillers or sweeteners), texture (powders or syrups) and nutritional value (caloric or non-caloric) [1,4]. Sweeteners, despite having few or no calories, have a strong sweet flavour which allows their use at low concentrations, thus their impact on the total caloric value become negligible [4,5]. Artificial sweeteners are a class of highly sweet compounds that are non-nutritive and non-caloric, since they provide little or no calories and do not increase blood sugar. Among the main artificial sweeteners approved for use as food additives are acesulfame-potassium (E950), aspartame (E951), saccharin (E954) and sucralose (E955) [7]. The most commonly natural sweeteners used are carbohydrates, but also stevia and thaumatin, known as natural zero calorie sweeteners, are widely used. Carbohydrates are a diverse group of compounds that have the major goal of provide quickly digestible energy (nutritive sweeteners), but they can also be a source of dietary fiber and regulate satiety and hunger [4]. Polyols or sugar alcohols are included

in the group of low-digestible carbohydrates, which means that they are partially or non-absorbed in the small intestine, but they can be fermented in the large intestine by bacteria [8,9]. Sugar alcohols are used as sugar substitutes that resulted from the hydrogenation of reducing sugars, with the presence of an alcohol group in the place of the carbonyl group [3]. They are naturally present in small amounts in fruits, vegetables, mushrooms, but also in the human organism [4,5,8]. Polyols are stable compounds at high temperature and pH changes [5]. This type of sweeteners has attracted interest and attention in scientific community due to their high sweeting power, lower caloric content and lower glycemic index, in comparison to sugars. Given this, polyols can be consumed by hypercaloric patients, diabetic patients or another patient's with specific diseases where caloric intake needs to be controlled. They are also associate with the decrease of caries (anti-caries effect), since the cariogenic bacteria cannot ferment them [3]. Nowadays, and according to European Union legislation, there are seven polyols that are defined as nutritive sweeteners and commonly used in food industry, they are sorbitol (E420), mannitol (E421), isomalt (E953), maltitol (E965), lactitol (E966), xylitol (E967), and erythritol (E968).

1.2. Erythritol

Erythritol (E968), also known as erythrite, meso-erythritol, tetrahydroxybutane or (2R,3S)-1,2,3,4-butanetetrol, is a linear four-carbon compound with the molecular formula $C_4H_{10}O_4$ (Figure 1) [1]. It has the smallest molecular weight of all sugar alcohols, being it of 122.12 $g \cdot mol^{-1}$ [10]. This polyol can be found in nature, being specially isolated from vegetables, fruits (grape, melon and pear), mushrooms, fermented food and alcoholic drinks, such as soy sauce, wine and beer, but it also exists in biofluids and tissues of humans and animals [11,12]. Its production had an enormous increase in the 80's decade, due to a major demand for natural and healthier food [11]. This polyol is currently used as an ingredient in many food products, such as baked goods, glazed goods, food coatings, chocolate, fermented milk and candies, but is also consumed directly in its natural form [3].

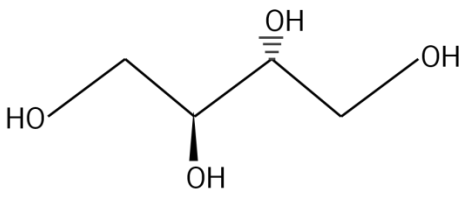


Figure 1 - 2D chemical structure of erythritol.

Erythritol is characterized for having around 60 % to 80 % of the sucrose relative sweetness and similar taste and texture, a sweet clean, fresh taste and no after taste [3,13].This sugar alcohol is stable at high temperatures (up to 160 °C) in both alkaline and acidic environments, and it is a non-hygroscopic substance, that crystallizes rapidly in its white odorless form [1]. When dissolved, erythritol present a strong cooling effect due to its negative heat of solution [10]. Erythritol was considered a safe sweetener after many specific tests regarding carcinogenicity and toxicity [5]. One of the main advantages of erythritol is that it is a very small molecule, so it is rapidly absorbed through the small intestine and excreted unchanged, in the urine, since it is not metabolized [14]. Thus, it is not fermented in the intestines, being improbable possible laxative effects usually associated with the consumption of sweeteners [1]. Moreover, the absorption of erythritol does not induce changes on insulin and glucose levels in blood, which demonstrates the significant potential that erythritol has as a substitute of sugar for diabetic patients [3,7]. Besides being a zero-calorie sweetener, non-glycemic and non-cariogenic, erythritol has proven to be an excellent radical scavenger with membrane-protecting properties and it can be used to suppress biofilms formation of some cariogenic bacteria [1,15]. This polyol has also antioxidant properties once it is a free radical scavenger that can act while is circulating throughout the body, and likewise it exhibit endothelium-protective and non-acidogenicity properties [3,7].

Erythritol can be used in a wide range of liquid and solid formulations, including consumer-friendly lozenges, granulated powders, medicated chewing gum and syrups. Due to its humectant properties, as well as, its enjoyable taste, its non-cariogenic function and its sweetness, this polyol can be worn in oral care products as base for toothpaste and mouthwash recipes. It is mainly used as a sweetener to balance the finished sugar product in terms of its sensory characteristics, such as texture, flavour and colour, but it is mostly used to produce no-sugar added, reduced-sugar or sugar-free food alternatives [10].

The extraction of this polyol from its natural sources, like fruits or vegetables, is not viable due to their low erythritol content. Erythritol can be synthetized through a chemical reaction that use as substrate dialdehyde starch, arabinoic or ribonic acid, 2-butene-1,4-diol or L-tartaric-acid, at high temperatures in the presence of a nickel catalyst. However, this synthesis is very complex, has low efficiency and high production costs, that makes the chemical synthesis economically unprofitable [11,16,17]. Due to the disadvantages of production *via* chemical synthesis, the biotechnological production of erythritol became an alternative since it is safer and more environmentally friendly.

Nowadays, the erythritol commercialized is mainly produced by fermentation using osmophilic fungi, which produce this compound in response to osmotic stress [16,18]. The most commonly osmophilic fungi used in the erythritol production belong to the genus *Aureobasidium*, *Trichosporonoides*, *Moniliella*, *Trichosporon*, *Torula*, *Pseudozyma*, *Trignopsis* and *Yarrowia* [11,16]. Although, the lactic acid bacteria like *Oenococcus oeni*, *Leuconostoc mesenteroides*, and *Lactobacillus sanfranciscencis* can also produce this polyol through fermentation processes [1]. Several other microorganisms like *Pichia*, *Zygopichia*, *Candida* and *Torulopsis* can produce erythritol as well, but thanks to their high production level of by-products, they are inappropriate for industrial process [13,15]. Some of the previous productions are already being developed at an industrial scale, although they also produced unwanted by-products like organic acids and mannitol, which cause a more expensive and challenging downstream process that makes the bioprocess, as a whole, expensive [16]. After fermentation, erythritol is recovered by membrane filtration of the fermented broth to separate the microorganisms, followed by a concentration and an ion exchange chromatography and, finally, it goes with a treatment with activated carbon and crystallization [7]. The main carbon source used in the production of erythritol is glucose, commonly obtained through chemical and enzymatic hydrolyses of corn and wheat starches [13]. However, glucose is not the only carbon source used to produce erythritol and others carbon sources have been already studied, namely glycerol, that can be used by the yeasts *Yarrowia lipolytica* and *Yarrowia divulgata* [19,20]. Once the retail prices of erythritol are still high (average market price around 15.95 € *per* kg), despite of the use of biotechnological *via*, there is an increasing demand for replacements in the process that will reduce the production costs. The use of an alternative low-cost substrate it is an option that its more and more studied. Since glycerol is a carbon source that *Y. lipolytica* and *Y. divulgata* can metabolized, the use of crude glycerol, a by-product from the biodiesel industry that is cheaper than glucose, could be an alternative carbon source to the production of erythritol by these yeasts [21].

1.3. *Yarrowia lipolytica*

Yarrowia lipolytica is one of the most extensively studied non-conventional yeasts, that is well known for its unusual metabolic properties [16]. It has received popularity, among researches, academies and industrialists, as a biotechnological workhorse in an array of diverse and unique applications [17].

Y. lipolytica is a eukaryotic organism (Fungi kingdom) and a hemiascomycetous dimorphic fungus that belongs to the class of *Ascomycetes*, subclass *Hemiascomycetes* and to the order of *Saccharomycetales*. The species name "*lipolytica*", remits to the capability that this yeast has to degrade lipidic carbon sources. It is a non-pathogenic microorganism and most of the processes that use this yeast are Generally Recognized As Safe (GRAS) by FDA (Food and Drug Administration) [22]. With this status, *Y. lipolytica* becomes an attractive microbial host for the manufacture of dietary supplements, fermented food and nutraceuticals [23]. The species *Y. lipolytica* is a strictly aerobic and dimorphic microorganism, capable of growing in the oval or hyphae form (Figure 2). Once it is simple to differentiate between the two morphological forms, this yeast has been considered a viable model for dimorphism studies. Several investigations reported that innumerable environmental factors such as carbon source, pH of the medium, nitrogen source, temperature and dissolved oxygen concentration (DOC) can affect the morphological form of different *Y. lipolytica* strains [24].

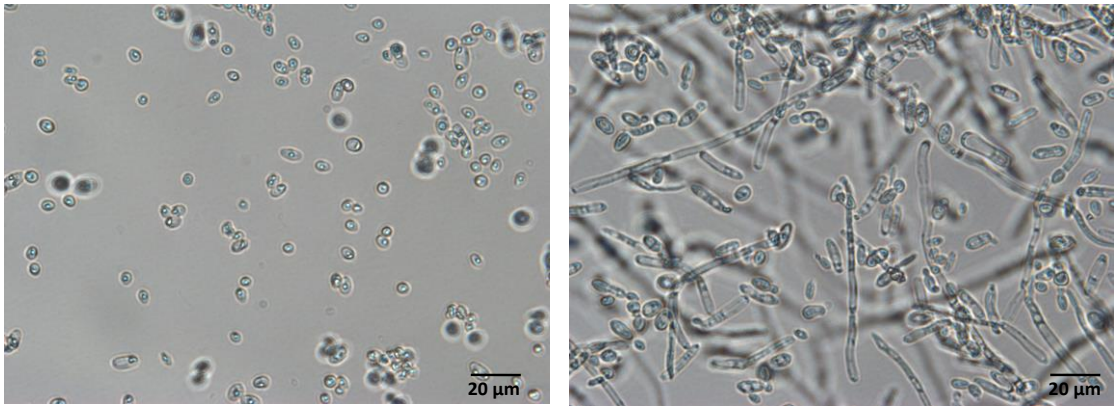


Figure 2 - Cell morphology of *Yarrowia lipolytica* W29: single oval form (left) and filamentous hyphae (right) (magnification 400x).

The oleaginous yeast *Y. lipolytica* have gained a strong interest in the past few years due to its high tolerance to a variety of organic compounds, elevated salt concentrations and pH levels which subsequently simplify bioprocess optimization and favours the use of non-glucose based feedstocks [23]. This yeast has the capacity to grow in environments rich in hydrophobic compounds such as

n-alkanes and oils, or marine and hypersaline environments [25]. Despite of the hydrophobic substrates, it is also able to metabolise other carbon sources such as alcohols (ethanol and glycerol) [26,27], organic acids (lactic acid, acetic acid and propionic acid) and sugars (glucose and fructose) [20]. Nevertheless, the cellular membrane of *Y. lipolytica* is almost impermeable to sucrose, once this yeast does not have the sucrose-cleaving enzyme invertase [28]. Due to the ability of this yeast to degrade several types of carbon sources, *Y. lipolytica* can use efficiently various agro-industrial wastes or by-products, like crude glycerol from biodiesel industry [29], animal fats [30], olive mill waste water [31], waste cooking oils [32], and vegetable oil refinery residues [19]. The versatility of its metabolism is reflected by the diversity of environments where it can be naturally found, such as cheese, yoghurts, kefir, soy sauce, meat, shrimp salads, soil, sewage and polluted environments by oils and fats [25]. *Yarrowia lipolytica* has attracted a huge interest due to its ability to produce and secrete several metabolites that have biotechnological importance and extensive industrial applications [16]. Among all the metabolites that this microorganism can produce, the ones that stand out are enzymes (lipases, esterases and proteases) [22], aromas (lactones) [33], organic acids [29], biosurfactants [22], microbial lipids [30] and polyols like erythritol [16].

1.4. *Yarrowia divulgata*

Yarrowia divulgata is a recently described species in literature that can be found in animal origin foods like processed meat, bacon and chicken liver, but also in marine sources (deep seawater and ocean fish) [34]. The species name *"divulgata"*, remits to the diversity of habitats where it can be found. This yeast is considered a eukaryotic organism (Fungi kingdom) that belongs to the order of *Saccharomycetales* and to the family of *Dipodascaceae* [35]. In spite of this yeast cells growth occurs at 30 ºC, Nagy *et al.* [34] described that strains of this species are unable to grow in temperatures above 35 ºC. The species *Y. divulgata* is a dimorphic microorganism capable of growing in the oval, subspheroid, ellipsoidal or hyphae form (Figure 3). In the study of Nagy *et al.* [34] they reported that nutrients limitation in the culture medium or environmental factors such as carbon source, nitrogen source and temperature can affect the morphological form of *Y. divulgata* strains.

Being a recent reported species, the studies on this field are scarce and few. Although, it was reported that this yeast is able to assimilate carbon compounds such as D-glucose, D-ribose,

ethanol, glycerol, erythritol, organic acids and hexadecane [34,36]. The production of erythritol by strains of *Y. divulgata* from glycerol has already been described [20].

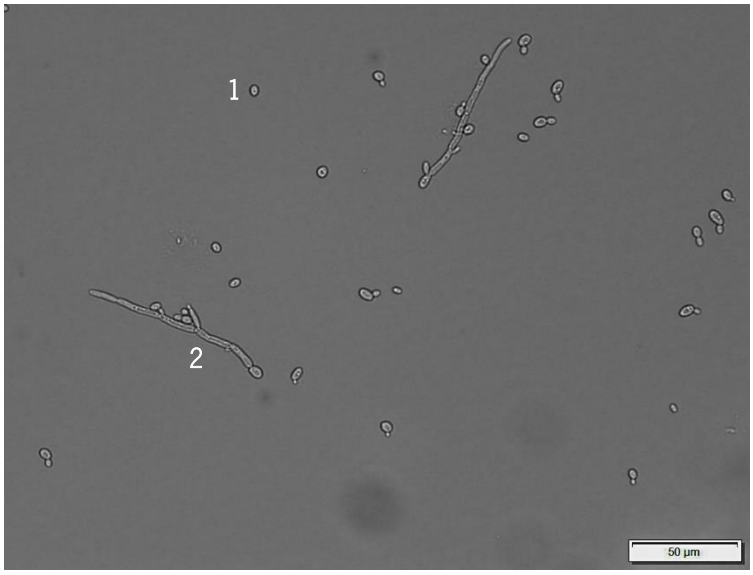


Figure 3 - Cell morphology of *Yarrowia divulgata* M445/4: single oval form (1) and filamentous hyphae (2) (magnification 200x).

1.5. Erythritol production by *Yarrowia* species

Erythritol is produced and accumulated by osmophilic/osmotolerant microorganisms as a response of an external osmotic pressure, induced by the presence of high salt/sugar concentrations or other carbon source used in the culture medium, particularly during stationary phase [11,13]. Polyols, namely erythritol, can function as osmolytes, soluble compounds stored inside cells, that play a role in maintaining the cell's fluid balance and correct protein folding [37]. The capability of *Yarrowia* species to tolerate these conditions makes them viable for the production of erythritol.

High concentration of carbon source can increase osmotic pressure, resulting in higher erythritol production. Also, the type of carbon source can influence the amount of erythritol produced. Yang *et al.* [38] studied the production of erythritol from crude glycerol and glucose and observe that when the carbon source used was glucose, lower yields were obtained when compared to glycerol. The highest yields obtained in the presence of glycerol in the medium, as a carbon source, are probably justified by the higher osmotic stress created by this substrate, thus acting as a stress factor [38]. Monteiro *et al.* [39] described that crude glycerol is mainly constituted by glycerol, water, catalysts, free fatty acids, alcohol and dissolved salts, being the last one

compound, probably the reason why, in the presence of crude glycerol, the erythritol production is higher.

In yeasts, the first steps involved in the erythritol production may vary depending on the carbon source used. Then, the erythritol precursor, erythrose-4-phosphate, is produced *via* non-oxidative phase of the pentose phosphate pathway (PPP) (Figure 4) [11,13]. This pathway is very important in eukaryotic organisms not only because it can create reducing power, in the form of NADPH, needed for cellular reactions, but also because it generates precursors of amino acids synthesis, like ribose-5-phosphate and erythrose-4-phosphate, nucleotides such as RNA and DNA, coenzymes (ATP, $FADH_2$ and coenzyme A), and on its own as compatible solute to protect and stabilize enzymes to facilitate cellular functions under osmotic conditions [10,40].

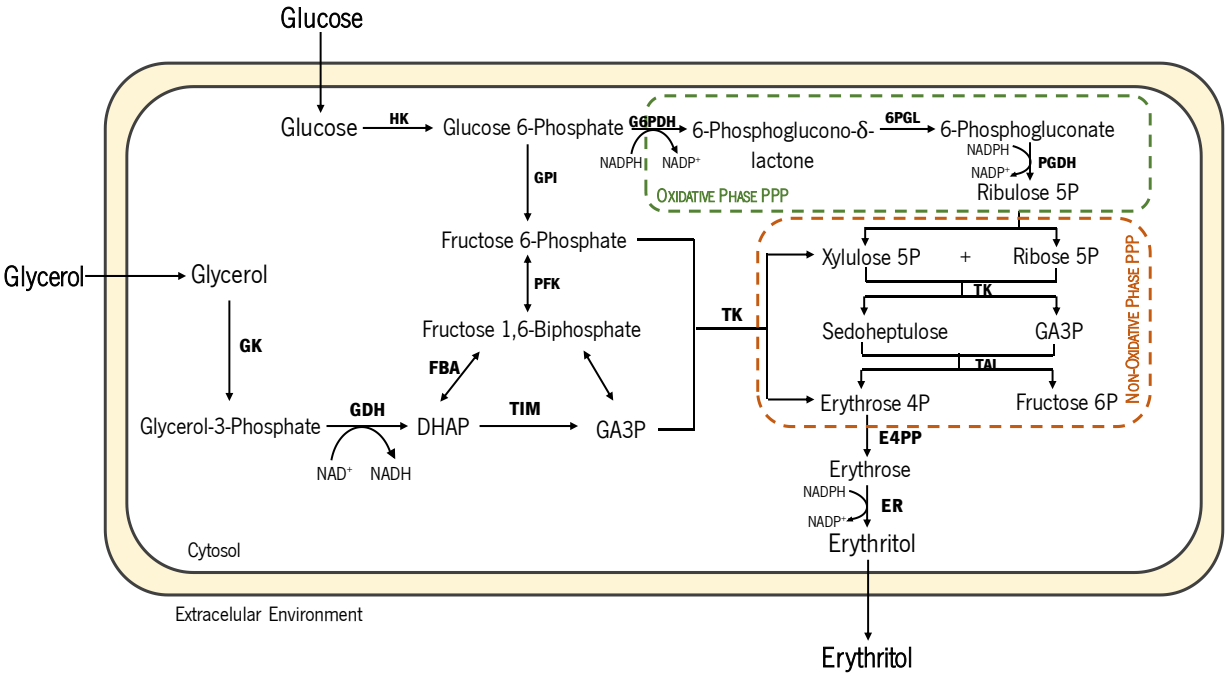


Figure 4 – Overview of the metabolic pathway used by *Y. lipolytica* to synthesize erythritol and derivates from glycerol and glucose. Abbreviations: DHAP- dihydroxyacetone phosphate; GA3P- glyceraldehyde-3-phosphate; GK- glycerol kinase; GDH- glycerol-3-phosphate dehydrogenase; TIM- triosephosphate isomerase; FBA- fructose-biphosphate aldolase; TK- transketolase; E4PP- erythrose-4-phosphate phosphatase; ER- erythrose reductase; PFK- phosphofructokinase; GPI- glucose-6-phosphate isomerase; HK- hexokinase; G6PDH: glucose-6-phosphate dehydrogenase; 6PGL- 6-phosphonogluconolactonase; PGDH- phosphogluconate dehydrogenase; TAL- transaldolase (adapted from [7,14,16]).

When the carbon source available is glycerol, it crosses the yeast cell membrane by active transport, mainly with Glycerol/H^+ antiporters, or by facilitated diffusion, and then it's channelled *via* the aerobic route into the central carbon metabolism for biomass and energy production [41].

The erythritol biosynthesis is a multi-step metabolic pathway that occur in two distinct phases: the first one an oxidative phase where NADPH and ribulose-5-phosphate are produced, and the second phase where the final product is erythrose-4-phosphate, that is produce *via* a non-oxidative phase (Figure 4). Firstly, glycerol it's phosphorylated by a glycerol kinase (GK), before being subsequently dehydrogenated by a glycerol-3-phosphate dehydrogenase (GDH), giving rise to dihydroxyacetone phosphate (DHAP) [16]. DHAP is then converted to glyceraldehyde-3-phosphate (GA3P), by a triosephosphate isomerase (TIM), and after that, with also the incorporation of fructose-6-phosphate they enter in the pentose phosphate pathway, where a transketolase (TK) converts both into erythrose-4-phosphate and xylulose-5-phosphate [16]. Lastly, erythrose-4-phosphate is dephosphorylated by an erythrose-4-phosphate phosphatase (E4PP) and then reduced by an erythrose reductase (ER), with the application of one molecule of NADPH, forming erythritol [16]. Some amount of GA3P and DHAP might also be raised to fructose-1,6-biphosphate, that will be convert to fructose-6-phosphate by a phosphofructokinase (Figure 4). This fructose-6-phosphate it is the one that is incorporated in the non-oxidative phase of PPP.

The key enzyme in the biosynthesis of erythritol is ER, the enzyme that catalyses the final step of the pathway [42]. This enzyme catalyses the reduction of erythrose to erythritol in the presence of NADPH as a cofactor [17]. Recently, Mironczuk *et al.* [43] reported that the TK enzyme also plays an important role in erythritol production since the gene overexpression encoding this enzyme resulted in an enhancement of erythritol concentration in flasks experiments.

Depending on experimental conditions, *Y. lipolytica* can also use erythritol as carbon source. In this catabolic pathway erythritol is first oxidized into erythrulose by an erythritol dehydrogenase and then phosphorylated into erythrulose-phosphate by an erythrulose kinase [11]. The entire catabolic pathway is not yet known but Carly *et al.* [16] have recently highlighted that this pathway in *Y. lipolytica* is similar to those present is other yeasts, including *Lipomyces starkeyi*.

1.5.1. Effect of culture conditions

The cellular growth and metabolites production by yeasts like *Yarrowia* are strongly influenced by the culture conditions, such as pH, medium composition, temperature and DOC. Erythritol production by *Yarrowia* species are related with its growth, once this production occurs during cell growth and substrate consumption, as an integral part of normal growth processes [19,44]. The erythritol production by this yeast is influenced by different culture conditions and characteristics of the culture medium. It has been described that high salt concentrations in the medium stimulate

the erythritol production, as well as, a low pH value [11,19]. There are several approaches available that can be used to increase erythritol production. The first and probably the simplest option is the optimization of the culture medium composition, followed by the optimization of environmental culture conditions. A more complex approach would pass by genetic modification of existing strains or search for new isolated species and strains [37].

Considering the medium composition optimization, the carbon source selected is an important parameter. For the erythritol production by *Y. lipolytica* strains, several carbon sources such as glucose [18], glycerol [19,27], maltose [18], waste cooking oils [44,45], malto-dextrin [18], molasses [46] and inulin [47] were tested and compared. In the study of Yang *et al.* [38] glucose and pure glycerol were the substrates that led to higher erythritol yields when used by *Y. lipolytica* A16, a mutant strain, and compared to the use of fructose, sucrose and maltose. The researchers also observed that the biomass production was comparable when glucose and pure glycerol were used as carbon source but the maximum synthesis of erythritol (50.4 $g \cdot L^{-1}$) was obtained with pure glycerol. Also, Rymowicz *et al.* [19] studied the production of erythritol using glucose and raw glycerol as carbon sources, and demonstrated that glucose was less efficient, being also the productivity and yield in erythritol obtained lower than with raw glycerol. Higher production of erythritol in glycerol can be justified by the fact that it creates a higher osmotic stress, which favours erythritol production. In order to determine which culture conditions were better for erythritol biosynthesis by *Y. lipolytica* Wratislavia K1, Rywińska *et al.* [48] performed several batch experiments using pure and raw glycerol as carbon sources. They observed that the highest erythritol production (58.2 $g \cdot L^{-1}$), yield (0.38 $g \cdot g^{-1}$) and productivity (0.78 $g \cdot L^{-1} \cdot h^{-1}$) were obtained when raw glycerol was used [48]. The use of raw glycerol proves the potential of using low-cost carbon sources as alternative substrate to produce erythritol, turning the process much more sustainable. This technology also follows the model of a circular economy due to the ability of turning by-products into valuable feedstocks for bio-based industries [27]. Likewise, for *Y. divulgata* strains glycerol was described as the best carbon source that produce the higher erythritol concentrations, when compared with other carbon sources like glucose and fructose [20].

The nature of nitrogen source and its concentration are other important parameters for the erythritol production. Different studies were performed to select the better source of nitrogen, between organic and inorganic sources. Rywińska *et al.* [49] tested the effect of different nitrogen sources (organic and inorganic) in erythritol production by *Y. lipolytica* Wratislavia K1, and observed that the highest erythritol yield and productivity was obtained when the medium was supplemented

with ammonium sulfate ($(NH_4)_2SO_4$), an inorganic nitrogen source. The researchers also observe that the better organic nitrogen source was yeast extract, being this corroborate also by Tomaszewska *et al.* [49,50]. Likewise, Rakicka *et al.* [27] studied several nitrogen sources, inorganic and organic, at different concentrations in the production of this sugar alcohol by *Y. lipolytica* Wratislavia K1 (a mutant strain) and concluded that the medium with 4.6 $g \cdot L^{-1}$ of inorganic nitrogen ($(NH_4)_2SO_4$) had the highest erythritol production of 103.4 $g \cdot L^{-1}$. Once nitrogen sources like yeast extract, peptone and ammonium sulphate have high costs, their use in industrial erythritol production should be avoided. Thus, Tomaszewska *et al.* [51] tested the use of corn steep liquor (CSL), an economic and effective substitute for the traditionally applied nitrogen sources, and concluded that the application of 40.0 $g \cdot L^{-1}$ of CSL resulted in a significant improvement in erythritol production. They observed that this organic by-product of the corn steeping process of the maize-starch industry can be used as an alternative to traditional nitrogen sources once it can accurately stimulate the cell growth and erythritol production.

Nitrogen limitation is the most commonly used trigger for metabolite production in *Y. lipolytica* [41]. A high C:N ratio is essential to redirect carbon source to polyol synthesis, rather than biomass formation or other metabolic pathways [11]. This ratio can be also used to shift between lipid formation or production of organic acids and polyols. Rywińska *et al.* [49] analysed the effect of different C:N ratio on erythritol production and concluded that the best yield of erythritol production, 0.25 - 0.26 $g \cdot g^{-1}$ was achieved when the ratio ranged from 150:1 to 75:1. Although, the highest erythritol productivity of 0.37 $g \cdot L^{-1} \cdot h^{-1}$ was obtained at a ratio of 75:1 [49]. In order to tested the effect of nitrogen concentration on erythritol production, Xiaoyan *et al.* [45] performed several experiments were the medium contains various amounts of $(NH_4)_2C_2O_4$, with the C:N ratios range from 44:1 to 244:1. The researchers reported that the increase in C:N ratio decreased the biomass level from 9.8 $g \cdot L^{-1}$ to 5.7 $g \cdot L^{-1}$, and the highest erythritol productivity, 0.3 $g \cdot L^{-1} \cdot h^{-1}$, was obtained at a ratio of 87:1 [45].

Beyond the nitrogen and carbon sources, some studies proved that phosphate concentration also influences the erythritol productivity. Rywińska *et al.* [49] tested different concentrations of potassium dihydrogen phosphate (KH_2PO_4), up to 2 $g \cdot L^{-1}$, to evaluate its effect in the production of erythritol, by *Y. lipolytica* Wratislavia K1. They observed that for the higher phosphate concentrations the erythritol production was lower and this one decreased as the phosphate concentration increase. The highest erythritol production (26 $g \cdot L^{-1}$) was obtained when the KH_2PO_4

concentration used was 0.25 g·L^{-1}. It was also observed that higher concentrations of phosphate led to mannitol production stimulation [49].

Furthermore, pH of culture medium is one of the parameters described as having a key role in the erythritol production. Rymowicz *et al.* [19] studied the effect of pH in erythritol production by *Y. lipolytica* Wratislavia K1 from crude glycerol, testing the growth and the production of erythritol, citric acid and mannitol at different pH values within the range 2.5 and 6.5. In this study, it was observed that an increase in pH results in a decrease of erythritol concentration and in an increase of citric acid concentration, being the amount of citric acid exceed by the amount of erythritol at pH 5. The highest erythritol concentration (170 g·L^{-1}) was obtained at pH 3, and in this condition citric acid was not detected, however there was some residual production of mannitol (12 g·L^{-1}) [19]. A little change from a higher pH of 5 to a pH of 3 leads to a redistribution of the carbon flux in the direction of polyols production [41]. In general, all the studies performed evidence that the optimum pH for the production of erythritol is around 3, and using this low pH value it is observed a highest erythritol yield and lowest levels of by-products, such as mannitol and citric acid [19,52].

Being the erythritol production a response to osmotic stress, and since the strains that produce higher amounts of erythritol are described as osmotolerants, the osmotic pressure is an important parameter in the erythritol production process. To increase osmotic pressure in the medium, besides to the high substrate concentration, the addition of salt can be used. Tomaszewska *et al.* [53] evaluated the effect of different concentrations of sodium chloride (NaCl (0 g·L^{-1}; 25 g·L^{-1}; 32.5 g·L^{-1})), on erythritol production by *Y. lipolytica* Wratislavia K1. The highest amount of erythritol was obtained when the salt concentration used was 25 g·L^{-1}. It was also observed that the addition of NaCl to the medium increase erythritol production, while mannitol production decrease [53]. Likewise, Rywińska *et al.* [49] studied the effect of several NaCl concentrations in erythritol production by *Y. lipolytica* Wratislavia K1, from glycerol. Salt concentration were tested up to 90 g·L^{-1}, and the highest yield and productivity of erythritol were obtained in the medium supplemented with 30 g·L^{-1} of NaCl [49]. The researchers also found that for higher sodium chloride concentrations (60 g·L^{-1} and 90 g·L^{-1}), the biomass concentration was lower, decreasing from 14 g·L^{-1} to 4 g·L^{-1} [49]. In another research, Tomaszewska *et al.* [51] studied the effect of NaCl in enzymes activity, namely ER and TK, the key enzymes of the metabolic pathway used to synthesize erythritol. They noted that ER and TK activities were enhanced to a greater extend by salt presence in the medium which corroborates earlier studies that showed that erythritol production by *Y. lipolytica* is enhanced by the addition of salt to medium. Once chloride ions have a corrosive effect

in the constitutive material of industrial reactor vessels, in their study Da Silva *et al.* [54] used polyethylene glycol (PEG) instead of NaCl, as an osmotic stress agent for the synthesis of erythritol by *Y. lipolytica*. They observed that this polymer can be used as an alternative to NaCl once it can accurately modulate osmotic pressure [54].

1.5.2 Glycerol as a carbon source

Glycerol, also known as glycerine, glycerin or 1,2,3-propanetriol, is a small, symmetrical, uncharged and simple alcohol molecule with the molecular formula $C_3H_5(OH)_3$ (Figure 5). It is a viscous liquid with sweet taste and hygroscopic properties, that is non-toxic, odorless and colourless.

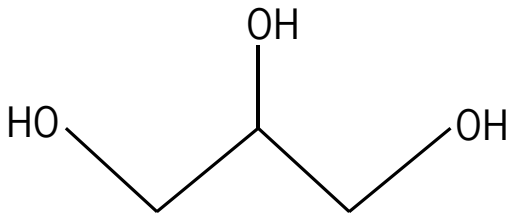


Figure 5 - 2D chemical structure of glycerol.

The three hydrophilic alcoholic hydroxyl groups are responsible for its entire miscibility with water and its highly hygroscopic nature. Under normal atmospheric pressure and in its pure anhydrous state, glycerol has a melting point of 18.2 °C, a specific gravity of 1.261 $g \cdot m^{-3}$ and a boiling point of 290 °C [55]. Glycerol is a versatile renewable raw material that is used mostly in the chemical industry, but it is also used as a humectant in sweets, cakes, cheeses and meats, and as a solvent, sweetener and preservative in foods and beverages. This alcohol can also be used in a wide range of products such as pharmaceutical preparations, personal and oral care products and in textile and paint goods. This wide variety of glycerol applications is due to the diversity of chemical reactions that can be used to convert it into materials with higher added value or to synthetic precursors. Nevertheless, possibilities of use are strictly linked and limited by the degree of purity of glycerol [39]. Glycerol could be produced in three ways: by chemical synthesis, from petrochemical feedstock; by microbial fermentation; or as a by-product from soap manufacturing, alcoholic beverage or biodiesel industries.

Global biodiesel production has been growing in the past few years once biodiesel can be used as a renewable fuel that is an alternative to fossil fuels in transportation sector. In 2019, the

countries of European Union produced around 16.09 million m^3 of biodiesel, the equivalent of 34.7 % of the global biodiesel production [56]. The four basic production technologies used to obtain biodiesel are microemulsification, thermal cracking, direct use and blending with petroleum-derived diesel fuel or by transesterification. Among these four, transesterification is the process that most of the facilities use once it is the less expensive technology. In this process, biodiesel is produced by a base-catalysed transesterification reaction with vegetable oils or animal fats (triglycerides) as feedstocks (Figure 6). During the transesterification process, triglycerides of fatty acids reacted with an alcohol molecule (methanol or ethanol), that act as an acyl acceptor, in the presence of a catalyst, to produce monomethyl esters (biodiesel) and glycerol as a side product [57]. The major bottleneck in the biodiesel production by transesterification is the generation of the by-product glycerol once this reaction produces biodiesel and glycerol at a volumetric ratio of 10:1 (*per* 10 kg of biodiesel is generated 1 kg of glycerol). In order to ensure the sustainability of global production of biodiesel, the discovery of new uses for crude glycerol is imperative [39].

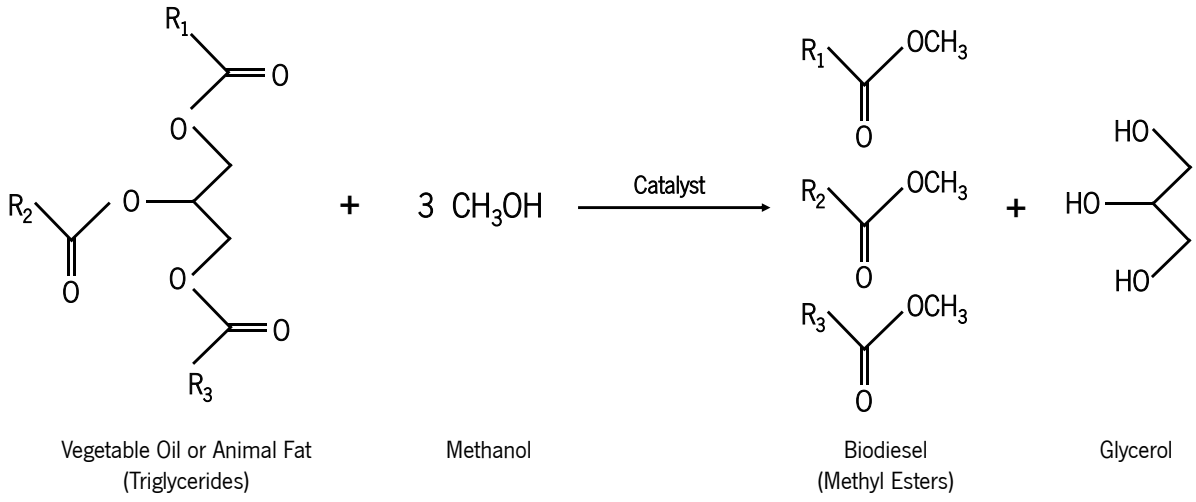


Figure 6- Transesterification reaction of vegetable oils or animal fats for biodiesel production.

The increment production in biodiesel industry is directly associated with an increase in surplus production of glycerol, which leads some companies to treat crude glycerol as an industrial waste. Crude glycerol from the biodiesel industry is a brownish, viscous liquid with a glycerol content of around 50 % to 80 %. Beyond glycerol, crude glycerol generally contains five main components: water, dissolved salts, fatty acids, alcohol (usually methanol) and catalysts. Due to its impurities, that can vary from 20 % to 50 %, the use of crude glycerol in other industries like chemical and pharmaceutical industry is limited by its degree of purity, what would claim a high cost purification process [39,55]. Taking in consideration the high amounts of crude glycerol generate every single

year and the high cost of its purification, it is urgent the discovering of other processes that can use this mixture as it is produced. Biotechnological conversion of the crude glycerol in value-added products is one of the possible applications of this surplus by-product [58]. A variety of studies targeted to the production of valuable compounds by microbial fermentation of crude glycerol have been developed. Among all the compounds that could be produced from fermentation of crude glycerol, the ones that stand out are propionic acid produced by *Propionibacterium freudenreichii* [59], poly(hydroxyalkanoates) (PHA) generated by *Paracoccus denitrificans* and *Cupriavidus necator* [60] and 1,3-propanediol produced by *Colostridium butyricum* or by *Klebsiella pneumonia* [61,62]. Similarly, the production of citric acid, lipids and polyols by *Y. lipolytica* using crude glycerol as carbon source are already reported in shake flasks and bioreactor experiments. Table 1 lists some studies where the value-added compound erythritol is produced by *Y. lipolytica* using crude glycerol as a carbon source. It should be noted that most of the strains cited in Table 1 are mutant strains, being only the strains A-15, A-6, 1.22 and A-101 the wild-type strains used to produce erythritol from crude glycerol.

Table 1 - Erythritol production by biotechnological conversion of crude glycerol by *Yarrowia lipolytica*.

Operation Mode	***Y. lipolytica* strain**	**Yield** ($g \cdot g^{-1}$)	**Productivity** ($g \cdot L^{-1} \cdot h^{-1}$)	**Reference**
Batch	Wratislavia K1	0.49	1.00	[53]
	A-15	0.39	0.72	
	A UV'1	0.34	0.81	
	Wratislavia K1	0.27- 0.34	0.59 – 0.72	[48]
		0.40	0.60	[52]
		0.40	0.66	[63]
	A-6	0.45	1.04	[51]
Fed-Batch	Wratislavia K1	0.21 – 0.32	0.60 – 0.70	[64]
		0.56	1.00	[19]
	Wratislavia AWG7	0.41	0.78	
	Wrastislavia 1.31	0.44	0.79	
	8661 UV1	0.38	0.68	
	1.22	0.31	0.51	
	A-101	0.46	0.68	
Repeated Batch	Wratislavia K1	0.27 – 0.57	0.25 – 0.59	[65]
Continuous	Wratislavia K1	0.40	0.90	[27]

1.6 Operation in bioreactors: operation modes

The scale-up of the process of production, such as erythritol production, and a better process monitoring can be ensured by the use of bioreactors. A bioreactor can operate in different operation modes in order to achieve the production of a desired metabolite. These modes can be batch, continuous and fed-batch.

A batch process is a closed system that is characterized for not having any input or output streams crossing the system frontiers [66]. In this operation mode, all the needed components are added at the beginning of the process, and the product required and co-products are removed when the process is finished or when there is depletion of all substrates [37]. The batch culture is the simplest and easiest operation mode to perform, although the productivity and concentration of the desired metabolite are low compared with other operation modes [37]. Tomaszewska *et al.* [53] studied erythritol production from crude glycerol in batch cultures in bioreactor using the *Y. lipolytica* strains Wratislavia K1, A UV'1 and A-15, and the best performance was obtained with *Y. lipolytica* Wratislavia K1. In this study it was also proved that the scale-up of the process, from flasks to bioreactor, resulted in a notorious increase of erythritol concentration, for all the strains [53].

In contrast, continuous process does not operate as a closed system, once the inputs and outputs flows continuously throughout the duration of the process, which means that during all the process the bioreactor is supplied with fresh medium while, at the same rate, the medium and cells present in bioreactor are extracted [66]. In the case of erythritol production in continuous fermentation mode, few studies have been reported so far. Rakicka *et al.* [27] tested erythritol production in a chemostat culture with crude glycerol by *Y. lipolytica* Wratislavia K1 and obtained a productivity of 0.9 $g \cdot L^{-1} \cdot h^{-1}$ and a erythritol production of 81.8 $g \cdot L^{-1}$.

Like continuous cultures, fed-batch mode is an open system where, at a given time of the fermentation, a certain amount of medium or substrate is added into the bioreactor [37]. Fed-batch cultures can be handled at a variable volume where the fresh medium is joined to bioreactor with no medium removal [67]. The fed-batch cultures with fixed medium volume are known as repeated batch cultures and in these experiments, portions of the culture medium are extracted and replaced with new fresh medium, at regular periods of time. The fed-batch cultures where the medium culture varied, can be done at a continuous feeding rate or by pulse addition (stepwise fed-batch) [11]. Rywińska *et al.* [49] studied the erythritol production from glycerol by *Y. lipolytica* Wratislavia

K1, in fed-batch with pulse additions of glycerol and come up to an erythritol production of 132 $g \cdot L^{-1}$ and a productivity of 1 $g \cdot L^{-1} \cdot h^{-1}$, that matches to a 2.8 and 1.2-fold increase, respectively. In another study conducted by Tomaszewska *et al.* [50] erythritol production by the same strain, but using continuous feeding and pulse additions of glycerol in separate studies, was observed, and it was proved that the highest amount of erythritol (201.2 $g \cdot L^{-1} \cdot h^{-1}$) was obtained with pulse addition. Mirończuk *et al.* [15] evaluated erythritol production in repeated batch culture by *Y. lipolytica* MK1 where 30 % or 40 % (v/v) of the culture medium was replaced, periodically, by newly fresh medium. They compared the results to the ones obtained in batch cultures and noted that repeated batch mode led to a 2.7-fold and 1.4-fold increase in erythritol concentration and yield, respectively, but a 1.6-fold decrease in erythritol productivity was reported. The highest level of erythritol produced (224 $g \cdot L^{-1}$) was obtained when 30 % (v/v) of the culture medium was replaced [15]. This method can improve the process efficiency by extending the effective production phase [17].

The most common reactors used to cell cultivations and fermentations are stirred tank reactors (STR). In these ones, air is injected at the bottom of the vessel and a Rushton turbine is commonly located above the air injector in order to reduce the bubble size and increase the oxygen transfer rate to liquid phase. To preserve a constant DOC in the culture medium, aeration and agitation rates must be controlled [58]. Most of the studies that describes erythritol production by *Y. lipolytica* from crude glycerol use STR bioreactors.

1.7 Oxygen mass transfer rate

Yarrowia lipolytica is a strictly aerobic yeast, thus the oxygen concentration available in the medium is one of the fundamental parameters for the bioprocess performed with this yeast. A high oxygen supply is imperative for aerobic microorganisms in order to generate ATP for the metabolism [21]. Furthermore, the DOC in the medium could directly affect the amount and type of compounds produced by the yeast, but also is a critical factor that has a substantial influence on the morphology and metabolism of *Y. lipolytica* [17,68].

Oxygen is a key substrate in any aerobic bioprocess, since it is a crucial nutrient for microbial growth, maintenance and metabolites production. A continuous supply of oxygen to the culture medium is needed due to its low solubility in aqueous medium [69]. The DOC in a suspension of aerobic microorganisms depends on the oxygen transfer rate (OTR) from the gas phase to the liquid, on the rate at which oxygen is transported into the cells and on the oxygen uptake rate (OUR), by the organism for growth, maintenance and production (Figure 7) [69].

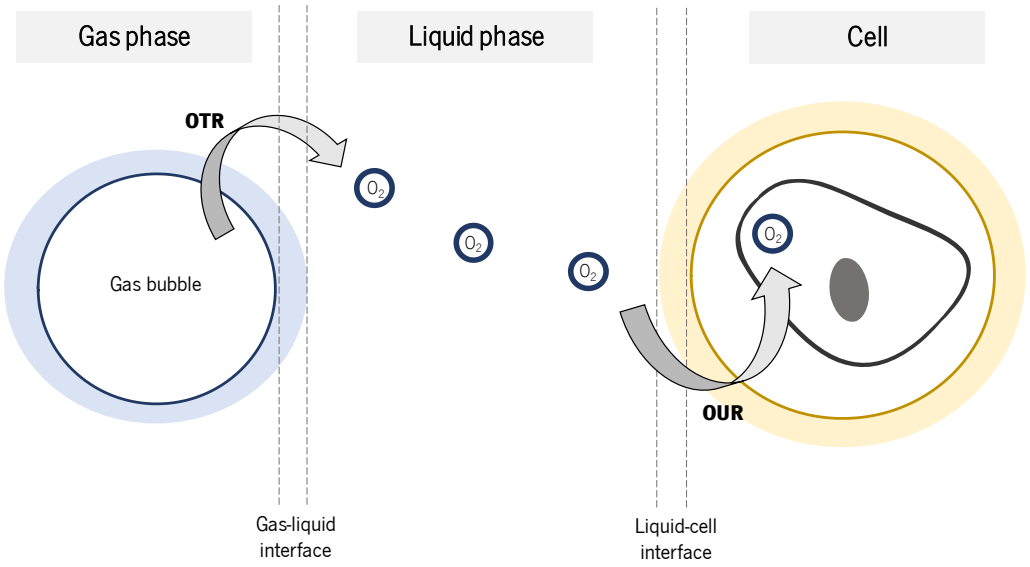


Figure 7 - Diagram of oxygen transfer from gas phase to liquid and from liquid phase to the cells. Abbreviations: OTR- oxygen mass transfer rate; OUR- oxygen uptake rate.

OTR can be strongly affected by the hydrodynamic conditions in the bioreactors. These conditions are known to be a function of energy dissipation that depends on several factors and parameters, such as physical properties of gas and liquid phases, operating conditions, geometrical characteristics of the bioreactor and also by the presence of cells [69,70]. Knowing or predicting the volumetric oxygen mass transfer coefficient (k_La) for different operating conditions, it is very important to prevent oxygen limitation in the culture medium [29]. k_La is a parameter that determines the magnitude of the OTR, once this can be described as proportional to the driven force for mass transfer [69]. OTR and k_La can be related by equation 1:

$$\mathrm{OTR} = k_{\mathrm{L}}a\ (\mathrm{C}^* - \mathrm{C}) \qquad \text{Equation 1}$$

where k_La is the mathematical product of mass transfer coefficient (k_L) and interfacial area (a), C* is the solubility of oxygen and C is the dissolved oxygen concentration in the liquid phase.

Many empirical correlations have been preponed to determine k_La, depending on the bioreactor configuration [69]. For a STR the most common function is given by equation 2 [29]:

$$k_L a = \alpha \left(\frac{P_g}{V}\right)^{\beta} v_s^{\gamma} \qquad \text{Equation 2}$$

where Pg is the power input to the aerated system, V is the working volume, v_s represents the superficial gas velocity and α, β and γ are the dimensionless constants.

The correct measurement and prediction of k_La is a crucial step in the optimum design, operation and scale-up of bioreactors [29].

The oxygen concentration available is directly related with the oxygen mass transfer phenomenon and some studies have showed the effect of this phenomenon in products formation by *Y. lipolytica* [29]. The raise of oxygen transfer from gas phase to the culture medium lead to an increase of cellular growth, lipase production [30], γ-decalactone secretion [33] and citric acid production [29]. Once erythritol production by *Y. lipolytica* from crude glycerol is an aerobic process, oxygen availability is a crucial parameter in the microorganism cultivation, substrate uptake rate and erythritol accumulation.

Although several works regarding the production of erythritol from crude glycerol have been published, data on oxygen mass transfer rate and DOC required to support the production in this medium is still limited. Rywińska *et al.* [48] studied the effect of different agitation and aeration rates on erythritol production by *Y. lipolytica* Wratislavia K1, reporting that the highest yield of erythritol was obtained at an agitation rate of 800 rpm and an aeration rate of 0.36 or 0.6 vvm for pure glycerol or crude glycerol, respectively. In our best knowledge this is the only study that somehow evaluates the effect of oxygen in erythritol productivity by *Y. lipolytica*. This yeast needs a high and continuous demand for oxygen, which is conceived as a major hindrance in the scale-up of the production process [17].

2. AIMS

This section presents the main goals of this work.

The consumption of food with the addition of refined sugars has been augmenting over the past years. This event had leading to an increase in the obesity rate and some associated diseases. These health problems caused by the excessive sugar consumption have been generating a growing demand for healthier options without losing the sweet taste. The use of non-caloric sweeteners can be seen as an alternative. Erythritol is a four-carbon sugar alcohol that has about 70 % of the sweetness of sucrose, it does not cause cavities, it has no calories and its rapidly absorbed and excreted unchanged in the urine. Considering that erythritol chemical synthesis has high production costs and low efficiency, this sugar alcohol is mostly produced from glucose using osmophilic/osmotolerant fungi.

Yarrowia lipolytica is one of the osmotolerant yeasts described as erythritol producer. This yeast has been well studied in the production of several metabolites of interest using different carbon sources including agro-industrial wastes and by-products like crude glycerol, from biodiesel industry. The production of erythritol by strains from *Yarrowia* genus can be influenced by several parameters, like medium composition, pH, operation mode and operational conditions, that should be taken in consideration. Since *Y. lipolytica* is an aerobic microorganism, the oxygen concentration available in the medium is one of the fundamental parameters for the bioprocess. The DOC in a suspension of aerobic microorganisms depends on k_La value.

Thus, the main goals of this present work are:

- Evaluation of growth and production of erythritol by strains of the genus *Yarrowia*, in culture media with crude glycerol, from the biodiesel industry;
- Scale-up of the bioprocess in a stirred tank reactor and evaluation of the effect of operational parameters in the dissolved oxygen concentration and in erythritol production;
- Exploring different operating modes, e.g., stepwise fed-batch operation, for the improvement of the erythritol production.

3. MATERIALS AND METHODS

In this section, the general methods, operation conditions and equipment used in this work are presented.

3.1 Yeast strains

In this work, two species of the genus *Yarrowia*, *Y. lipolytica* and *Y. divulgata* were used. From the species *Y. lipolytica*, three strains were tested: *Y. lipolytica* W29 (ATCC 20460) isolated from soil, *Y. lipolytica* Ch 3/4 and *Y. lipolytica* Ch 1/5, both isolated from cheese. From the species *Y. divulgata*, two different strains were used, *Y. divulgata* M445/4 and *Y. divulgata* 5257/2, isolated from minced meat. The strains *Y. lipolytica* Ch 1/5, *Y. lipolytica* Ch 3/4, *Y. divulgata* M445/4 and *Y. divulgata* 5257/2 were provided by Doctor Edina Nagy from the Faculty of Food Science of Corvinus University of Budapest [34].

All strains were preserved on yeast extract, peptone, dextrose and agar medium (YPDA) and kept at 4 °C, for a maximum of two weeks. The YPDA medium composition ($g \cdot L^{-1}$) was: glucose 20, yeast extract 10, peptone 20, and agar 20. For longer storage, the strains were kept in 20 % (v/v) glycerol and stored at - 80 °C.

3.2 Erythritol production experiments: medium and culture conditions

All strains were pre-grown in yeast extract, peptone, glycerol medium (YPG) composed by pure glycerol 20 $g \cdot L^{-1}$, yeast extract 10 $g \cdot L^{-1}$, and peptone 20 $g \cdot L^{-1}$. The cells were incubated overnight at 27 °C and 200 rpm in 500 mL Erlenmeyer's flasks with 200 mL of culture medium. After pre-growth, the cells were collected and centrifuged and finally resuspended in 200 mL of production medium, in order to obtain a starting cellular concentration of 1 $g \cdot L^{-1}$. The production medium was composed by: crude glycerol 100 $g \cdot L^{-1}$, yeast extract 1 $g \cdot L^{-1}$, NH_4Cl 3 $g \cdot L^{-1}$, NaCl 25 $g \cdot L^{-1}$, KH_2PO_4 0.2 $g \cdot L^{-1}$, and $MgSO_4 \cdot 7H_2O$ 1 $g \cdot L^{-1}$. The compounds were dissolved in 0.72 M potassium phosphate buffer at pH 3. The erythritol production experiments on flasks were carried out in 500 mL baffled flasks containing 200 mL of production medium and incubated at 27 °C and 200 rpm, over seven days. The pH of the medium was maintained at 3 by adding 5 M KOH. The glycerol used in the production medium was crude glycerol from the biodiesel industry, provided by Prio Energy – Prio Biocombustíveis, SA that have the following composition: glycerol 81.8 %, water 11.8 %, sodium chloride 4.7 % and methanol < 0.01 %.

The results presented are the average values of two independent experiments. The mean values were reported and compared by variance analysis (One-Way ANOVA). The differences between the conditions were tested using the Tukey test. All analyses were performed for $p \leq 0.1$ using GraphPad Prism program, version 7.00.

3.3 Bioreactor experiments

The two strains that presented the best results in the flasks experiments were selected for erythritol production in bioreactor.

All the bioreactor experiments were accessed in a 3.7 L stirred tank reactor (RALF PLUS SOLO, Bioengineering, Switzerland) using different operation modes (batch and stepwise fed-batch). The bioreactor has 31 cm height and 17 cm diameter and contain an agitator with two Rushton impellers 6-blade, 6 cm outside diameter (Figure 8). The medium pH and the DOC in the medium were monitored using the BioScadaLab software. The medium pH was measured using a pH probe (405-DPAS-SC-K8S/225 Mettler Toledo) and maintained at 3 with the addition of 2 M KOH through Peripex peristaltic pumps (Bioengineering, Switzerland). Oxygen concentration dissolved in the medium was monitored by a polarographic-membrane probe (InPro 6000, Mettler Toledo, USA).

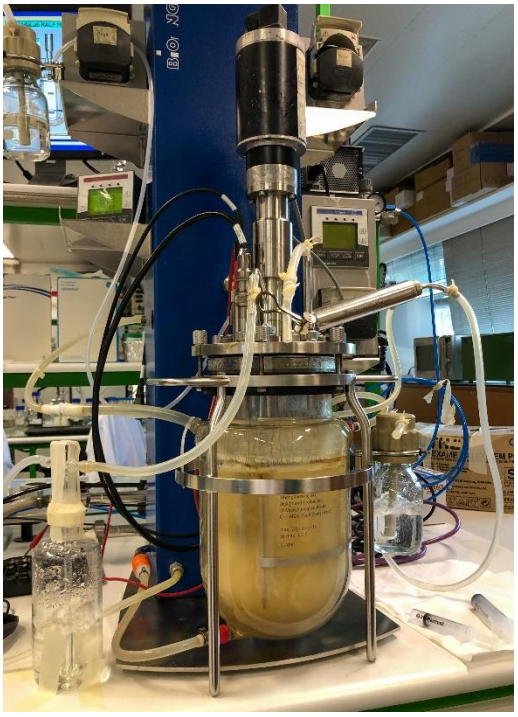


Figure 8 – Stirred tank reactor (RALF PLUS SOLO, Bioengineering, Switzerland) with production medium.

In all the experiments, the yeast cells were pre-grown overnight (16 to 18 hours) at 27 ºC and 200 rpm, in 1 L Erlenmeyer's flask containing 500 mL of YPG medium. Afterwards, cells were centrifuged and resuspended in production medium (described in section 3.2). 1.7 L of medium production was inoculated with an initial cellular concentration of 1 $g \cdot L^{-1}$. All experiments in bioreactor were performed at 27 ºC and at pH 3.

3.3.1 k_La calculation: static gassing-out technique

In order to evaluate the effect of aeration and stirring rates on k_La values, several experiments were carried out varying the aeration rate from 1 vvm to 3 vvm and changing the stirring rate from 400 rpm to 900 rpm. Each experiment was replicated thrice to ensure the reproducibility and the repeatability of the results.

For experimental k_La determination, the static gassing-out technique was used. This technique allows the evaluation of the effect that operational parameters, such as stirring and aeration rates, could have in the oxygen transfer efficiency [71]. After a preliminary gassing-out with compressed nitrogen, to remove the oxygen from the medium, the aeration was switched on at specific conditions of aeration and stirring rates until saturation [58]. All the experiments were carried out in 1.7 L production medium at 27 ◦C and pH 3.

The method is based in the oxygen mass balance equation (Equation 3) which, in the absence of cells and in batch mode, is simplified to the equality between the time variation of the DOC $\left(\frac{dC}{dt}\right)$ and the oxygen transfer rate from the gas to the liquid [29].

$$\frac{dC}{dt} = k_L a(C^* - C) \qquad \text{Equation 3}$$

Integrating this equation, the value of k_La was obtained, which is equal to the symmetrical slope of the plot of $\ln(C^* - C)$ *vs* time [72].

3.3.2 Batch experiments

All batch cultures were conducted with a working volume of 1.7 L of production medium.

To evaluate the oxygen effect in erythritol production, different constant aeration and stirring rates were tested, varying the aeration rate from 1 vvm to 3 vvm and changing the stirring rate from 400 rpm to 900 rpm. In this strategy, three different conditions were tested: low stirring and aeration rates (400 rpm and 1 vvm), medium stirring and aeration rates (600 rpm and 2 vvm) and high stirring and aeration rates (900 rpm and 3 vvm). The experiments with low and medium stirring and aeration rates were only done for the strain *Y. lipolytica* W29 once it was the best producer of erythritol in flask experiments. The batch experiments in high stirring and aeration rates, that were the most promising operational conditions, were done for the strains *Y. lipolytica* W29 and *Y. lipolytica* Ch 3/4.

The operational conditions, stirring and aeration rates, that presented the better results in batch experiments were selected for erythritol production in bioreactor in different operation modes.

3.3.3 Stepwise Fed-Batch experiments

All stepwise fed-batch cultures were initiated with a working volume of 1.7 L of production medium and were carried out with the best operational conditions (aeration and stirring rates) determined in previous batch cultures experiments (section 3.3.2).

The stepwise fed-batch experiments were initiated with a batch culture, as described above. After a batch period of 72 hours, a pulse of crude glycerol was added to the bioreactor when glycerol concentration was close to zero, in order to obtain a concentration near to 100 $g \cdot L^{-1}$.

3.4 Analytical methods

Samples were periodically collected to determine the concentration of biomass, glycerol, erythritol and mannitol. Biomass concentration was measured by optical density (O.D) at 600 nm and later converted to dry cell mass *per* liter, through a calibration curve for each strain (Table 1, Annex 1). Glycerol, erythritol and mannitol concentrations were determined by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H ion-exchange column (300 x 7.8 mm and 8 μm size particle) attached to a refractive index (RI) detector (Jasco RI-4030). The column temperature was 60 °C and it was eluted with H_2SO_4 5 mM at 0.5 $mL \cdot min^{-1}$. To HPLC analysis, 2 mL of each collected sample was centrifuged and therefore the respective supernatant was filtered through a syringe filter with a pore size of 0.22 μm.

3.5 Kinetic and yield parameters

For all the experiments in flask and bioreactor, different parameters were calculated from experimental data, such as biomass and erythritol yields, as well as, erythritol productivity .

Biomass yield ($Y_{X/S}$), expressed in grams of biomass produced *per* grams of substrate consumed, was calculated through the Equation 4:

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \qquad \text{Equation 4}$$

where ΔX is the variation of biomass concentration produced and ΔS is the variation of glycerol concentration (glycerol consumed) in the same period.

Product yield or erythritol yield ($Y_{E/S}$), expressed in grams of erythritol produced *per* grams of substrate consumed, was calculated using the Equation 5:

$$Y_{E/S} = \frac{\Delta E}{\Delta S} \qquad \text{Equation 5}$$

where ΔE is the variation of erythritol concentration produced.

Erythritol productivity (Q_E), express in grams of erythritol *per* liter, *per* hour, was calculated according the Equation 6:

$$Q_E = \frac{\Delta E}{\Delta t} \qquad \text{Equation 6}$$

where Δt is the variation of time in hours.

4. RESULTS AND DISCUSSION

In the present work the performance for erythritol production of three strains of *Y. lipolytica* and two of *Y. divulgata* was compared in flasks batch cultures. Erythritol production was monitored and *Y. lipolytica* species proved to be the best erythritol producer. The strains *Y. lipolytica* W29 and *Y. lipolytica* Ch 3/4 were selected for the scale-up of the bioprocess.

The effect of different k_La values on erythritol production in batch cultures in a STR bioreactor were analysed. A k_La value of 162 h^{-1}, obtained at 3 vvm of aeration rate and 900 rpm of stirring rate was found to be optimal for erythritol production and for optimal oxygenation. With the scale-up of the bioprocess from flask to bioreactor, a 2.4-fold and a 3.3-fold increase on productivity was obtained for the strain W29 and the strain Ch 3/4, respectively, reducing the experiment time from 168 hours to only 72 hours.

The increase of initial glycerol concentration from 100 $g \cdot L^{-1}$ to 200 $g \cdot L^{-1}$ in batch experiments was attempted but the yeast cells did not grow and produced erythritol. Thus, a stepwise fed-batch experiment with a supplementary addition of glycerol, allowed to reach almost the double of erythritol concentration for both strains, without decreasing productivity. In this operation mode the highest titer of erythritol (64 $g \cdot L^{-1}$), that correspond to 0.42 $g \cdot g^{-1}$ yield and 0.45 $g \cdot L^{-1} \cdot h^{-1}$ productivity, was obtained with *Y. lipolytica* W29.

4.1 *Yarrowia* strain selection

The production capacity of a given metabolite depends not only on the culture conditions, but also on the species and strain used. The species *Y. lipolytica* has been widely studied for the production of several metabolites of interest, having already been described as an erythritol producer [16,44,73]. Similarly, erythritol production has also been studied for other species of the genus *Yarrowia* [20]. Nevertheless, there are still a recent interest in finding new natural strains with improved performance on crude glycerol utilization and on polyols production [74].

Motivated by the nowadays interest on this issue, the ability to produce erythritol from crude glycerol was evaluated in strains belonging to two species of the genus *Yarrowia*: three strains of the species *Y. lipolytica* (W29, Ch1/5 and Ch 3/4) and two of the species *Y. divulgata* (M445/4 and 5257/2). Glycerol has selected as the carbon source, once it has been described as the carbon source from which a higher erythritol production by *Yarrowia* strains was obtained [20,38]. Yang *et al.* [38] described glycerol as a better carbon source than glucose since it promotes an increase in osmotic pressure, which is a premise for higher erythritol production. The glycerol used in this study was crude glycerol, a by-product of the biodiesel industry. The use of this by-product aims to make the process more sustainable and profitable.

To evaluate erythritol production capacity, five *Yarrowia* strains grew up under optimal conditions for the production of erythritol in baffled flasks for seven days (168 hours). Periodic sampling was performed every 24 hours in order to determine the cellular growth, glycerol uptake and erythritol production profiles for each strain (Figure 9).

Under the culture conditions used, all strains were able to grow (Figure 9A) and produce erythritol (Figure 9C), however none of the strains were able to consume the totally of glycerol in the time of the experiment (Figure 9B). The strain that attained the lowest biomass concentration (Figure 9A) was *Y. lipolytica* Ch3/4 (13 ± 3 $g \cdot L^{-1}$), while the growth of the other four strains was similar. This lower growth may be due to the culture conditions used, not being the best conditions for the growth of this specific strain, or the strain may have been shifting its metabolism to the production of erythritol and other metabolites at the expense of growth. The glycerol consumption profile (Figure 9B) was similar for the strains of *Y. lipolytica* species, but the two strains of *Y. divulgata* showed a lower glycerol consumption, than *Y. lipolytica*. Despite the lower consumption rate of glycerol, the growth of *Y. divulgata* strains were similar to *Y. lipolytica* but the erythritol production were lower. The strain that produced a higher erythritol concentration in flask (Figure 9C) was *Y. lipolytica* W29 (34 ± 6 $g \cdot L^{-1}$), followed by *Y. lipolytica* Ch 3/4 (25 ± 6 $g \cdot L^{-1}$) that also

stood out. *Y. lipolytica* W29 it was the one that also has the higher and faster consumption of glycerol overtime, probably associated with the higher erythritol production (Figure 9B). For all the strains only residual amounts of the by-product mannitol (0.8 - 2.1 g·L^{-1}) were detected in the culture medium after seven days (data not shown).

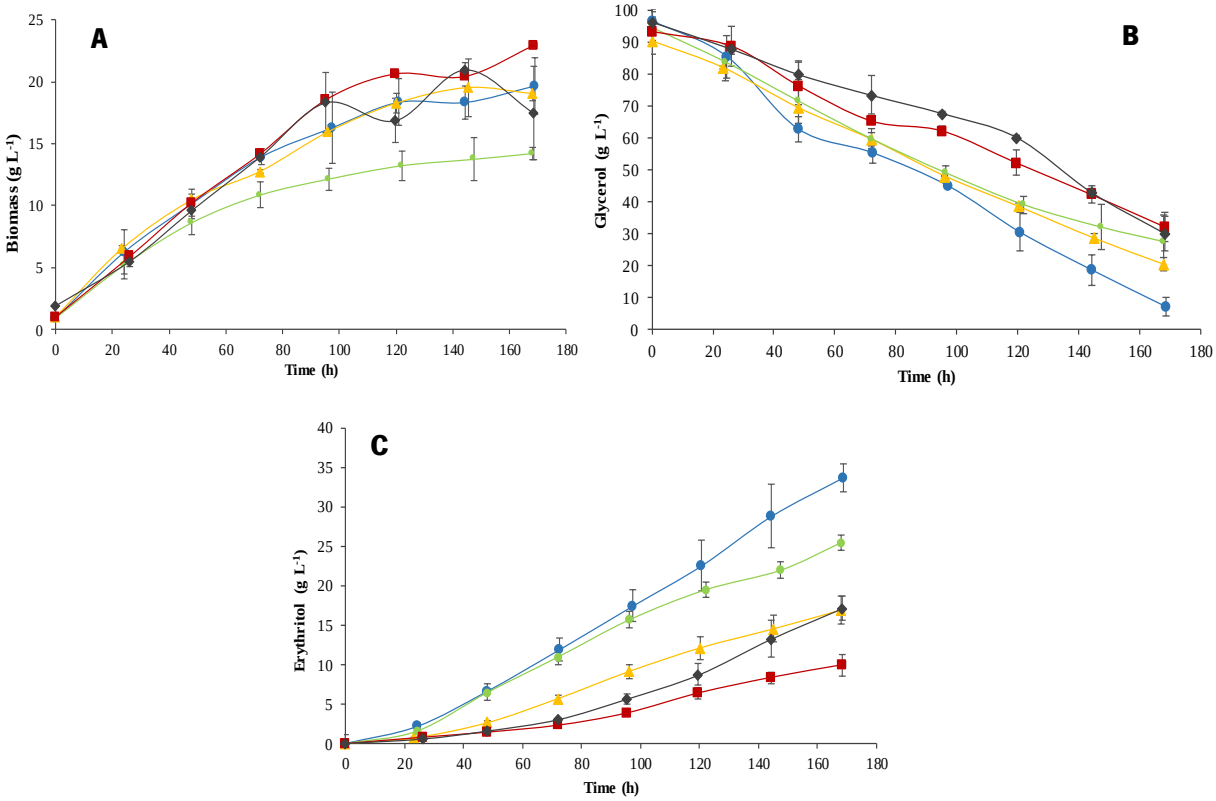


Figure 9 – Time course of cellular growth (**A**), glycerol consumption (**B**) and erythritol production (**C**) in batch cultures in flasks of *Y. lipolytica* strains W29 (●), Ch1/5 (▲), Ch3/4 (●) and *Y. divulgata* strains M445/4 (▪) and 5257/2 (◆). The error bars represent the standard deviation of two independent replicates.

For a more accurate comparison of erythritol production, the parameters of biomass yield, erythritol yield and erythritol productivity by all strains were calculated (Table 2). It is clear from the values on table 2 that *Y. lipolytica* strain W29 and strain Ch 3/4 were the strains for which the yields were the highest, being statistically equal between both, but statistically superior than the values obtained for the other strains. Although, for the strains Ch 1/5, 5257/2 and Ch 3/4 there were no significant differences between them, relatively to erythritol productivity. Despite this, the erythritol concentrations obtained for *Y. lipolytica* Ch 1/5 (17 ± 2 g·L^{-1}) and *Y. divulgata* 5257/2 (17 ± 2 g·L^{-1}), were lower than the one obtained by the *Y. lipolytica* Ch 3/4 strain. Regarding to biomass yield (Table 2), only the strain M445/4 showed statistically significant difference with the strains W29 and Ch 3/4, once these strains present the lowest biomass yields, unlike the M445/4

strain that showed the highest biomass yield. Even though, the strain M445/4 was the one that produced the lowest erythritol concentration which is reflected in the low values obtained for product yield and productivity. The strain *Y. lipolytica* W29 was the one that reach the highest erythritol productivity, but it proved to be statistically equal to the value obtained in experiments with the strain *Y. lipolytica* Ch 3/4 (Table 2).

Table 2 – Biomass yield ($Y_{X/S}$), erythritol yield ($Y_{E/S}$) and erythritol productivity (Q_E) for *Y. lipolytica* strains W29, Ch 1/5, Ch 3/4 and *Y. divulgata* strains M445/4, 5257/2 in batch cultures. The values are presented as the average and standard deviation of two independent experiments. Different letters above the results represent statistically significant differences between strains ($p < 0.1$).

	Y. lipolytica			***Y. divulgata***	
	W29	**Ch 1/5**	**Ch 3/4**	**M445/4**	**5257/2**
$\mathbf{Y_{X/S}}$ ($g \cdot g^{-1}$)	$(0.22 \pm 0.06)^{a}$	$(0.26 \pm 0.02)^{ab}$	$(0.19 \pm 0.03)^{a}$	$(0.36 \pm 0.05)^{b}$	$(0.24 \pm 0.08)^{ab}$
$\mathbf{Y_{E/S}}$ ($g \cdot g^{-1}$)	$(0.375 \pm 0.005)^{a}$	$(0.24 \pm 0.02)^{b}$	$(0.38 \pm 0.06)^{a}$	$(0.16 \pm 0.09)^{b}$	$(0.26 \pm 0.04)^{b}$
$\mathbf{Q_E}$ ($g \cdot L^{-1} \cdot h^{-1}$)	$(0.20 \pm 0.03)^{a}$	$(0.10 \pm 0.01)^{bc}$	$(0.15 \pm 0.04)^{ab}$	$(0.059 \pm 0.008)^{c}$	$(0.102 \pm 0.009)^{bc}$

Comparing the two species studied, the strains of the species *Y. lipolytica* demonstrated better results than those of the *Y. divulgata* species. Rakicka *et al.* [20] studied the erythritol production by strains of several species of the genus *Yarrowia*, including a strain of *Y. lipolytica* and another one of *Y. divulgata*. When they compared the results of these two species, they observed that *Y. divulgata* produced higher erythritol concentrations than *Y. lipolytica*. In this study, the experiments were performed using different culture media to stimulate the production of different metabolites: a control medium, a medium for the production of citric acid and another one for the production of erythritol. The highest erythritol concentration observed by *Y. divulgata* was obtained in the control medium, being this same medium used to stimulate the production of erythritol in flask [20]. This control medium was quite different from the production medium used in this present work once it had a different pH and did not contain NaCl. These slightly changes in culture conditions may justify this difference in the most productive species. It should also be noted that in this present study we used the optimal medium and conditions to stimulate the production of erythritol for strains of *Y. lipolytica*, which means acidic pH and high salt concentration, that could not be the optimal culture conditions for *Y. divulgata*. Finally, in the study of Rakicka *et al.* [20], the *Y. lipolytica* strain was not exposed to high osmotic pressures, contrary to what happened in this present study, which may explain the low erythritol concentration reported by them for this species.

4.2 Erythtitol production in bioreactor

4.2.1 Batch cultures

According to the results obtained in flask experiments, the better erythritol producer's strains were *Y. lipolytica* W29 and *Y. lipolytica* Ch 3/4. Since the higher erythritol concentration was obtained with the strain W29, this was the strain selected to study the effect of oxygenation conditions, assessed by k_La values for different aeration and stirring rates and also to explore different operation modes in a STR bioreactor. Previous studies conducted by the group [58,75] showed that for bioprocesses with *Y. lipolytica* the operation at constant stirring and aeration rates, thus at variable DOC in the medium was more suitable than keeping DOC controlled, so in this present study in bioreactor experiments variable DOC conditions were studied.

Firstly, k_La values were determined at aeration and stirring rates selected (Table 3), in experiments carried out in a 3.7 L stirred tank reactor.

Table 3 - Experimental k_La values determined by static gassing-out method under different experimental conditions. The values are presented as the average and standard deviation of three independent experiments.

Experimental conditions		**k_La (h^{-1})**
Aeration rate (vvm)	**Stirring rate (rpm)**	
1	400	15 ± 1
2	600	70 ± 1
3	900	162 ± 3

As expected in a STR bioreactor, the continuous increase of aeration and stirring rates led to an enhancement of k_La value. By incrementing the aeration rate from 1 vvm to 3 vvm and the stirring rate from 400 rpm to 900 rpm, an 11-fold improvement in k_La values were obtained. In a previous work Ferreira *et al.* [29], in the same bioreactor, but with a citric acid production medium, obtained the same behaviour for the increment of k_La with the increase of aeration and stirring rates. They also observed an 18-fold improvement in k_La values by increasing the aeration rate from 1 vvm to 3 vvm and the stirring rate from 200 rpm to 600 rpm [29]. Besides the differences in the stirring rate range used in both works, the differences may also be explained by the fact that culture medium used in both experiments are not the same (lower pH and higher salt concentration in the present work).

Since *Y. lipolytica* is a strictly aerobic yeast, oxygen availability in the medium influences the production of erythritol. However, to our best knowledge, only one work is available in literature, addressing this topic. Thus, in this work different operational conditions were tested to evaluate the influence of DOC in erythritol production, namely the use of constant aeration and stirring rates, according with Table 3. Several batch experiments were performed with *Y. lipolytica* W29 under these conditions and the results obtained are depicted in Figure 10.

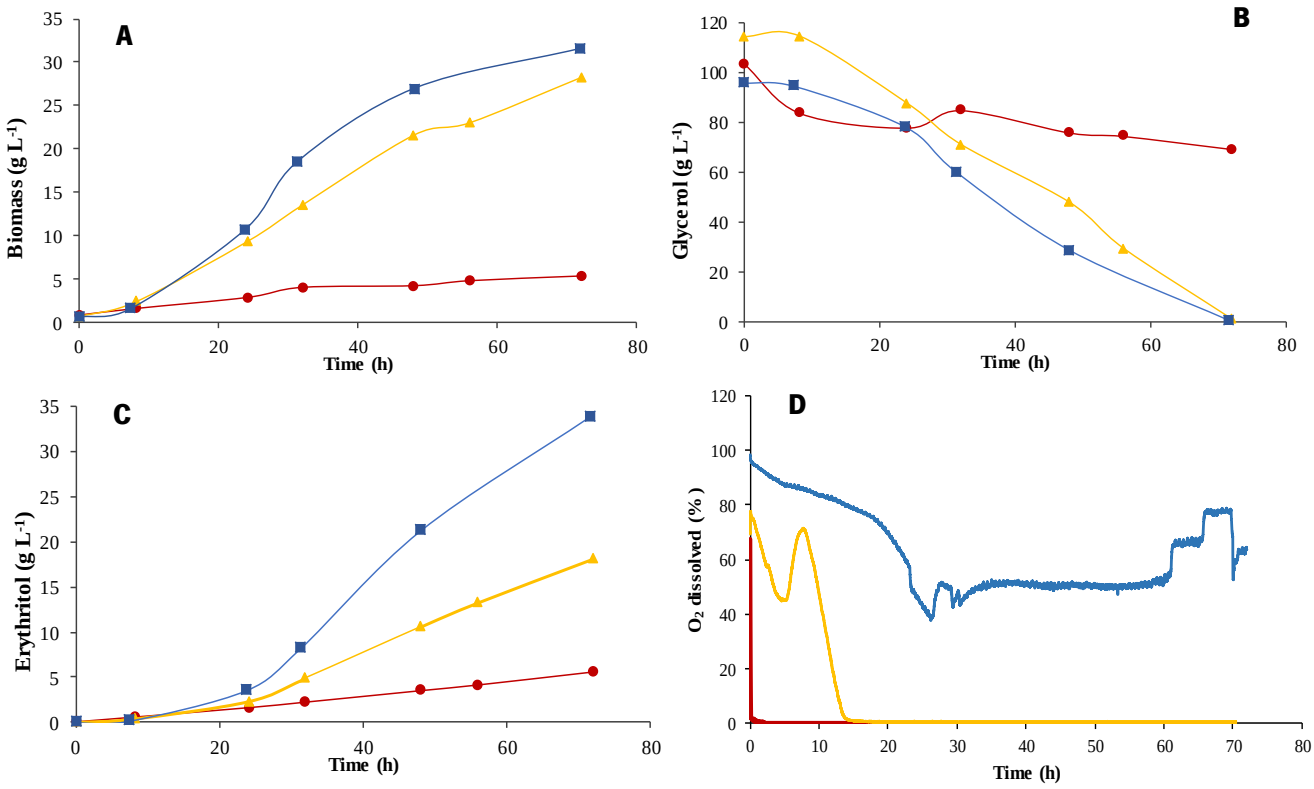


Figure 10 – Time course of cellular growth (**A**), glycerol consumption (**B**), erythritol production (**C**) and dissolved oxygen concentration (**D**) in batch cultures in bioreactor of *Y. lipolytica* W29, at different k_La values: 15 h^{-1} (●), 70 h^{-1} (▲) and 162 h^{-1} (■).

The raise of aeration and stirring rates from 1 vvm to 3 vvm and from 400 rpm to 900 rpm, respectively, had a clearly positive impact on erythritol production (Figure 10C), where an increase of k_La from 15 h^{-1} to 162 h^{-1} (10.8-fold increase) led to a 7-fold improvement of erythritol production. At the lowest k_La (15 h^{-1}), the cellular growth, glycerol consumption and erythritol production were also the lowest. This can be explained by the completely depletion of oxygen from the medium observed throughout all the process (Figure 10D), which cause no oxygen availability for yeast cells metabolism. The depletion of oxygen from the medium was also observed in the experiment with the k_La value of 70 h^{-1}, at 13 hours of operation (Figure 10D). Additionally, glycerol

consumption and cellular growth profiles were similar for the experiments at middle and maximum k_La values (70 h^{-1} and 162 h^{-1}, respectively) (Figure 10A and 10B). When compared with the flask experiments, glycerol uptake was faster for the bioreactor cultures operating at the two highest k_La values, thus reducing the total operation time from 168 hours to 72 hours. Like in the flask experiments, residual amounts of the by-product mannitol (0.32 - 3.2 $g{\cdot}L^{-1}$) were detected in all the three tested conditions.

The results shown are in accordance with the different profiles of DOC in the medium (Figure 10D) observed, under different conditions. Through the first hours of yeast cultivation, that corresponds to the exponential growth phase, a sharpest decrease of DOC in the medium was observed, particularly for the lowest k_La condition tested. In the experiment with the highest k_La, DOC in the medium dropped to 40 % in the first 24 hours of the experiment, but later increased slightly and stabilized around 55 %, during the erythritol production (Figure 10D). In the phase of erythritol production, that occurs after the nitrogen source had been entirely consumed, the oxygen requirement is lower and a raise of DOC in the medium is expected [68]. The augmented DOC in the last hours of the batch experiment is due to the deceleration of cells metabolism, that corresponds to the decline of growth rate, entering the cells in the beginning of stationary phase. Contrary to what was observed for the other two conditions, with a higher k_La, that means higher aeration (3 vvm) and stirring (900 rpm) rates, the depletion of dissolved oxygen concentration from the medium was prevented.

In the batch experiment where the k_La was the highest (162 h^{-1}), after 8 hours of lag phase, where glycerol consumption and erythritol production were null, yeast cells grew to a maximum concentration of 30.8 $g{\cdot}L^{-1}$, registered at 72 hours (Figure 10A). This lag phase is due to the culture conditions that cells are initially exposed to once the transfer of yeast cells to a medium with acidic pH and higher osmotic pressure force cells to undergo in a longer adaptation phase to culture medium. This higher number of hours of the lag phase, associated to the increase of the osmotic pressure was described by Tomaszewska-Hetman and Rywińska [76], when they studied the effect of increased NaCl concentration on cellular growth and on erythritol production.

The increase of k_La to higher values increased, as expected, the DOC in the medium (Figure 10D). This augmented availability of oxygen concentration dissolved in the medium resulted in a higher biomass concentration and, thereafter, in a higher erythritol production (Figure 10). The erythritol produced in the batch experiment with higher k_La exceed in 28 $g{\cdot}L^{-1}$ the erythritol titer produced in the experiments with lower k_La value. The higher erythritol concentration (34 ± 2 $g{\cdot}L^{-}$

$^{-1}$), and in turn the highest product yield and productivity, were observed in the conditions of the highest k_La, in the batch experiments (Table 4).

Table 4 - Erythritol yield ($Y_{E/S}$) and productivity (Q_E) for *Y. lipolytica* W29 in batch experiments under different k_La values.

Experimental conditions - k_La (h^{-1})	$Y_{E/S}$ ($g \cdot g^{-1}$)	Q_E ($g \cdot L^{-1} \cdot h^{-1}$)
15	0.16	0.08
70	0.16	0.25
162	0.35 ± 0.03	0.47 ± 0.03

A 2.2-fold and a 5.9-fold improvement in erythritol yield and productivity, respectively, was observed increasing the k_La from 15 h^{-1} to 162 h^{-1} (Table 4). The lowest erythritol yield (0.16 $g \cdot g^{-1}$) and productivity (0.08 $g \cdot L^{-1} \cdot h^{-1}$) were reported for the batch experiments with the lowest k_La, where a completely depletion of oxygen from the medium was observed (Figure 10D). Comparing the results obtained in the bioreactor experiments with the highest k_La with those in flask experiments, the glycerol conversion into their products is similar but the biomass yield is higher. Furthermore, glycerol consumption was drastically higher and faster in the bioreactor experiments (Figure 9B and 10B). With the scale-up of the bioprocess, a 2.35-fold improvement in erythritol productivity was observed, however the erythritol yields remained equal. With all these results it is plausible to affirm that the oxygen availability in the medium is a crucial parameter in the erythritol production optimization. Rywińska *et al.* [48] evaluated the effect of different aeration and stirring rates in erythritol production, using a mutant strain *Y. lipolytica* Wratislavia K1 and crude glycerol as carbon source. They observed that with the increase of the aeration and stirring rates from 0.36 vvm to 0.6 vvm and from 600 rpm to 800 rpm, respectively, the erythritol concentration increased about 16 $g \cdot L^{-1}$, like the results obtained in the present work. In this study, they also reported a product yield (0.38 $g \cdot g^{-1}$) similar to the one obtained in this present work, however with a higher productivity (0.78 $g \cdot L^{-1} \cdot h^{-1}$). Rywińska *et al.* [48] described, as well, a 1.3-fold increase in productivity when the aeration rate used was 0.6 vvm and the stirring rate was 800 rpm. Likewise, in another study from Tomaszewska *et al.* [52], where *Y. lipolytica* Wratislavia K1 and crude glycerol as carbon source were used, they reported that at an aeration rate of 0.36 vvm and a stirring rate of 800 rpm led to an erythritol yield of 0.4 $g \cdot g^{-1}$ and an erythritol productivity of 0.6 $g \cdot L^{-1} \cdot h^{-1}$. It is noteworthy that the results obtained by Tomaszewska *et al.* [52], are similar to the ones observed in the present work.

Although in the herein reported study higher aeration rate was used, the values of erythritol yield and productivity are similar to those obtained in the works of Rywińska *et al.* [48] and Tomaszewska *et al.* [52], probably because the strain that they used is a mutant strain, unlike the one that was used here that is a wild-type strain.

Considering all the results obtained, further experiments were performed with constant aeration and stirring rates of 3 vvm and 900 rpm, respectively, once with these conditions the depletion of DOC in the medium can be avoid.

Taking into account that in flask experiments the better erythritol producer's strains were *Y. lipolytica* W29 and *Y. lipolytica* Ch 3/4, batch experiments with the better stirring and aeration conditions were also performed for *Y. lipolytica* Ch 3/4. This batch experiments were performed in order to understand if there were significant differences between the erythritol production by the two strains at high scale. The cellular growth, the glycerol consumption, the erythritol production and the DOC in the medium profiles are represented in Figure 11.

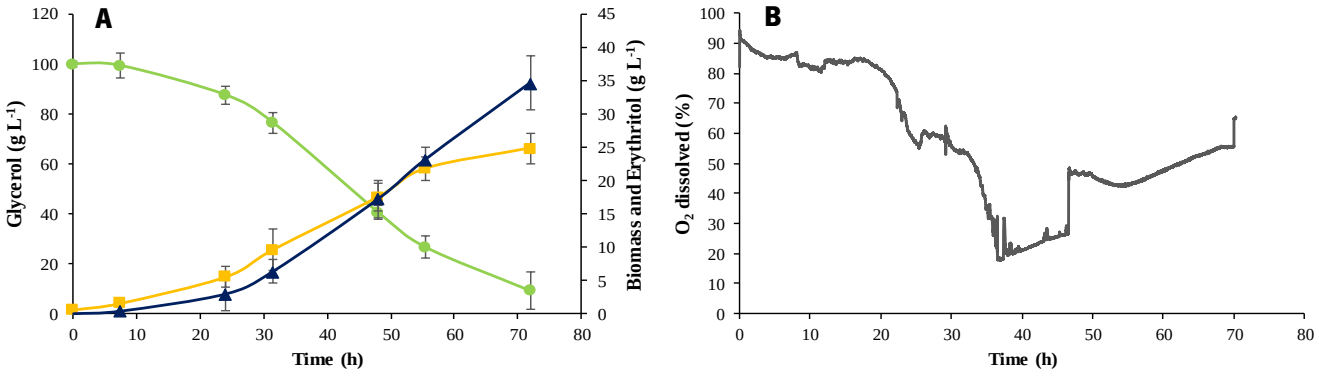


Figure 11 - (A) Time course of cellular growth (■), glycerol consumption (●) and erythritol production (▲) and **(B)** dissolved oxygen profile in batch culture in bioreactor of *Y. lipolytica* Ch 3/4, which occurred in the following constant conditions: a stirring rate of 900 rpm and an aeration rate of 3 vvm. The error bars represent the standard deviation of two independent replicas.

In this batch experiment, in the first 12 hours no cellular growth was observed, which was reflected in the low consumption of glycerol and in the null production of erythritol (Figure 11A). Once again, this big lag phase can be justified by the higher osmotic pressure and acidic pH that cells are exposed to when transferred to the production medium. After this lag phase, yeast cells started to grow exponentially and a continuous erythritol production was observed until a maximum concentration of 35 ± 4 $g \cdot L^{-1}$, registered at 72 hours (Figure 11A). This erythritol production translated to a product yield of 0.38 ± 0.09 $g \cdot g^{-1}$, and a productivity of 0.5 ± 0.2 $g \cdot L^{-1} \cdot h^{-1}$. Contrary to what was observed for the batch experiments with *Y. lipolytica* W29, in the same conditions, the

glycerol was not totally consumed after 72 hours. Like in the last batch experiments, mannitol was produced at low concentrations but at higher titers (6.6 ± 0.4 $g \cdot L^{-1}$) than the ones achieved by *Y. lipolytica* W29.

Throughout the experiment it was not observed any kind of depletion of DOC in the medium, but the DOC profile was distinct from that one obtained with the strain W29 (Figure 10D and 11B). A gradual decrease in DOC was observed during the first 38 hours of experiment, that can be justified by the higher oxygen need from yeast cells over time once they are starting to grow exponentially. After this period, the DOC in the medium stabilized close to 20 %, only for 12 hours. As seen before, in the last hours of the experiment, the DOC in the medium augmented, due to cells metabolism that are reaching the stationary phase. Comparing the results obtained in bioreactor with those obtained in flask, with the scale-up there was an increase of 10 $g \cdot L^{-1}$ in the erythritol concentration produced by the strain Ch 3/4 (Figure 9C and 11A). However, the biggest highlight goes to erythritol productivity once the batch time was reduced, resulting in a 3.3-fold increase of the erythritol productivity. The biomass yield (0.27 ± 0.08 $g \cdot g^{-1}$) was similar to the one obtained in flask and the glycerol conversion into erythritol remains the same. The results obtained with the strains *Y. lipolytica* W29 and *Y. lipolytica* Ch 3/4 are comparable with other studies that apply *Y. lipolytica* strains in batch mode experiments, using crude glycerol as a carbon source. However, the strains used in these works are mutants, unlike the W29 and Ch3/4 that are wild-type strains. In the literature, the yield values reported range from 0.34 $g \cdot g^{-1}$ to 0.56 $g \cdot g^{-1}$, but higher productivities are reported, varying between 0.60 $g \cdot L^{-1} \cdot h^{-1}$ and 1.00 $g \cdot L^{-1} \cdot h^{-1}$ [15,48,53,63]. It should be noted that the majority of these erythritol productivities were obtained using mutant strains.

The erythritol concentration produced by both strains was practically the same, however the biomass yield obtained in bioreactor experiments was higher for the strain W29. Regarding to erythritol productivity and glycerol conversion into the respective product (erythritol), the obtained values were quite similar for the two strains. Hereupon, with the scale-up of the biotechnological process both strains were capable to produce erythritol in batch experiments, reaching similar values between them, both for erythritol concentration, erythritol yield and for erythritol productivity. It has also been proven that there were no significant differences between the two strains in batch cultures.

During the batch experiments, the erythritol production was always increasing overtime without a stabilization or decreasing in the production rate. This profile leaves open the hypothesis that erythritol production would continue if the carbon source had not run out. Considering that erythritol

production ends when glycerol is completely consumed, experiments using a higher initial glycerol concentration (200 g·L^{-1}), in batch culture, were performed. The biomass concentration, glycerol consumption, erythritol production and DOC profiles for both strains (W29 and Ch3/4) are represented in Figure 12.

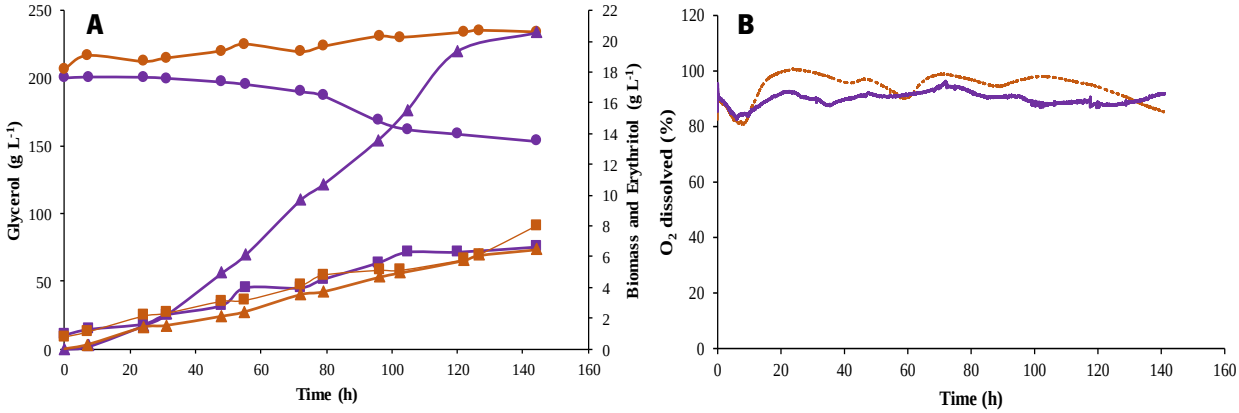


Figure 12 - (A) *Y. lipolytica* W29 cellular growth (■), glycerol consumption (●) and erythritol (▲) production profiles, *Y. lipolytica* Ch 3/4 cellular growth (■), glycerol consumption (●) and erythritol (▲) production profiles and **(B)** dissolved oxygen profile in batch culture for *Y. lipolytica* W29 (- - -) and for *Y. lipolytica* Ch 3/4 (—), which occurred in the following conditions: a stirring rate of 900 rpm, an aeration rate of 3 vvm and an initial glycerol concentration of 200 g·L^{-1}.

The raise of initial glycerol concentration from 100 g·L^{-1} to 200 g·L^{-1}, had a clearly negative impact on cellular growth and on erythritol production for both strains (Figure 12A). For *Y. lipolytica* W29, the carbon source was not consumed at all, maintaining its glycerol consumption profile constant over the 148 hours of the experiment. The residual biomass and erythritol concentrations produced during all the experiment for the strain W29 were 8.0 g·L^{-1} and 6.5 g·L^{-1}, respectively. Also, the DOC in the medium remained almost the same overtime, near the 100 %, which corroborates the results obtained for cellular growth (Figure 12B).

The same behaviour concerning DOC in the medium profile was observed for *Y. lipolytica* Ch 3/4, but in this case, cells grow up to a maximum of 6.7 g·L^{-1} after 148 hours of yeast cultivation (Figure 12A). 80 Hours after the start of the experiment, the concentration of crude glycerol decreased, being consumed about 46.7 g·L^{-1} until the end of the experiment, with the concomitant erythritol production of 20.6 g·L^{-1}. This result indicates that the strain Ch 3/4 seems to be more resistant to high crude glycerol concentrations than the strain W29 once this strain was more metabolically inhibited for erythritol production than the strain Ch 3/4. Tomaszewska *et al.* [53] studied the ability of *Y. lipolytica* strains Wratislavia K1, A UV'1 and A-15, to produce erythritol in

bioreactor batch cultures, using crude glycerol as carbon source. The best performance was obtained with *Y. lipolytica* Wrastislavia K1, with an erythritol yield of 0.49 $g \cdot g^{-1}$ and a productivity of 1.00 $g \cdot L^{-1} \cdot h^{-1}$. In this study the initial crude glycerol concentration was only of 150 $g \cdot L^{-1}$, 50 $g \cdot L^{-1}$ less than the concentration used in this present work, and the NaCl concentration in the medium was, in total, 25 $g \cdot L^{-1}$. This means that they only supplemented the production medium with 17.6 $g \cdot L^{-1}$ of NaCl, being the remaining concentration present in crude glycerol impurities fraction. This production medium was quite different from the one used in this present work, once the final NaCl concentration used here was approximately 40 $g \cdot L^{-1}$. These changes in culture conditions, that could affect the yeast growth, may justify the low production of erythritol observed. Lastly, in the study of Tomaszewska *et al.* [53], the strains that attained a better performance were Wratislavia K1 and A UV'1, two mutant strains, unlike those used in this present work that are wild-type strains.

In several another studies available in the literature [19,48,64], the erythritol production in different operation modes using an initial crude glycerol concentration higher than 150 $g \cdot L^{-1}$, is only reported when they do not supplement the production medium with NaCl. For instance, Nikolaou *et al.* [77] analysed the relative sensitivity of different fungal species, namely *Y. lipolytica*, to osmotic stress imposed by the presence of diverse NaCl concentrations in the medium, up to 175 $g \cdot L^{-1}$. They observed that the growth of this species was slightly inhibited by 58 $g \cdot L^{-1}$ NaCl, and no growth was observed for concentrations above 116 $g \cdot L^{-1}$ NaCl. Comparing with other species, *Y. lipolytica* has a high resistance to NaCl, being *Saccharomyces cerevisiae*, *Kluyveromyces lactis* and *Ashbya gossypii* much more sensitivity once their growth is totally inhibited by 88 $g \cdot L^{-1}$ NaCl. In this present study we used a NaCl concentration of approximately 45 $g \cdot L^{-1}$, that can justify the slightly cellular growth inhibition during all the experiment, and therefore the residual erythritol concentration produced. Using an initial crude glycerol concentration of 200 $g \cdot L^{-1}$ and supplementing the production medium with 25 $g \cdot L^{-1}$ NaCl, the cells will suffer a high osmotic stress, being therefore unable to grow and produce erythritol. Although *Y. lipolytica* withstanding high osmotic pressures, the pressure at which this yeast cells are subjected in these present batch experiments was too high.

4.2.3 Stepwise fed-batch cultures

As the initial concentration of 200 g·L^{-1} crude glycerol reduced cell growth and erythritol production, the hypothesis that erythritol production would continue if the carbon source had not run out in batch mode, it is still open. Considering these, experiments using stepwise fed-batch, a fed-batch with the addition of one pulse of crude glycerol, were performed. The stepwise fed-batch started after a 72 hours batch culture, when the crude glycerol concentration was already close to zero. At this time a pulse of 100 g·L^{-1} (approximately 195 mL) of crude glycerol was added. Since crude glycerol would be added directly to the culture medium, it is expected a very low dilution of the biomass once the dilution will be 1:1.12. The cellular growth, glycerol consumption and erythritol production profiles are shown at Figure 13.

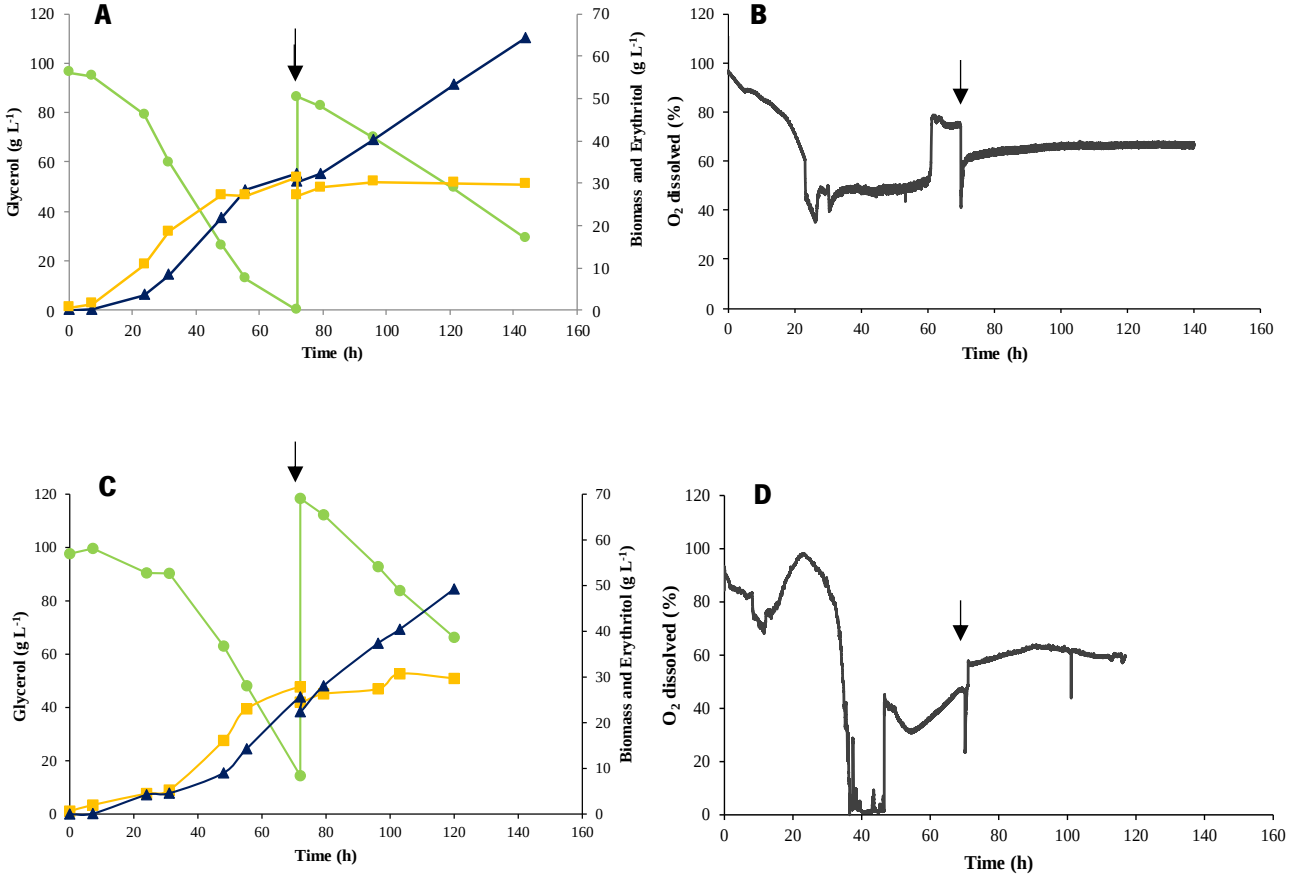


Figure 13 - (A) *Y. lipolytica* W29 cellular growth (■), glycerol consumption (●) and erythritol (▲) production profiles, **(B)** dissolved oxygen profile in stepwise fed-batch culture of *Y. lipolytica* W29, **(C)** *Y. lipolytica* Ch 3/4 cellular growth (■), glycerol consumption (●) and erythritol (▲) production profiles and **(D)** dissolved oxygen profile in stepwise fed-batch culture of *Y. lipolytica* Ch 3/4, which occurred in the following constant conditions: a stirring rate of 900 rpm and an aeration rate of 3 vvm. The arrow indicates the moment when 100 g·L^{-1} of crude glycerol was added into the bioreactor.

The cellular growth and production profiles, during the first 72 hours of experiment (Figure 13), were very similar to the experiment in batch mode for both strains (Figure 10 and 11). For the strain Ch 3/4 between the 35 and 50 hours of batch experiment, a sharp cellular growth was observed, which was later reflected in the accentuated decrease of DOC in the medium (Figure 13C and 13D). In the last 16 hours of the batch phase, for both experiments, a deceleration on cellular growth rate was observed (Figure 13A and 13C), which can explain the increase of the DOC in the medium (Figure 13B and 13D). At the end of the batch period, for the strain W29 and the strain Ch 3/4 a maximum biomass of 31 $g \cdot L^{-1}$ and 28 $g \cdot L^{-1}$ were attained and the concentration of erythritol produced were 32 $g \cdot L^{-1}$ and 25 $g \cdot L^{-1}$, respectively.

The crude glycerol pulse occurred after 72 hours of batch culture, which resulted in a change in the glycerol concentration present in the medium from 0 to 86 $g \cdot L^{-1}$ in the case of W29 strain and from 14 to 118 $g \cdot L^{-1}$ for the strain Ch 3/4 (Figure 13A and 13C). With the addition of the crude glycerol pulse to the medium, the DOC in the medium decreases instantly but increases immediately thereafter, remaining in both cases at 60 - 70 % (Figure 13B and 13D). After the addition of the crude glycerol pulse, the erythritol production continued to increase until the end of the experiment, reaching an erythritol concentration of 64 $g \cdot L^{-1}$ for the strain W29 and 49 $g \cdot L^{-1}$ for Ch 3/4 strain. During the fed-batch phase the biomass concentration remained almost constant and unalterable, in both cases (Figure 13A and 13C). The absence of cellular growth may be explained by the fact that the pulse added to the culture medium consists only of crude glycerol, thus leading to a limitation of nitrogen and phosphate that are necessary to cellular growth. During this phase of erythritol production with constant cell concentration a constant profile of DOC in the medium was observed overtime, for both strains (Figure 13B and 13D). Thus, the previous selected oxygen transfer conditions in batch cultures were still adequate to prevent oxygen depletion in the stepwise fed-batch cultures. Moreover, the requirements of oxygen were lower after the pulse since DOC was only consumed for erythritol production but not needed for cellular mass biosynthesis, since cell concentration was constant. Mannitol concentration detected in the final of the experiments were higher than the one obtained in batch cultures, although the concentrations for the strain W29 and the strain Ch 3/4 were still very low (2.23 $g \cdot L^{-1}$ and 10.05 $g \cdot L^{-1}$, respectively). However, it should be noted that with the strain W29 a more specific erythritol production is confirmed than for the strain Ch 3/4.

For a more accurate comparison of erythritol production between the two strains in study, in the different phases of the experiment, the parameters erythritol yield and erythritol productivity were calculated (Table 5).

Table 5 - Erythritol yield ($Y_{E/S}$) and erythritol productivity (Q_E) calculated for the stepwise fed-batch experiment and for each phase (batch phase and fed-batch phase), for *Y. lipolytica* W29 and *Y. lipolytica* Ch 3/4.

	***Y. lipolytica* W29**			***Y. lipolytica* Ch 3/4**		
	Batch phase (until 72 hours)	Fed-batch phase (after 72 hours)	Stepwise Fed-Batch (144 hours)	Batch phase (until 72 hours)	Fed-batch phase (after 72 hours)	Stepwise Fed-Batch (120 hours)
$\mathbf{Y_{E/S}}$ ($g \cdot g^{-1}$)	0.34	0.56	0.42	0.31	0.45	0.36
$\mathbf{Q_E}$ ($g \cdot L^{-1} \cdot h^{-1}$)	0.45	0.45	0.45	0.21	0.20	0.34

In the fed-batch phase, after the addition of crude glycerol, a 1.6-fold and 1.5-fold in erythritol yield was observed for the strain W29 and Ch 3/4, respectively (Table 5). This increase is due to the glycerol deviation for the production of erythritol in detriment of cell growth, once in the phase after the pulse no cellular growth was observed, so more glycerol was available to be converted into erythritol (Figure 13A and Figure 13C). In spite of a higher yield being reported in the fed-batch phase, the values of productivity for the two strains in study were equal in both phases, 0.45 $g \cdot L^{-1} \cdot h^{-1}$ for the strain W29 and 0.20 $g \cdot L^{-1} \cdot h^{-1}$ for Ch 3/4 strain (Table 5). In a study carried out by Mirończuk *et al.* [15], where they used *Y. lipolytica* MK1, a mutant strain, similar results were reported. They evaluated the performance of this yeast to produce erythritol, in repeated batch cultures, where a percentage (30 % or 40 %) of culture medium was punctually replaced by the same amount of fresh medium [15]. In this study, and during the 2nd batch phase, they observed an increase in erythritol yield, in comparison to the 1st batch phase, while the erythritol productivity values remained similar for both phases. Likewise, Tomaszewska *et al.* [50] evaluated the erythritol production by *Y. lipolytica* Wratislavia K1, another mutant strain, in a fed-batch mode with pulsed additions of crude glycerol. They described a slightly different scenario once, for the same biomass concentration produced (29.0 $g \cdot L^{-1}$), they observed a higher erythritol yield (0.6 $g \cdot g^{-1}$) and a higher productivity (1.2 $g \cdot L^{-1} \cdot h^{-1}$), and not an equal productivity for both phases [50]. This higher

productivity was probably due to the strain used in this work, once it is a mutant strain, unlike those used in the present work.

In a general way, the divided addition of crude glycerol (100 $g \cdot L^{-1}$ at the beginning and 100 $g \cdot L^{-1}$ after 72 hours of batch experiment) it is more advantageous than the use of a single initial high crude glycerol concentration, for both strains. The strain that demonstrated the better erythritol production (64 $g \cdot L^{-1}$), erythritol yield (0.42 $g \cdot g^{-1}$) and erythritol productivity (0.45 $g \cdot L^{-1} \cdot h^{-1}$) was *Y. lipolytica* W29. These results demonstrate that yeast cells can continue to produce erythritol at the same rate, as long as they continued to have the carbon source available. Consequently, with this operation mode, it would be possible to extend the production of erythritol without loss of cells performance.

5. CONCLUSIONS AND FUTURE WORK PERSPECTIVES

In this section, the general conclusions of the work are presented, as well as, perspectives for future work related with this field of research.

5.1 Final Conclusions

Y. lipolytica is the most well-known studied strain of the genus *Yarrowia* for the production of various metabolites of industrial interest, including the erythritol production. In this work, it was also proven that the recently described species *Y. divulgata* is capable of producing erythritol, however the concentrations obtained were lower than those reported by *Y. lipolytica*. This may be due to the fact that culture conditions were not optimized for this new species. In flask experiments the strain that produced a higher amount of erythritol was *Y. lipolytica* W29, thus also attaining the highest productivity. Since the highest erythritol concentrations were obtained by the species of *Y. lipolytica*, and the lowest by the species of *Y. divulgata*, we can assess that under the conditions used, the species *Y. lipolytica* is the most indicated for the production of erythritol. The strains *Y. lipolytica* W29 and *Y. lipolytica* Ch 3/4 were selected to perform the scale-up of the bioprocess, to evaluate the effect of oxygen availability in erythritol production, and to optimize the production of erythritol.

In a STR bioreactor, different operational conditions were used to obtain different k_La values and analyse its effect in the production of erythritol. It was demonstrated that the oxygen availability in the medium affects erythritol productivity, being the optimal oxygenation conditions a k_La of 162 h^{-1} attained at aeration and stirring rates of 3 vvm and 900 rpm, respectively. The scale-up of the bioprocess from flask to STR bioreactor favoured the production of erythritol by the strains *Y. lipolytica* W29 and *Y. lipolytica* Ch 3/4, increasing erythritol productivity.

To extend the erythritol production, two different strategies were performed: batch with initial crude glycerol concentration of 200 $g \cdot L^{-1}$ and stepwise fed-batch with 200 $g \cdot L^{-1}$ crude glycerol added in two fractions of 100 $g \cdot L^{-1}$. It was proven that the raise of initial glycerol concentration from 100 $g \cdot L^{-1}$ to 200 $g \cdot L^{-1}$, had a clearly negative impact on cellular growth and on erythritol production for *Y. lipolytica* W29 and *Y. lipolytica* Ch 3/4. On the other hand, the stepwise fed-batch strategy had better results, being proven that the yeasts are able to produce erythritol and extend its production without losing efficiency, as long as they continue to have an available carbon source.

As final conclusion, two wild-type strains of *Y. lipolytica* were proven to have great potential for the production of erythritol, a sweetener with important value for food industry, using a low-cost and sustainable substrate, crude glycerol from biodiesel industry.

5.2 Future Work Perspectives

The present work accomplished with yeasts of the species *Y. lipolytica* and *Y. divulgata* brings new perspectives on the erythritol production field, contributing for the optimization of some relevant parameters. Nevertheless, there are still some new ideas and questions that could be developed and studied in future works.

In this work it was proven that *Y. divulgata* is capable of producing erythritol in flask, however the erythritol concentrations observed were lower than the ones obtained by *Y. lipolytica*. Therefore, it would be interesting the optimization of culture medium conditions for the production of erythritol by *Y. divulgata* strains.

With this present work it was observed that an initial crude glycerol concentration of 200 $g \cdot L^{-1}$ with supplementation of 25 $g \cdot L^{-1}$ NaCl to the production medium made cells suffer a high osmotic stress, being therefore unable to grow and produce erythritol. Thus, it would be interesting to perform batch cultures at high glycerol concentration but without NaCl supplementation to the medium. Also, strains genetically modified with enhanced toleration to high salt concentrations could be tested [78].

Nevertheless, and once the stepwise fed-batch strategy demonstrated that erythritol production could continue with the addition of glycerol in pulses, it would be interesting to perform stepwise fed-batch studies with more punctual additions of crude glycerol, in order to assess if erythritol production could be extended and higher concentrations could be obtained.

Moreover, since these results showed that *Y. lipolytica* can produce erythritol with cells in resting phase, it would be interesting to study the production of this sweetener using immobilised cells in a continuous operation mode for substrate and product.

6. REFERENCES

This section lists all the references that contribute to the elaboration of this written work.

[1] Grembecka, M., Sugar Alcohols as Sugar Substitutes in Food Industry, in: J.M. Merillon, K. Ramawat (Eds.), Sweeteners, : pp. 1–27.

[2] Drewnowski, A., Mennella, J.A., Johnson, S.L., and Bellisle, F., Sweetness and Food Preference, J. Nutr. 142 (2012) 1142S-1148S.

[3] Martău, G.A., Coman, V., and Vodnar, D.C., Recent advances in the biotechnological production of erythritol and mannitol, Crit. Rev. Biotechnol. 40 (2020) 608–622.

[4] Grembecka, M., Natural sweeteners in a human diet, Rocz. Państwowego Zakładu Hig. 66 (2015) 195–202.

[5] Carocho, M., Morales, P., and Ferreira, I.C.F.R., Sweeteners as food additives in the XXI century: A review of what is known, and what is to come, Food Chem. Toxicol. 107 (2017) 302–317.

[6] Faruque, S., Tong, J., Lacmanovic, V., Agbonghae, C., Minaya, D.M., and Czaja, K., The Dose Makes the Poison: Sugar and Obesity in the United States – a Review, Polish J. Food Nutr. Sci. 69 (2019) 219–233.

[7] Felipe Hernández-Pérez, A., Jofre, F.M., De Souza Queiroz, S., Vaz De Arruda, P., Chandel, A.K., and Felipe, M.D.G.D.A., Biotechnological production of sweeteners, in: Biotechnol. Prod. Bioact. Compd., Elsevier, : pp. 261–292.

[8] Grembecka, M., Sugar alcohols—their role in the modern world of sweeteners: a review, Eur. Food Res. Technol. 241 (2015) 1–14.

[9] Grabitske, H.A., and Slavin, J.L., Low-Digestible Carbohydrates in Practice, J. Am. Diet. Assoc. 108 (2008) 1677–1681.

[10] Regnat, K., Mach, R.L., and Mach-Aigner, A.R., Erythritol as sweetener—wherefrom and whereto?, Appl. Microbiol. Biotechnol. 102 (2018) 587–595.

[11] Carly, F., and Fickers, P., Erythritol production by yeasts: a snapshot of current knowledge, Yeast. 35 (2018) 455–463.

[12] Guo, J., Li, J., Chen, Y., Guo, X., and Xiao, D., Improving Erythritol Production of *Aureobasidium pullulans* from Xylose by Mutagenesis and Medium Optimization, Appl. Biochem. Biotechnol. 180 (2016) 717–727.

[13] Moon, H.J., Jeya, M., Kim, I.W., and Lee, J.K., Biotechnological production of erythritol and its applications, Appl. Microbiol. Biotechnol. 86 (2010) 1017–1025.

[14] Fickers, P., Cheng, H., and Lin, C.S.K., Sugar alcohols and organic acids synthesis in *Yarrowia lipolytica*: Where are we?, Microorganisms. 8 (2020) 1–22.

[15] Mirończuk, A.M., Dobrowolski, A., Rakicka, M., Rywińska, A., and Rymowicz, W., Newly isolated mutant of *Yarrowia lipolytica* MK1 as a proper host for efficient erythritol biosynthesis from glycerol, Process Biochem. 50 (2015) 61–68.

[16] Carly, F., Vandermies, M., Telek, S., Steels, S., Thomas, S., Nicaud, J.M., and Fickers, P., Enhancing erythritol productivity in *Yarrowia lipolytica* using metabolic engineering, Metab. Eng. 42 (2017) 19–24.

[17] Bilal, M., Xu, S., Iqbal, H.M.N., and Cheng, H., *Yarrowia lipolytica* as an emerging biotechnological chassis for functional sugars biosynthesis, Crit. Rev. Food Sci. Nutr. 0 (2020) 1–18.

[18] Lin, S.J., Wen, C.Y., Liau, J.C., and Chu, W.S., Screening and production of erythritol by newly isolated osmophilic yeast-like fungi, Process Biochem. 36 (2001) 1249–1258.

[19] Rymowicz, W., Rywińska, A., and Marcinkiewicz, M., High-yield production of erythritol from raw glycerol in fed-batch cultures of *Yarrowia lipolytica*, Biotechnol. Lett. 31 (2009) 377–380.

[20] Rakicka, M., Kieroń, A., Hapeta, P., Neuvéglise, C., and Lazar, Z., Sweet and sour potential of yeast from the *Yarrowia* clade, Biomass and Bioenergy. 92 (2016) 48–54.

[21] Liu, X., Yu, X., Gao, S., Dong, X., Xia, J., Xu, J., He, A., Hu, L., Yan, Y., and Wang, Z., Enhancing the erythritol production by *Yarrowia lipolytica* from waste oil using loofah sponge as oil-in-water dispersant, Biochem. Eng. J. 151 (2019).

[22] Coelho, M.A.Z., Amaral, P.F.F., and Belo, I., *Yarrowia lipolytica*: an industrial workhorse, Appl. Microbiol. Microb. Biotechnol. (2010) 930–944.

[23] Miller, K.K., and Alper, H.S., *Yarrowia lipolytica*: more than an oleaginous workhorse, Appl. Microbiol. Biotechnol. 103 (2019) 9251–9262.

[24] Bellou, S., Makri, A., Triantaphyllidou, I.E., Papanikolaou, S., and Aggelis, G., Morphological and metabolic shifts of *Yarrowia lipolytica* induced by alteration of the dissolved oxygen concentration in the growth environment, Microbiol. (United Kingdom). 160 (2014) 807–817.

[25] Gonçalves, F.A.G., Colen, G., and Takahashi, J.A., *Yarrowia lipolytica* and its multiple applications in the biotechnological industry, Sci. World J. 2014 (2014).

[26] Chernyavskaya, O.G., Shishkanova, N. V., Il'chenko, A.P., and Finogenova, T. V., Synthesis of α-ketoglutaric acid by *Yarrowia lipolytica* yeast grown on ethanol, Appl. Microbiol. Biotechnol. 53 (2000) 152–158.

[27] Rakicka, M., Rukowicz, B., Rywińska, A., Lazar, Z., and Rymowicz, W., Technology of efficient continuous erythritol production from glycerol, J. Clean. Prod. 139 (2016) 905–913.

[28] Pereira-Meirelles, F.V., Rocha-Leão, M.H.M., and Sant' Anna Jr., G.L., A stable lipase from *Candida lipolytica*: Cultivation Conditions and Crude Enzyme Characteristics, Appl. Biochem. Biotechnol. 63–65 (1997) 73–85.

[29] Ferreira, P., Lopes, M., Mota, M., and Belo, I., Oxygen mass transfer impact on citric acid production by *Yarrowia lipolytica* from crude glycerol, Biochem. Eng. J. 110 (2016) 35–42.

[30] Lopes, M., Gomes, A.S., Silva, C.M., and Belo, I., Microbial lipids and added value metabolites production by *Yarrowia lipolytica* from pork lard, J. Biotechnol. 265 (2018) 76–85.

[31] Fakas, S., Papanikolaou, S., Aggelis, G., Komaitis, M., and Galiotou-Panayotou, M., Citric acid production by *Yarrowia lipolytica* cultivated on olive-mill wastewater-based media, Bioresour. Technol. 99 (2007) 2419–2428.

[32] Lopes, M., Miranda, S.M., Alves, J.M., Pereira, A.S., and Belo, I., Waste Cooking Oils as Feedstock for Lipase and Lipid-Rich Biomass Production, Eur. J. Lipid Sci. Technol. 121 (2019) 1–9.

[33] Braga, A., and Belo, I., Production of γ-decalactone by *Yarrowia lipolytica*: Insights into experimental conditions and operating mode optimization, J. Chem. Technol. Biotechnol. 90 (2015) 559–565.

[34] Nagy, E., Niss, M., Dlauchy, D., Arneborg, N., Nielsen, D.S., and Péter, G., *Yarrowia divulgata* f.a., sp. nov., a yeast species from animal-related and marine sources, Int. J. Syst. Evol. Microbiol. 63 (2013) 4818–4823.

[35] UniProt Consurtium, Taxonomy - *Yarrowia divulgata* (species), https://www.uniprot.org/taxonomy/479095 (acessed in December 29, 2020).

[36] Nagy, E.S., Bujna, E., Sipiczki, G., Farkas, C., Belo, I., Lopes, M., Braga, A., Mesquita, D.P., Ferreira, P., and Nguyen, Q.D., Optimization of lipase production by *Yarrowia divulgata*, Third Int. Conf. Food Sci. Technol. 70 (2018) 136–137.

[37] Rzechonek, D.A., Dobrowolski, A., Rymowicz, W., and Mirończuk, A.M., Recent advances in biological production of erythritol, Crit. Rev. Biotechnol. 38 (2018) 620–633.

[38] Yang, L.B., Zhan, X.B., Zhu, L., Gao, M.J., and Lin, C.C., Optimization of a low-cost hyperosmotic medium and establishing the fermentation kinetics of erythritol production by *Yarrowia lipolytica* from crude glycerol, Prep. Biochem. Biotechnol. 46 (2016) 376–383.

[39] Monteiro, M.R., Kugelmeier, C.L., Pinheiro, R.S., Batalha, M.O., and César, A. da S., Glycerol from biodiesel production: Technological paths for sustainability, Renew. Sustain. Energy Rev. 88 (2018) 109–122.

[40] Nelson, D.L., and Cox, M.M., Penthose Phosphate Pathway of Glucose Oxidation, in: LEHNINGER Princ. Biochem., 4th ed., Sara Tenney, : pp. 549–553.

[41] Russmayer, H., Egermeier, M., Kalemasi, D., and Sauer, M., Spotlight on biodiversity of microbial cell factories for glycerol conversion, Biotechnol. Adv. 37 (2019) 1–15.

[42] Ghezelbash, G.R., Nahvi, I., and Emamzadeh, R., Improvement of erythrose reductase activity, deletion of by-products and statistical media optimization for enhanced erythritol production from *Yarrowia lipolytica* mutant 49, Curr. Microbiol. 69 (2014) 149–157.

[43] Mirończuk, A.M., Biegalska, A., and Dobrowolski, A., Functional overexpression of genes involved in erythritol synthesis in the yeast *Yarrowia lipolytica*, Biotechnol. Biofuels. 10 (2017) 1–12.

[44] Liu, X., Lv, J., Xu, J., Xia, J., He, A., Zhang, T., Li, X., and Xu, J., Effects of osmotic pressure and pH on citric acid and erythritol production from waste cooking oil by *Yarrowia lipolytica*, Eng. Life Sci. 18 (2018) 344–352.

[45] Xiaoyan, L., Xinjun, Y., Jinshun, L., Jiaxing, X., Jun, X., Zhen, W., Tong, Z., and Yuanfang, D., A cost-effective process for the coproduction of erythritol and lipase with *Yarrowia lipolytica* M53 from waste cooking oil, Food Bioprod. Process. 103 (2017) 86–94.

[46] Mirończuk, A.M., Rakicka, M., Biegalska, A., Rymowicz, W., and Dobrowolski, A., A two-stage fermentation process of erythritol production by yeast *Y. lipolytica* from molasses and glycerol, Bioresour. Technol. 198 (2015) 445–455.

[47] Rakicka, M., Lazar, Z., Rywińska, A., and Rymowicz, W., Efficient utilization of inulin and glycerol as fermentation substrates in erythritol and citric acid production using *Yarrowia lipolytica* expressing inulinase, Chem. Pap. 70 (2016) 1452–1459.

[48] Rywińska, A., Tomaszewska, L., and Rymowicz, W., Erythritol biosynthesis by *Yarrowia lipolytica* yeast under various culture conditions, African J. Microbiol. Res. 7 (2013) 3511–3516.

[49] Rywińska, A., Marcinkiewicz, M., Cibis, E., and Rymowicz, W., Optimization of medium composition for erythritol production from glycerol by *Yarrowia lipolytica* using response surface methodology, Prep. Biochem. Biotechnol. 45 (2015) 515–529.

[50] Tomaszewska, L., Rywińska, A., and Rymowicz, W., High selectivity of erythritol production from glycerol by *Yarrowia lipolytica*, Biomass and Bioenergy. 64 (2014) 309–320.

[51] Tomaszewska-Hetman, L., Rymowicz, W., and Rywińska, A., Waste conversion into a sweetener-development of an innovative strategy for erythritol production by *Yarrowia lipolytica*, Sustain. 12 (2020).

[52] Tomaszewska, L., Rakicka, M., Rymowicz, W., and Rywińska, A., A comparative study on glycerol metabolism to erythritol and citric acid in *Yarrowia lipolytica* yeast cells, FEMS Yeast Res. 14 (2014) 966–976.

[53] Tomaszewska, L., Rywińska, A., and Gladkowski, W., Production of erythritol and mannitol by *Yarrowia lipolytica* yeast in media containing glycerol, J. Ind. Microbiol. Biotechnol. 39 (2012) 1333–1343.

[54] da Silva, L.V., Coelho, M.A.Z., Amaral, P.F.F., and Fickers, P., A novel osmotic pressure strategy to improve erythritol production by *Yarrowia lipolytica* from glycerol, Bioprocess Biosyst. Eng. 41 (2018) 1883–1886.

[55] Pagliaro, M., Properties, Applications, History, and Market, in: M. Pagliaro (Ed.), Glycerol Renew. Platf. Chem., Elsevier, : pp. 1–21.

[56] Kotrba, R., 2020 EU biodiesel production remains stable, consumption down 6%, Biodiesel Mag.,http://www.biodieselmagazine.com/articles/2517090/2020-eu-biodiesel-production-remains-stable-consumption-down-6 (accessed in November 23, 2020).

[57] Win, S.S., and Trabold, T.A., Sustainable waste-to-energy technologies: Transesterification, in: T.A. Trabold, C.W. Babbitt (Eds.), Sustain. Food Waste-to-Energy Syst., Academic Press, : pp. 89–109.

[58] Ferreira, P.M.A., Biotechnological approaches of crude glycerol use: optimization of citric acid production by *Yarrowia lipolytica*, PhD Thesis in Chemical and Biological Engineering, University of Minho, 2015.

[59] Ruhal, R., and Choudhury, B., Use of an osmotically sensitive mutant of *Propionibacterium freudenreichii* subspp . *shermanii* for the simultaneous productions of organic acids and trehalose from biodiesel waste based crude glycerol, Bioresour. Technol. 109 (2012) 131–139.

[60] Mothes, G., Schnorpfeil, C., and Ackermann, J.-U., Production of PHB from Crude Glycerol, Eng. Life Sci. (2007) 475–479.

[61] González-Pajuelo, M., Andrade, J.C., and Vasconcelos, I., Production of 1,3-propanediol by *Clostridium butyricum* VPI 3266 using a synthetic medium and raw glycerol, J. Ind. Microbiol. Biotechnol. 31 (2004) 442–446.

[62] Mu, Y., Teng, H., Zhang, D.-J., Wang, W., and Xiu, Z., Microbial production of 1,3-propanediol by *Klebsiella pneumoniae* using crude glycerol from biodiesel preparations, Biotechnol. Lett. (2006) 1755–1759.

[63] Rywińska, A., Marcinkiewicz, M., Cibis, E., and Rymowicz, W., Optimization of medium composition for erythritol production from glycerol by *Yarrowia lipolytica* using response surface methodology, Prep. Biochem. Biotechnol. 45 (2015) 515–529.

[64] Rymowicz, W., Rywińska, A., and Gładkowski, W., Simultaneous production of citric acid and erythritol from crude glycerol by *Yarrowia lipolytica* Wratislavia K1, Chem. Pap. 62 (2008) 239–246.

[65] Mirończuk, A.M., Furgała, J., Rakicka, M., and Rymowicz, W., Enhanced production of erythritol by *Yarrowia lipolytica* on glycerol in repeated batch cultures, J. Ind. Microbiol. Biotechnol. 41 (2014) 57–64.

[66] Felder, R.M., Rousseau, R.W., and Bullard, L.G., 2005, Elementary Principles of Chemical Processes, 4th Editio, WILEY, .

[67] Macauley-Patrick, S., and Finn, B., Modes of Fermenter Operation, in: B. McNeil, L.M. Harvey (Eds.), Pract. Ferment. Technol., : pp. 69–95.

[68] Rywińska, A., Musiał, I., Rymowicz, W., Arowska, B., and Boruczkowski, T., Effect of agitation and aeration on the citric acid production by *Yarrowia lipolytica* grown on glycerol, Prep. Biochem. Biotechnol. 42 (2012) 279–291.

[69] Garcia-Ochoa, F., and Gomez, E., Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview, Biotechnol. Adv. 27 (2009) 153–176.

[70] Suresh, S., Srivastava, V.C., and Mishra, I.M., Techniques for oxygen transfer measurement in bioreactors: A review, J. Chem. Technol. Biotechnol. 84 (2009) 1091–1103.

[71] Wise, W.S., The measurement of the aeration of culture media, J. Gen. Microbiol. 5 (1951) 167–177.

[72] Stanbury, P.F., Whitaker, A., and Hall, S.J., Aeration and Agitation, in: Princ. Ferment. Technol., Second, Pergamon, : pp. 243–275.

[73] Rakicka-Pustułka, M., Mirończuk, A.M., Celińska, E., Białas, W., and Rymowicz, W., Scale-up of the erythritol production technology – Process simulation and techno-economic analysis, J. Clean. Prod. 257 (2020) 1–11.

[74] Papanikolaou, S., Diamantopoulou, P., Blanchard, F., Lambrinea, E., Chevalot, I., Stoforos, N.G., and Rondags, E., Physiological Characterization of a Novel Wild-Type *Yarrowia lipolytica* Strain Grown on Glycerol : Effects of Cultivation Conditions and Mode on Polyols and Citric Acid Production, Appl. Sci. 10 (2020) 1–24.

[75] Machado, A.R.A., Production of the sweetener erythritol by *Yarrowia lipolytica* strains, Master Thesis in Biotecnology, University of Minho, 2018.

[76] Tomaszewska-Hetman, L., and Rywińska, A., Erythritol biosynthesis from glycerol by *Yarrowia lipolytica* yeast: Effect of osmotic pressure, Chem. Pap. 70 (2016) 272–283.

[77] Nikolaou, E., Agrafioti, I., Stumpf, M., Quinn, J., Stansfield, I., and Brown, A.J., Phylogenetic diversity of stress signalling pathways in fungi, BMC Evol. Biol. 9 (2009) 1–18.

[78] de Caria, J.P.F., Estudo de estratégias de melhoramento de estirpes de *Yarrowia lipolytica* para a produção de ácido cítrico e lipase, Master Thesis in Bioengineering, University of Minho, 2015.

7. APPENDIX

Appendix 1 – Calibration Curves of Biomass

Table 1 – Equations of the biomass calibration curve with a confidence interval of 95 % for the five strains used. Abbreviations: O.D.- Optical Density; LOD- Limit of detection; LOQ- Limit of Quantification.

Strain	Calibration Curve	LOD ($g \cdot L^{-1}$)	LOQ ($g \cdot L^{-1}$)
Y. lipolytica W29	O.D. = (0.56 ± 0.04) Dry weight (g/L) - (0.0002 ± 0.0319)	0.07	0.25
Y. lipolytica Ch 1/5	O.D. = (1.16 ± 0.03) Dry weight (g/L) + (0.02 ± 0.01)	0.01	0.04
Y. lipolytica Ch 3/4	O.D. = (0.48 ± 0.04) Dry weight (g/L) + (0.03 ± 0.04)	0.10	0.32
Y. divulgata M445/4	O.D. = (1.08 ± 0.04) Dry weight (g/L) + (0.03 ± 0.01)	0.01	0.04
Y. divulgata 5257/5	O.D. = (0.91 ± 0.07) Dry weight (g/L) + (0.03 ± 0.03)	0.04	0.14