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Production of the sweetener erythritol by *Yarrowia lipolytica* strains

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Work developed under the guidance of:

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É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA DISSERTAÇÃO (da página v até à página vii), APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

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ABSTRACT

Nowadays, the demand for alternatives to sugar has become a priority. Erythritol is a sugar alcohol with nearly 70 % of sucrose sweetness, with zero calories, non-cariogenic and non-glycemic. This sweetener is produced through fermentation from glucose mostly by osmophilic microorganisms. The osmotolerant yeast *Yarrowia lipolytica* can produce erythritol from crude glycerol, a by-product of biodiesel industry. The production of erythritol by this yeast can be affected by several parameters, such as yeast strain, medium composition and operating condition. The main objective of the present work was the development of a process for erythritol production by *Y. lipolytica* using crude glycerol as carbon source.

Initially, a screening for osmotolerant strains of *Y. lipolytica* was performed using two different methodologies: assessment of cell growth in agar plates containing 1 M of NaCl and miniaturized assays in liquid medium supplemented with NaCl. Nine strains that grew better in the medium with salt were selected and their capability to produce erythritol was tested. The strains W29, UV15-31, UV15-93 and EMS75-162 produced the higher concentrations of erythritol.

The scale-up of the process in a stirred tank reactor was performed only with the strain *Y. lipolytica* W29. The effect of dissolved oxygen in the production of erythritol was assessed, using two different strategies: constant aeration and stirring rates and controlled dissolved oxygen concentration. The results showed that dissolved oxygen concentration in the medium is an important parameter that influences the erythritol productivity. The highest concentration of erythritol (32 $g \cdot L^{-1}$), yield (0.43 $g \cdot g^{-1}$) and productivity (0.44 $g \cdot L^{-1} \cdot h^{-1}$) were obtained in the batch performed at high constant stirring and aeration rates, 900 rpm and 3 vvm, respectively.

Some strategies of fed-batch operation were also performed to evaluate if the erythritol production could be extended with glycerol feeding to *Y. lipolytica* culture. The different strategies tested were fed-batch at constant feeding rates with completed and concentrated medium, fed-batch at constant feeding rate only with addition of crude glycerol, and a step-wise fed-batch with the addition of crude glycerol. It was observed that erythritol accumulation in the medium was extended with the addition of only crude glycerol to the bioreactor. The step-wise fed-batch proved to be the best strategy among the tested ones, leading to highest yield (0.42 $g \cdot g^{-1}$) and productivity (0.45 $g \cdot L^{-1} \cdot h^{-1}$).

KEYWORDS: Erythritol; *Yarrowia lipolytica*; Crude glycerol

RESUMO

Atualmente, a procura por alternativas ao açúcar tornou-se uma prioridade. O eritritol é um álcool de açúcar com cerca de 70 % da doçura da sacarose, com zero calorias, não-cariogénico e não-glicémico. Este adoçante é produzido através da fermentação da glucose maioritariamente por microrganismos osmofílicos. A levedura osmotolerante *Yarrowia lipolytica* consegue produzir eritritol, usando como fonte de carbono o glicerol bruto, um subproduto da indústria do biodiesel. A produção de eritritol por esta levedura pode ser influenciada por diversos parâmetros, tais como a estirpe de levedura utilizada, a composição do meio e as condições de operação. O principal objetivo deste trabalho foi o desenvolvimento de um processo para a produção de eritritol pela levedura *Y. lipolytica*, usando-se glicerol bruto como fonte de carbono.

Inicialmente, foi realizado um rastreio para estirpes osmotolerantes de *Y. lipolytica*, usando duas metodologias diferentes: avaliação do crescimento celular em meio com sal em placas de agar e em microplacas usando meio líquido. Nove estirpes, que apresentaram um melhor crescimento nos meios com sal, foram selecionadas para avaliar a sua capacidade de produção de eritritol, sendo que as estirpes W29, UV15-31, UV15-93 e EMS75-162 produziram eritritol em maiores concentrações.

O aumento de escala do processo num reator de tanque agitado foi realizado apenas com a estirpe W29. Foi avaliado o efeito do oxigénio dissolvido na produção de eritritol usando 2 estratégias diferentes: taxas de agitação e arejamento constantes e concentração de oxigénio dissolvido constante. Observou-se que a concentração de oxigénio dissolvido no meio é um parâmetro importante com influência na produtividade de eritritol. A concentração mais elevada de eritritol (32 $g \cdot L^{-1}$), o maior rendimento (0.43 $g \cdot g^{-1}$) e a maior produtividade (0.44 $g \cdot L^{-1} \cdot h^{-1}$) foram obtidos no ensaio em modo descontínuo realizado com taxas de agitação (900 rpm) e arejamento (3 vvm) constantes.

Foram ainda exploradas algumas estratégias de operação em semi-contínuo para avaliar se a produção de eritritol poderia ser prolongada com a adição de glicerol. As diferentes estratégias testadas foram modo semi-contínuo com taxas de alimentação constantes com meio completo e concentrado, com taxa de alimentação constante com a adição de apenas glicerol bruto, e um modo descontínuo repetido com a adição pontual de glicerol bruto. Observou-se que a acumulação de eritritol no meio foi prolongada com a adição de apenas glicerol bruto. O modo descontínuo repetido demonstrou ser a melhor estratégia, obtendo–se o rendimento (0.42 $g \cdot g^{-1}$) e a produtividade (0.45 $g \cdot L^{-1} \cdot h^{-1}$) mais elevados.

PALAVRAS-CHAVE: Eritritol; *Yarrowia lipolytica*; Glicerol bruto.

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LIST OF ABBREVIATIONS

ALDO   Fructose-biphosphate aldolase

EMS   Ethyl methane sulfonate

EPK   Erythrose 4-phosphate kinase

ER   Erythrose reductase

FDA   Food and Drug Administration

GK   Glycerol kinase

GPD   Glycerol-3-phosphate dehydrogenase

GRAS   Generally recognized as safe

G6PD   Glucose-6-phosphate dehydrogenase

HK   Hexokinase

HPLC   High Performance Liquid Chromatography

PGD   6-phosphogluconate dehydrogenase

PGI   Glucose-6-phosphate isomerase

PGLS   6-phosphogluconolactonase

PFK   Phosphofructokinase

RPE   Ribulose-6-phosphate 3-epimerase

RPI   Ribose-5-phosphate isomerase

RPM   Revolutions per minute

STR   Stirred tank reactor

TAL   Transaldolase

TKT   Transketolase

TPI   Triose phosphate isomerase

VVM   Volume per volume per minute

YPDA   Yeast extract, peptone, dextrose and agar medium

YPG   Yeast extract, peptone and glycerol medium

YPGA   Yeast extract, peptone, glycerol and agar medium

1. INTRODUCTION

Erythritol is a polyol used as a sweetener in food industry. This compound is currently produced mostly by osmophilic yeast from glucose, but a constant interest in reducing erythritol retail prices encourages the search for production alternatives. *Yarrowia lipolytica* is capable to produce erythritol from crude glycerol, a by-product of biodiesel industry, and that can be an interesting alternative to the processes currently used in erythritol industry.

In this Chapter, the focus is erythritol and its production by the yeast *Y. lipolytica*. A brief overview on metabolic pathways involved in glycerol consumption and erythritol production is also presented.

1.1 Sweeteners

The human desire for sweet taste, common to all ages, races, and cultures, has been evolutionally shaped throughout history [1, 2]. Even though sugar has been present in the human diet since immemorial times, due to its natural occurrence in fruits, vegetables, and honey, nowadays an enormous diversity of food contains added sugar [1, 3]. This refined sugar, from sugar cane/beet or high fructose corn syrup prepared from corn starch, that is added during food processing and food preparation, frequently has low nutritional value, but high energy value [1, 3]. The preference for this type of food over a diet without added sugar has become a problem, since the excess of sugar can cause several health problems [2]. Nutrition is one of the most important environmental factors in human body development and well-being [2].

The increase of sugar consumption during the last century has attracted public and scientific interest concerning its adverse health effects. This higher consumption rate has been accompanied by an increase in metabolic disorders, such as overweight, obesity, insulin resistance and cardiovascular disease [2]. Although the obesogenic role of sugar is not yet well known, the epidemiological studies, carried during the last decades, have indicated an association between energy imbalance (energy intake higher than energy expenditure) and this disorder [2-4]. Thus, it became necessary to find healthier options to added sugars and low-calorie sweeteners, that maintain the sweet taste but have less energy value, could be an alternative [1].

Sweeteners are chemical compounds used as sweetening agents due to their sweet taste [2]. These compounds can be classified considering their origin (natural or synthetic agents), technological function (sweeteners and semisynthetic fillers), consistency (syrups and powders) and nutritional value (caloric and non-caloric) [2]. Carbohydrates are a diverse group of organic compounds widely recognized as natural sweeteners [2]. Its primary function is to provide energy, but they can also perform structural functions, be source of dietary fiber and regulate satiety and hunger [2]. Carbohydrates can be divided in three main groups: sugars (mono and disaccharides), oligosaccharides, and polysaccharides. Polyhydric alcohols, known as well as polyols or sugar alcohols, are also included in carbohydrates classification [2].

Polyols are low-digestible carbohydrates, which mean that they are partially or not absorbed in the small intestine but are at least fermented by bacteria in the large intestine [5, 6]. They are used as sugar substitutes, occurring naturally in fruits, vegetables, mushrooms and human organism [2, 7].

These sugar-alcohols are generally recognized as safe for food additives and polyols like sorbitol (E420), xylitol (E967), maltitol (E965), mannitol (E421), lactitol (E966), isomalt (E953) and erythritol (E968) are commonly used in food products [6]. Their lower caloric content, lower glycemic index comparing to sugars and anti-caries effects, since cariogenic bacteria cannot ferment them, have led to an increase of public interest in polyols [7].

1.2 Erythritol

Erythritol (E968), also called (2R,3S)-1,2,3,4-butanetetrol, erythrite, meso-erythritol or tetrahydroxybutane, is a linear four-carbon compound ($C_4H_{10}O_4$) (Figure 1) [2]. This sweetener can be found in vegetables, fruits, seaweeds and fermented foods, such as wine, beer, sake and soy sauce, and it also exists endogenously in human tissues and body fluids [2, 6]. This polyol is characterized for being nearly 60 to 70 % as sweet as sucrose, for having a clean sweet taste, no after taste and for the ability to mask certain unwanted after tastes of other sweeteners [2, 6]. When ingested, it provides a strong cooling effect in the mouth [2]. This sugar alcohol is stable at high temperatures (up to 160°C) as well as in acid or alkaline environments, it is a non-hygroscopic substance and crystallize rapidly [2]. In addition to being a zero-calorie sweetener, non-cariogenic, bulk sweetener and non-glycemic, erythritol has proven to be an exceptional radical scavenger with membrane-protecting properties and to suppress the formation of biofilms of some cariogenic bacteria [2, 8]. Erythritol is a very small molecule, so it is rapidly absorbed through the small intestine and, afterwards, excreted unchanged in the urine [2]. Since erythritol is not fermented by intestines' microorganisms, it is unlikely to observe laxative effects normally correlated with the consumption of sweeteners [2].
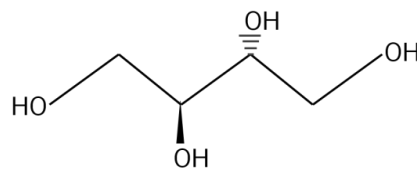
Figure 1 – Erythritol 2-dimensional chemical structure.

Erythritol can be synthetized through chemical reactions using as substrates 2-butene-1,4-diol, dialdehyde starch, L-tartaric-acid, or arabinoic or ribonic acid obtained by decarboxylation of C-6 sugars [9]. However, the low efficiency, high production costs and complexity of the process makes it

unfeasible [2, 6, 9, 10]. The biotechnological production became an alternative, since it is more environmentally friendly and safer than chemical production. Nowadays, industrial production of this polyol is achieved through fermentation processes, mostly conducted by osmophilic yeasts: *Moniliella pollinis, Trichosporonoides megachiliensis, Torula* sp., and, recently, *Yarrowia lipolytica* [6, 9, 11]. This polyol is also naturally produced by some lactic acid bacteria, like *Oenococcus oeni, Leuconostoc mesenteroides*, and *Lactobacillus sanfranciscencis* [6, 11]. Several other microorganisms can produce erythritol as well, like *Pichia, Zygopichia, Candida, Torulopsis, Trigonopsis, Moniliella tomentosa* var. *pollis*, but due to their high level of by-products production (glycerol and ribitol) are unsuitable for industrial process [8, 11]. After the production process, the recovery of erythritol consists in membrane filtration of the culture medium to separate the microorganisms, followed by ion exchange chromatography and, finally, crystallization [9, 11]. The carbon source used in the industrial production of erythritol is glucose, obtained through chemical and/or enzymatic hydrolyses of wheat and corn starches [9, 11]. Nonetheless, the retail prices of erythritol are high, thus there is an interest in reducing the production costs [9]. Since glucose is a valuable compound, the use of alternative low cost substrates could reduce the production costs [9]. Some authors already studied the production of erythritol by *Aureobasidium pullulans* from xylose [12]. Also, there are studies reporting this polyol production using glycerol as carbon source by *Yarrowia lipolytica* strains [8, 13-15], and also from *Moniliella megachiliensis* [16]. Considering glycerol as carbon source, the use of crude glycerol, a by-product from biodiesel industry much cheaper than glucose, in the production of erythritol by *Y. lipolytica* could be an alternative to the current erythritol production processes.

1.3 Yarrowia lipolytica

Yarrowia lipolytica, one of the most extensively studied "non-conventional" yeasts, is a strictly aerobic and non-pathogenic microorganism [17]. Most of the processes in which this yeast is used are classified as GRAS (Generally Recognized As Safe) by the FDA (Food and Drug Administration) [17]. This dimorphic yeast has the ability to grow in hydrophobic substrates [17, 18], such as n-alkanes [19], fatty acids [20], fats and oils, which makes it an interesting microorganism for bioremediation process [17]. Besides the hydrophobic substrates, other carbons sources like alcohols, such as ethanol [21] and glycerol [22], sugars (glucose and fructose) [23, 24] and organic acids [25] can be metabolized by *Y. lipolytica*. Moreover, this yeast can efficiently use agro-industrial wastes, like olive mill waste waters

[26], vegetable oil refinery residues [27], animal fats [28] and crude glycerol from biodiesel industry [13]. *Y. lipolytica* can be naturally found in cheeses, yoghurts, kefir, soy sauce, meat and shrimp salads and even polluted environments [17, 18]. These diversified habitats reflect the versatility of this yeast metabolism. From the different carbons sources, *Y. lipolytica* is capable of producing several important metabolites with biotechnological interest, such as lipase [29], γ-decalactone [30], organic acids [21, 31-33], single cell oil [28], biosurfactants [27] and erythritol [22]. Its intense secretory activity justifies the interest of this microorganism in industrial usage, in molecular biology and in genetics studies [17]. The metabolite produced strongly depends on growth conditions, depending on which carbon and nitrogen source is used and their respective concentrations in the medium, and other parameters like pH, temperature, oxygenation and micronutrients, the efficiency and type of metabolites production may be affected [18]. For instance, Tomaszewska *et al.* [34] studied the effect of pH in acetate-negative mutants of *Y. lipolytica* and demonstrated that citric acid synthesis is most effective at pH 5.0-5.5, but for the same conditions with a lower pH (3-3.5) the erythritol accumulation is favored and citric acid detected is residual.

1.4 Erythritol production by *Yarrowia lipolytica*

Erythritol is produced by osmophilic/osmotolerant yeasts to counterbalance the external osmotic pressure [11]. *Y. lipolytica* is described as able to grow in medium containing high levels of salts [35]. The ability of *Y. lipolytica* to tolerate these conditions makes it a potential erythritol producer.

Some authors have already studied the erythritol production using *Y. lipolytica* strains. Moreover, the carbon source most commonly used in these studies is glycerol, however there are studies reporting the use of glucose [13], inulin [36] and waste cooking oil [37]. These studies are predominantly carried out with random mutant strains and the most studied strain is *Y. lipolytica* Wratislavia K1. This mutant was isolated from a continuous citric acid fermentation with the parent strain *Y. lipolytica* Wratislavia 1.31, an acetate negative mutant [38]. Table 1 summarizes the results obtained in these studies of erythritol production by different *Y. lipolytica* strains.

Table 1 – Erythritol production by different *Y. lipolytica* strains.

Operation mode	Substrate	*Y. lipolytica* Strain	Yield range (g·g^{-1})	Productivity (g·L^{-1}·h^{-1})	Reference
Batch (flask)	Glycerol	Wratislavia K1; Wratislavia MK1; Wratislavia 1.31	0.31 - 0.39	0.33 – 0.40	[8]
	Glucose	UV Mutant 49	0.25	-	[39]
Batch	Glycerol	Wratislavia K1; MK1; A UV'1; A-15; JMY2900; FCY214; FCY218; A-3; A-6; A-8	0.34 - 0.56	0.40 – 1.03	[8, 14, 15, 34, 40-42]
	Waste cooking oil	M53	0.74	-	[37]
Fed-batch	Glycerol	Wratislavia K1; A UV'1; A-15; CICC 1675; Wratislavia 1.31; Wratislavia AWG7; A -101; 8661 UV1; 1.22	0.27 – 0.56	0.44 – 1.01	[13-15, 38, 43]
	Glucose	Wratislavia K1	0.14	0.09	[13]
Repeated-batch	Glycerol	Wratislavia K1; MK1;	0.41 - 0.66	0.3 – 0.49	[8, 22]
Continuous	Glycerol	Wratislavia K1; MK1;	0.4 - 0.57	0.9 – 1.12	[44, 45]

Erythritol is synthesized via the pentose phosphate pathway (Figure 2) [11]. Through this pathway, eukaryotic organisms can create not only reducing power, in the form of NADPH needed for cellular reactions, but also ribose-5-phosphate and erythrose-4-phosphate, precursors in the synthesis of amino acids and nucleotides [11]. Thus, the production of erythritol starts with the detour of glucose 6-phosphate, glycolysis' intermediate, to the pentose phosphate pathway. In the oxidative phase of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase catalyzes the dehydrogenation of glucose-6-phosphate to create 6-phosphoglucono-δ-lactone and NADPH. Then, the intramolecular ester 6-phosphoglucono-δ-lactone is hydrolyzed to 6-phosphogluconate by 6-phosphogluconolactonase [46]. In the next step, 6-phosphogluconate is dehydrogenated and decarboxylated to form ribulose-5-

phosphate, NADPH and release a CO_2 molecule, by 6-phosphogluconate dehydrogenase [47]. An isomerization reaction catalyzed by ribose-5-phosphate isomerase converts ribulose-5-phosphate into ribose-5-phosphate [46, 47]. In the non-oxidative phase of this pathway, transketolase, a thiamine pyrophosphate dependent enzyme, transfers two carbon atoms from xylulose-5-phosphate to ribose-5-phosphate, producing sedoheptulose-7-phosphate (a seven-carbon ketose) and glyceraldehyde-3-phosphate (a three-carbon aldose) [46, 47]. Afterwards, transaldolase catalyzes the transfer of a three-carbon fragment from sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate, forming the four-carbon aldose D-erythrose-4-phosphate [46, 47]. Then, transketolase catalyzes the reaction in which xylulose-5-phosphate gives a two-carbon fragment to erythrose-4-phosphate, originating fructose-6-phosphate and glyceraldehyde-3-phosphate [47]. The four-carbon aldose erythrose-4-phosphate can also be converted to erythrose by erythrose 4-phosphate kinase [11]. Finally, erythrose reductase uses NAD(P)H to catalyze the hydrogenation of erythrose 4-phosphate, originating erythritol [11].

Yarrowia lipolytica can synthetize erythritol from glycerol. After crossing cell membrane by facilitated diffusion or active transport, glycerol is phosphorylated by glycerol kinase, creating glycerol-3-phosphate [48]. Then, a NAD-linked dehydrogenase oxidizes glycerol-3-phosphate to dihydroxyacetone-3-phosphate, which is transformed to glyceraldehyde-3-phosphate by triose phosphate isomerase [48]. Afterwards, glyceraldehyde-3-phosphate is used in gluconeogenesis to produce glucose-6-phosphate, which is then used in the pentose phosphate pathway, resulting in the synthesis of erythritol [46].
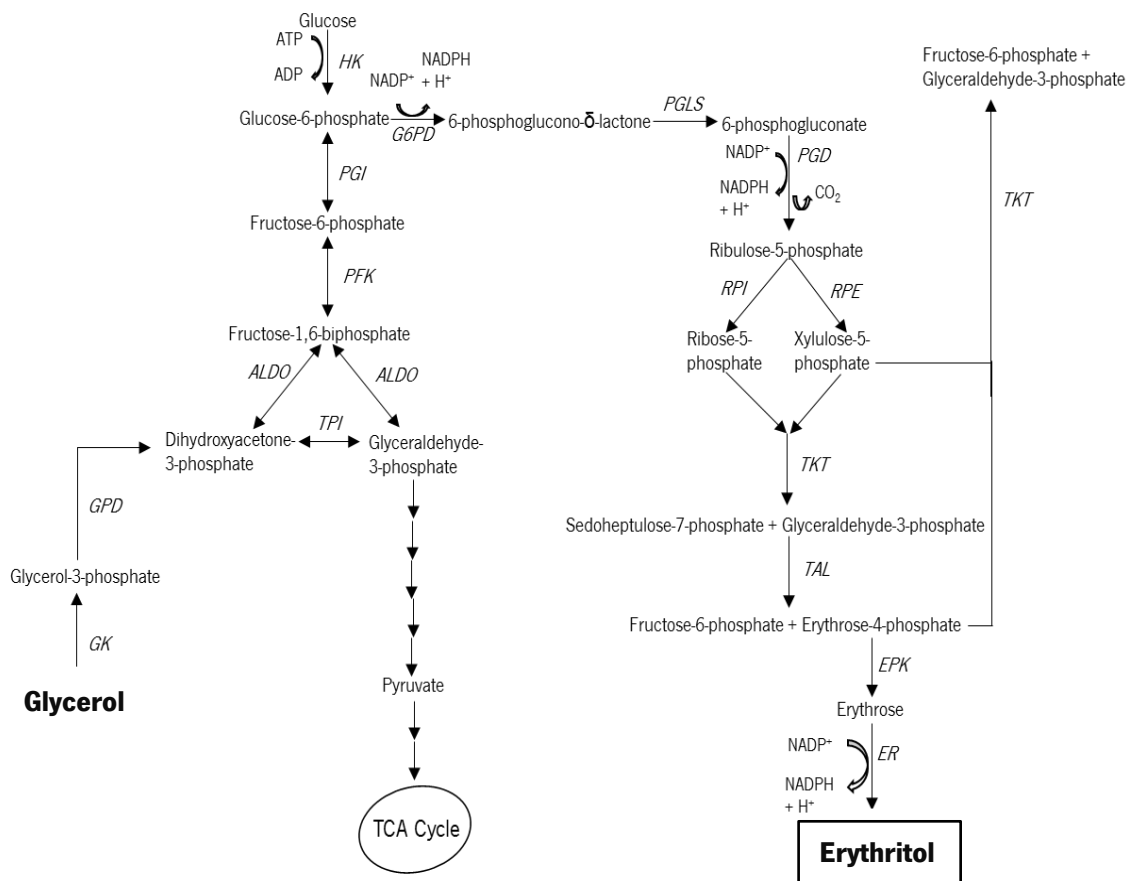
Figure 2 - Pathways involved in the production of erythritol by *Y. lipolytica*. Abbreviations: HK - Hexokinase; G6PD - Glucose-6-phosphate dehydrogenase; PGLS - 6-phosphogluconolactonase; PGD - 6-phosphogluconate dehydrogenase; RPI - Ribose-5-phosphate isomerase; RPE - Ribulose-6-phosphate 3-epimerase; TKT - Transketolase; TAL - Transaldolase; EPK - Erythrose 4-phosphate kinase; ER - Erythrose reductase; PGI - Glucose-6-phosphate isomerase; PFK - Phosphofructokinase; ALDO - Fructose-biphosphate aldolase; TPI - Triose phosphate isomerase; GPD - Glycerol-3-phosphate dehydrogenase; GK - Glycerol kinase.

1.5 Effect of culture conditions

Cultures conditions like medium composition, pH, temperature and dissolved oxygen concentration can affect the production of metabolites by *Y. lipolytica*, favoring a particular metabolite over another. The production of erythritol by *Y. lipolytica* is influenced by several culture conditions. Thus, selecting the proper culture conditions is the first step to improve the yeast performance.

The carbon source used has an important influence in the erythritol productivity. The erythritol production by *Y. lipolytica* strains from several carbon sources, such as glucose and other sugars [49], glycerol [13], waste cooking oil [37, 50], inulin [36], molasses [51] has been tested. However, the substrates glucose and glycerol provided the best results. Rymowicz *et al.* [13] tested the use of glucose and crude glycerol as carbon sources and demonstrated that glucose was the less efficient

substrate. In that work a 7.1 and 6.2-fold decrease on erythritol productivity and yield, respectively, was observed comparing to glycerol. Also, Yang *et al.* [49] studied the effect different carbon sources, glucose, fructose, sucrose, maltose, and glycerol, in erythritol production by *Y. lipolytica* A16. Glucose and glycerol were the carbon sources with better results and again higher titers were obtained with glycerol. These better titers obtained with glycerol are due to a higher osmotic stress created by the glycerol when compared to glucose, which favors the erythritol production [49].

Nitrogen source and its concentration is another important factor that affects the amount of erythritol produced. In the study of Rakicka *et al.* [45], different nitrogen sources, inorganic ($(NH_4)_2SO_4$) and organic (yeast extract), at different concentrations were tested in the production of this polyol by *Y. lipolytica* Wratislavia K1. The medium containing 4.6 $g·L^{-1}$ of $(NH_4)_2SO_4$ resulted in the highest production, while a lower amount of erythritol was obtained in the medium containing 9.75 $g·L^{-1}$ of yeast extract [45]. Also, Rywińska *et al.* [14] studied the effect of several nitrogen sources, organic and inorganic, on the production of erythritol by *Y. lipolytica* Wratislavia K1 and concluded that ammonium sulfate leads to higher erythritol productivity and yield.

Besides carbon and nitrogen sources, phosphate concentration also influences the erythritol production. An excessive amount of phosphate affects negatively the erythritol production [52]. Rywińska *et al.* [14] studied the effect of different concentrations of KH_2PO_4 (0-2 $g·L^{-1}$) in the production of this sweetener by *Y. lipolytica* Wratislavia K1. The highest erythritol production was obtained in the medium containing only 0.25 $g·L^{-1}$ of KH_2PO_4 and higher concentrations of phosphate led to stimulation of mannitol production accompanied by a decrease in erythritol production [14].

The better erythritol producing strains are described as osmophilic/osmotolerant, thereby salt concentration in the culture medium plays a very important role in the production of this sugar alcohol. Rywińska *et al.* [14] studied the effect of different NaCl concentrations on erythritol production by *Y. lipolytica* Wratislavia K1. The salt concentration ranged from 0 to 90 $g·L^{-1}$, and higher erythritol productivity and yield were obtained in the medium supplemented with 30 $g·L^{-1}$ of NaCl [14]. Also, Tomaszewska *et al.* [15] studied the effect of different concentrations of NaCl (0; 25; 32.5 $g·L^{-1}$) by *Y. lipolytica* Wratislavia K1. In that work, the highest amount of erythritol was obtained when a concentration of 25 $g·L^{-1}$ of salt was used [15]. It was also observed that the addiction of NaCl to the medium favors the erythritol production over mannitol that decreases [15].

Besides medium composition, also other culture parameters present a strong influence on erythritol production, for example the pH of the medium. Rymowicz *et al.* [13] studied the effect of pH on erythritol production by *Y. lipolytica* Wratislavia K1 from crude glycerol. In that work, different pH

values within the range 2.5 - 6.5 were tested [13]. For high pH values, it was observed a decrease in the production of erythritol and an increase in the production of citric acid, and the amount of citric acid exceeded the erythritol produced [13]. The highest erythritol titer was obtained at pH 3, and in this condition no citric acid was detected and some mannitol (12 g·L^{-1}) was produced [13]. Tomaszewska *et al.* [34] also studied the effect of pH on sugar alcohol production by different strains of *Y. lipolytica* (Wratislavia 1.31, WratislaviaAWG7, and Wratislavia K1) and the highest erythritol production was achieved at pH 3 with low levels of by-products (arabitol, citric and isocitric acid) detected.

Yarrowia lipolytica is a strictly aerobic yeast and the oxygen available to the cell is an important parameter. Previous studies have demonstrated that the dissolved oxygen concentration in the medium influences the production of several metabolites, such as γ-decalactone [53], lipase [54] and citric acid [31, 55]. However, to our best knowledge, only one study addressing the effect of dissolved oxygen in erythritol production was found. Rywińska *et al.* [42] studied the effect of different stirring and aeration rates and, consequently, the dissolved oxygen concentration on erythritol production by *Y. lipolytica* Wratislavia K1. In that study, it was demonstrated that the highest erythritol yield from crude glycerol was obtained with high stirring rate (800 rpm) and with aeration rate of 0.6 vvm.

1.6 Operation in bioreactor: operation modes

The production of metabolites, such as erythritol, in bioreactor can be achieved through the use of different operation modes: batch, fed-batch and continuous.

A batch process is a closed system, which means all the required substrates are added at the beginning, and the product and co-products are extracted when the fermentation is terminated [9, 56]. The batch culture is the simplest operation mode, making it a convenient method for the production of erythritol. In a study conducted by Tomaszewska *et al.* [15], a scale-up of erythritol production from pure glycerol was assessed in batch reactors, using the *Y. lipolytica* strains A UV'1, A-15 and Wratislavia K1. The scale-up of the batch from the flask to the bioreactor resulted in a significant increase in erythritol concentration for all strains.

Unlike batch cultures, fed-batch fermentations do not work as closed systems, since at a given point of the process, an amount of substrate is introduced into the bioreactor [56]. Fed-batch cultures can be carried out in different ways: at fixed volume, where a portion of the culture medium is substituted by fresh medium, or at variable volume, where the fresh medium is added into the

bioreactor with no medium removal [56]. The fed-batch cultures with fixed medium volume are named as repeated batch cultures. In this mode, portions of the culture medium are periodically removed and replaced with fresh medium [8]. Comparing to the traditional batch processes, this method can improve the process efficiency by extending the effective production phase [8, 57]. Mirończuk *et al.* [8] studied erythritol production by *Y. lipolytica* MK1 in repeated batch culture. Periodically 30 or 40 % of the medium was replaced and the culture was stable for 915 hours [8]. Comparing the results to batch cultures, it led to a 2.7 and 1.4-fold increase in erythritol concentration and yield, respectively, while an 1.6-fold decrease in productivity was observed [8]. The fed-batch cultures with variable medium volume can be performed at either continuous feeding or pulsed addition (step-wise fed-batch) [57]. Both strategies were studied in the erythritol production. Tomaszewska *et al.* [58] examined erythritol production by *Y. lipolytica* Wratislavia K1 using constant feeding, which resulted in a 2.4 and 1.1-fold increase in erythritol concentration and yield, respectively. In another study conducted by Rywińska *et al.* [14], fed-batch with pulsed additions by the same strain was performed and it resulted in a 2.8 and 1.2-fold increase in erythritol concentration and productivity, respectively.

The last operation mode is continuous process, that is an open system, during which the bioreactor is fed with fresh medium, while, at the same rate, medium and cells are removed [57]. There are a few studies dedicated to the erythritol production by *Y. lipolytica* using continuous culture. Rakicka *et al.* [45] evaluated erythritol production in a continues mode using Wratislavia K1 strain, and a productivity of 1.12 $g \cdot L^{-1} \cdot h^{-1}$ and a yield of 0.52 $g \cdot g^{-1}$ were achieved.

2. AIMS

This chapter presents the main goals of this work.

The outputs of the thesis also listed.

2.1 Aims

The amount of diseases related to sugar consumption has increased worldwide in the last decades. Consequently, the demand for healthier options to added sugars is on the raise, and low-calorie sweeteners, such as sugar-alcohols, have been seen as an alternative. Erythritol is a sweetener of great interest since it has low caloric value, is non-cariogenic, and non-glycemic. Since the chemical synthesis of erythritol has low efficiency and high production costs, its production is now achieved by fermentation processes using microorganisms.

The non-conventional yeast *Y. lipolytica* can use crude glycerol, from biodiesel industry, as carbon source to produce erythritol. However, several parameters can influence the amount of erythritol produced, such as strain, medium composition, and operating condition, so these parameters should be considered in production process implementation. Since the scale-up of the process is the major issue for industrial application, optimization strategies for erythritol production must include some relevant parameters, such as oxygen availability and reactor operation mode.

Therefore, the main goals of this work are:

- Screening of osmotolerant strains (wild type and mutants) of *Y. lipolytica* with high capability to produce erythritol;

- Scale-up the process in a stirred tank reactor and implementation of different operation mode (fed-batch).

2.2 Outputs of this thesis

Oral communication:

Ferreira, P., Ribeiro, M., Machado, A. R., Nagy, E., Nguyen, D. Q., Belo, I.; "Production of erythritol by Yarrowia species from crude glycerol"; 13th International Chemical and Biological Engineering Conference - CHEMPOR 2018. Aveiro, Portugal, 2-4 October 2018.

3. MATERIALS AND METHODS

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

3.1. Yeast strains

Five wild type strains of *Y. lipolytica* (W29 (ATCC 20460), NCYC 2904, CBS 2075, CBS 2073, and IMUFRJ) and thirty-eight acetate negative mutants of *Y. lipolytica* W29 were used in this study. The mutant strains were obtained in a previous work by exposing *Y. lipolytica* W29 to different treatments with mutagenic agents: UV-irradiation, ethyl methane sulfonate (EMS), and the combination of both treatments [59].

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

3.2. Screening of *Yarrowia lipolytica* strains for erythritol production

3.2.1 Salt tolerance

Two different methodologies were used to select the most osmotolerant strains. In both strategies, the growth of the strains in medium with different concentrations of NaCl was evaluated.

3.2.1.1 Selection in agar plates

All strains were pre-grown over-night in yeast extract peptone glycerol medium (YPG) at 27 °C and 200 rpm. The YPG medium was composed by (g·L^{-1}): yeast extract 10, peptone 20, and pure glycerol 20. The cells were diluted in NaCl 0.9 % solution to achieve an optical density (600 nm) of 0.6 and were, afterwards, serially diluted. Drops (3 µL) of each dilution (10^0 – 10^4) were spotted onto plates with YPGA medium and YPGA medium supplemented with NaCl (1 M). The cells growth was accessed after 24 hours of incubation at 27 °C. The pictures were taken using imaging equipment (ChemiDoc XR+, BioRad, USA).

3.2.1.2 Selection in miniaturized assays in liquid medium

A second methodology was performed to try to overcome the subjectivity of the strains selection carried out through picture observation.

The strains with better resistance to salt concentration in the previous methodology were pre-grown over night in YPG medium, at 27 °C and 200 rpm. Each strain was inoculated in 5 mL of YPG medium, supplemented with different NaCl concentrations (0, 1, and 2 M), with an initial cellular concentration of 0.1 g L^{-1}. Afterwards, 300 µL of each culture was plated into a microtiter plate with 96 sample wells and incubated in a rotary shaker at 27°C and 200 rpm. The optical density of the culture was measured after 0, 24 and 48 hours of incubation, at 600 nm using a microplate reader (BIO-TEK, Synergy HT).

In order to compare the results of the different strains, the equation 1 was applied:

$$Inhibition\ (\%) = 100 - \Delta X_{NaCl} \times \frac{100}{\Delta X_{Control}}$$
<div align="right">Equation 1</div>

Where ΔX_{NaCl} corresponds to the difference in optical density (600 nm) at 0 hours and 24/48 hours of strains grown in YPG medium containing NaCl, and $\Delta X_{Control}$ the difference in optical density (600 nm) at 0 hours and 24/48 hours of strains grown in YPG medium without NaCl.

3.2.2 Erythritol production assays

The yeast strains selected before were pre-grown overnight in YPG medium, at 27 °C and 200 rpm. Cells were centrifuged and resuspended in production medium to obtain a starting cellular concentration of 1 g·L^{-1}. The experiments were carried out in 500 mL baffled flasks containing 200 mL of production medium on a rotary shaker at 200 rpm and 27°C. The production medium was composed by (g·L^{-1}): crude glycerol 100, yeast extract 1, NH$_4$Cl 3, NaCl 25, KH$_2$PO$_4$ 0.2, MgSO$_4$·7H$_2$O 1 and 0.72 M potassium phosphate buffer at pH 3.0. The medium pH was maintained at 3 by adding KOH (2 M). The mean values, of two independent experiments, were reported and compared by analysis of variance (One-way ANOVA). The differences between the conditions were tested using the Tukey test. All analyses were performed at P ≤ 0.05 using GraphPad Prism, version 6.01.

3.3 Production of erythritol in bioreactor

The strain that presented the best result in the flasks experiments was selected and the erythritol production was accessed in a stirred tank bioreactor (STR) using different operation modes (batch, fed-batch and step-wise fed-batch). The experiments were performed in a 3.7 L bioreactor (RALF PLUS SOLO, Bioengineering, Switzerland) with 31 cm height and 17 cm diameter, which contained an agitator with two Rushton impellers, 6-blade, 6 cm outside diameter (Figure 3). The medium pH was maintained at 3 by adding KOH (2 M) using Peripex peristaltic pumps (Bioengineering, Switzerland). A polarographic-membrane probe (InPro 6000, Mettler Toledo, USA), associated with the BioScadaLab software, was used to measure the dissolved oxygen concentration.

Figure 3 – Bioreactor (RALF PLUS SOLO, Bioengineering, Switzerland) with production medium.

In all experiments, the cells were pre-grown overnight, in 1 L Erlenmeyer flask containing 500 mL of YPG medium, at 27 °C and 200 rpm. In order to achieve an initial cellular concentration of 1 g·L^{-1}, yeast cells were centrifuged and resuspended in the production medium. All experiments in bioreactor were performed at 27 °C.

3.3.1 Batch cultures

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 operation strategies were tested: constant aeration and stirring rates and controlled dissolved oxygen concentration.

In the constant aeration and stirring rates strategy, two different conditions were tested: low stirring and aeration rates and high stirring and aeration rates. In the low agitation and aeration rates condition, the experiment started at 200 rpm and 1 vvm, and after 48 hours the stirring rate was raised to 400 rpm. At high stirring and aeration rates, the conditions used were 900 rpm and 3 vvm.

In the constant dissolved oxygen strategy, the experiments were conducted at 20 % and 40 % of dissolved oxygen. The dissolved oxygen concentration in the culture medium was maintained by manipulating the stirring and aeration rates, through a cascade control mode. In the cascade mode, the stirring and aeration rate automatically varied between 400 rpm - 1200 rpm and 1 vvm – 3 vvm, respectively.

3.3.2 Fed-batch cultures

The fed-batch experiments were initiated with a batch culture, as described above, with stirring and agitation rates of 900 rpm and 3 vvm, respectively. After a batch period of 72 hours, the feeding was initiated. In order to assess the erythritol production in this operation mode, different approaches were studied: fed-batch at constant feeding rate with completed and concentrated medium, fed-batch at constant feeding rate only with glycerol and a step-wise fed-batch with the addition of crude glycerol.

In the experiments at constant feeding rate with completed medium, two feeding rates were tested, 6.3 mL·h^{-1} and 11.9 mL·h^{-1}. The composition of the feeding medium was similar to the production medium but concentrated. The feeding medium composition was (g·L^{-1}): crude glycerol 200, yeast extract 2, NH$_4$Cl 6, NaCl 50, KH$_2$PO$_4$ 0.4, MgSO$_4$·7H$_2$O 2. The feeding medium was added to the bioreactor at a constant flow rate through a peristaltic pump (Watson Marlow).

In the fed-batch culture at constant feeding rate only with glycerol, a glycerol solution (200 g·L^{-1} of crude glycerol) was added at constant feeding rate of 12.8 mL·h^{-1}.

In the step-wise fed-batch, crude glycerol was added to the bioreactor in order to obtain a final concentration close to 100 g·L^{-1}. This addition was performed after a 72 hours batch.

3.4 Analytical methods

Samples were periodically collected to measure biomass concentration, glycerol consumption, and erythritol and mannitol production. Biomass concentration was measured by optical density at 600 nm and converted to dry cell mass per liter. Glycerol, erythritol and mannitol concentration was 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 was eluted with H_2SO_4 5 mM at 0.5 mL·min^{-1} and the column temperature was 60 °C. To HPLC analyzes, 2 mL of each sample was centrifuged, the respective supernatant was filtered through a syringe filter with a pore size of 0.22 μm.

3.5 Kinetic parameters

For flask and bioreactor experiments, the kinetic parameters erythritol yield and productivity were calculated.

Erythritol yield, expressed in grams of erythritol produced per grams of substrate consumed, was calculated according to Equation 2:

$$Yield = \frac{\Delta E}{\Delta S}$$
Equation 2

where ΔE is the variation of erythritol concentration produced and ΔS is the variation of concentration of glycerol in the same period.

Erythritol productivity, expressed in grams of erythritol per liter per hour, was calculated using Equation 3:

$$Productivity = \frac{\Delta E}{t}$$
Equation 3

where t is the time period in hours.

4. RESULTS AND DISCUSSION

In this work, a screening of 43 *Y. lipolytica* strains (wild-type and mutant) was performed and 9 strains proved to be the most osmotolerant. Erythritol production of these 9 strains was evaluated and the strains W29, UV15-31, UV15-93 and EMS75-162 produced higher concentrations of erythritol (statistically similar). The wild-type *Y. lipolytica* W29 was the selected strain for the scale-up of the bioprocess.

The effect of oxygen in erythritol production was evaluated using different operation strategies in batch bioreactor experiments. The higher dissolved oxygen concentration enhanced erythritol productivity, while the lowest erythritol productivity was observed in conditions with almost 0 % of dissolved oxygen. Highest concentration of erythritol ((32 g·L^{-1}), yield (0.43 g·g^{-1}) and productivity (0.44 g·L^{-1}·h^{-1}), were obtained with stirring and aeration rates of 900 rpm and 3 vvm, respectively.

To evaluate if erythritol production could be extended with the addition of glycerol, several fed-batch strategies were tested (constant feeding rate with completed medium, constant feeding rate of a crude glycerol solution and a step-wise fed-batch with addition of crude glycerol. The best results were obtained when only glycerol was fed to culture. Nevertheless, the highest titer was obtained with the step-wise fed-batch strategy, 62 g·L^{-1} of erythritol that correspond to 0.42 g·g^{-1} yield and 0.45 g·L^{-1}·h^{-1} productivity.

4.1. Screening of *Yarrowia lipolytica* strains for erythritol production

4.1.1. Salt tolerance

The production of erythritol by osmophilic/osmotolerant yeasts occurs to cope with the high external osmotic pressure [60]. Therefore, the first selection method for *Y. lipolytica* strains potential better producers was based on salt tolerance. In order to select the most osmotolerant, the growth of the 43 strains exposed to different NaCl concentration was evaluated using two different methodologies.

In the first methodology, the salt sensitivity was evaluated in agar plates containing NaCl 1 M. The cellular growth in these plates was compared with the cellular growth in plates without salt (control). The cellular growth was assessed after 24 hours and the results are presented in Figure 4.

The images presented in Figure 4 show the sensitivity of each strain of *Y. lipolytica* to the osmotic stress caused by the presence of NaCl in the YPGA plates. It is possible to observe that some strains were more sensitive to NaCl, such as strains 16, 17, 40, 41 and 43. These strains had lower growth (less intense spots), even at a 10^0 dilution on medium plates with 1 M of NaCl. On the opposite, the strains 11, 13 were the most resistant to NaCl, since it is observable a slightly growth in the 10^3 dilution on medium plates containing NaCl.

After careful observation of each strain growth represented in the images of Figure 4, out of the 43 *Y. lipolytica* strains tested, the 20 strains that had denser spots at 10^2 dilution on plates with NaCl 1 M were selected as the most tolerant to the salt. The strains selected were: W29 (1), CBS 2075 (3), CBS 2073 (5), UV15-19 (8), UV15-27 (10), UV15-31(11), UV15-54 (12) UV15-93 (13), UV15-303 (14), UV15-86 (15), UV15-345 (21), UV15-352 (22), EMS50-72 (23), EMS50-53(27), UV5-42 (29), UV15-404 (30), EMS75-162 (31), EMS50-101 (32), UV5-71 (35), UV5-72 (36).
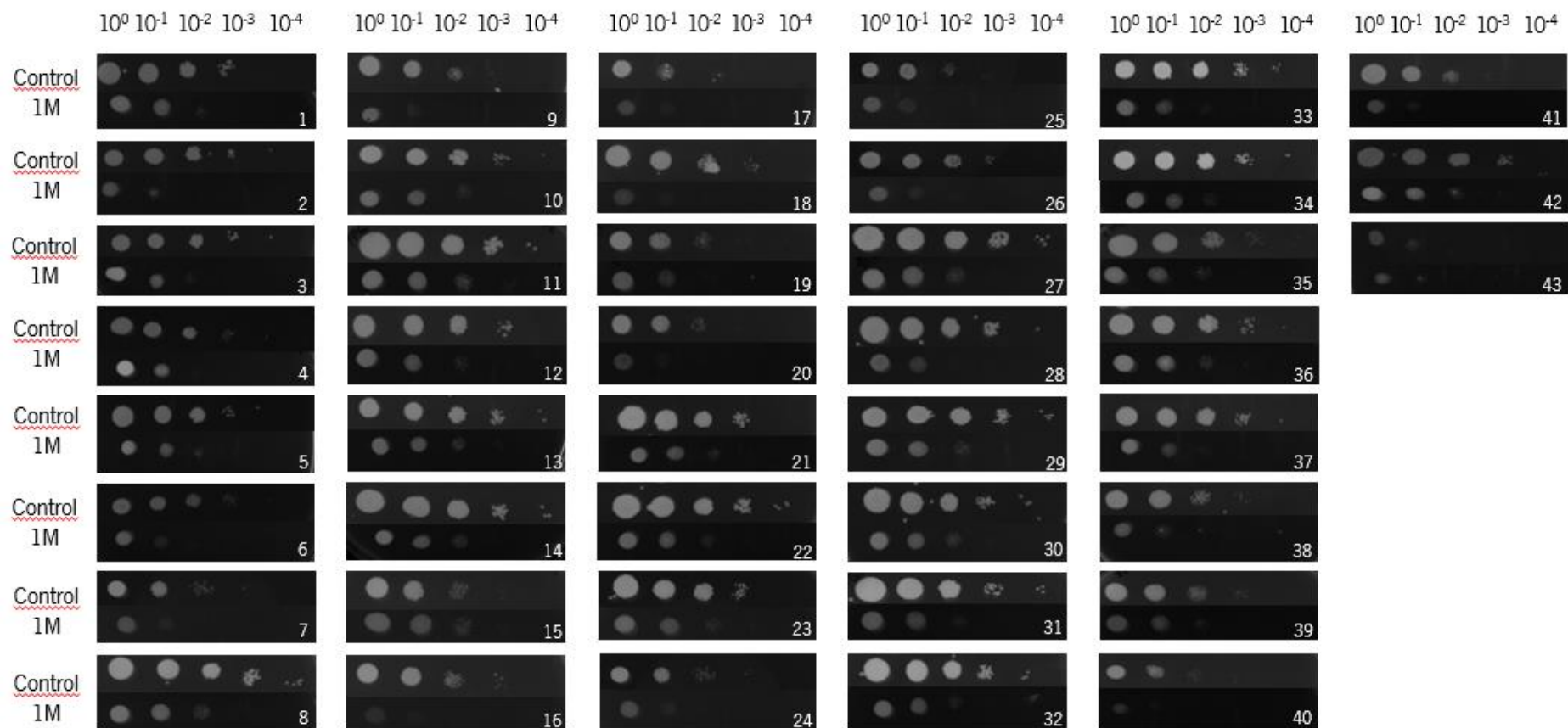
Figure 4 – Growth of *Y. lipolytica* strains serially diluted on YPGA plates containing 1 M of NaCl. Control plates of YPGA medium without NaCl. Images of the plates were taken after 24 hours of incubation. Each number corresponds to a different strain: 1 – W29; 2 – NCYC 2904; 3 – CBS 2075; 4 – IMUFRJ; 5 – CBS 2073; 6 – UV15-12; 7 – UV15-17; 8 – UV15-19; 9 – UV15-22; 10 – UV15-27; 11 – UV15-31; 12 – UV15-54; 13 – UV15-93; 14 – UV15-303; 15 – UV15-86; 16 – UV15-101; 17 – UV15-245; 18 – UV15-291; 19 – UV15-95; 20 – UV15-330; 21 – UV15-345; 22 – UV15-352; 23 – EMS50-72; 24 – EMS50-564; 25 - EMS50-132; 26 – EMS75-136; 27 – EMS50-53; 28 – EMS50-150; 29 – UV5-42; 30 – UV15-404; 31 – EMS75-162; 32 - EMS50-101; 33 – UV5-27; 34 – UV5-54; 35 – UV5-71; 36 – UV5-72; 37 – E50-UV15-3; 38 – UV15-E50-26; 39 – UV5-75; 40 – E50-UV15-59; 41 – E50-21; 42 – UV-EMS-10; 43 – UV15-E50-3.

The strain selection in the plate methodology is a poor screening strategy but allows a first indication of potential best group of strains. Thus, the salt tolerance of the 20 selected strains was further assessed using a different methodology, in miniaturized assays in liquid medium. In this methodology, the cells grew in a microtiter plate with 96 sample wells assay and their growth was followed over time. Cultivation was carried in YPG medium supplemented with 0 (control), 1 and 2 M of NaCl and cellular growth was measured at 0, 24 and 48 hours of incubation.

The following table, Table 2, resumes the percentage of growth inhibition obtained for each *Y. lipolytica* strain at 24 and 48 hours of incubation. As expected, higher concentration of salt caused higher growth inhibition of all strains. A higher concentration of NaCl corresponds to a higher osmotic pressure, and, by consequence, a higher stress experienced by the cells that affects the cellular growth.

In a general way, although more pronounced in medium supplemented with 1 M of NaCl, the inhibition percentage of each strain after 24 hours of incubation was higher than the observed at 48 hours. Comparing the growth in medium without NaCl, cells grown in medium supplemented with NaCl probably had longer lag phase, the cells needed more time to adjust to the new and stressful condition. However, after 48 hours, cells were able to recover from the initial shock experienced, which was reflected in a better cellular growth.

As shown in Table 2, the growth of *Y. lipolytica* strains in medium containing 1 M of NaCl was slightly inhibited by less than 90 % after 24 hours. However, only for two strains a 50 % cellular growth inhibition was observed. After 48 hours, the cells were able to adapt to the osmotic stress and for almost all strains the inhibition values were lower than 30 %, and for a few strains were even lower than 10 %.

Regarding the cell growth in YPG medium supplemented with 2 M NaCl, after 24 hours of incubation, almost 100 % of growth inhibition was observed, and only for four strains the inhibitory percentage was lower than 95 % (strains W29 (1), UV15-19 (8), EMS75-162 (31) and UV5-71 (35)). After 48 hours, the percentage of inhibition remained very high and only seven strains had inhibition values between 87 and 93 %.

The *Y. lipolytica* strains with growth inhibition lower than 70 % after 24 hours of growth in YPG medium with 1 M NaCl, and/or the lowest inhibition percentage after 48 hours of growth in YPG medium containing 2 M NaCl were selected to assess their ability to produce erythritol. Therefore, according to this methodology, the *Y. lipolytica* strains W29 (1), UV15-19 (8), EMS50-72 (23), UV5-42 (29), EMS75-162 (31), and UV5-72 (36) were selected.

Table 2 – Percentages of growth inhibition of *Y. lipolytica* strains grown in YPG medium supplemented with 1 and 2 M of NaCl at 24 and 48 hours of incubation.

Y. lipolytica strain	Growth Inhibition (%)			
	NaCl 1 M		NaCl 2 M	
	24 h	48h	24 h	48h
W29 (1)	65 ± 7	11 ± 3	94 ± 1	87 ± 0
CBS 2075 (3)	88 ± 2	1 ± 1	99 ± 0	98 ± 0
CBS 2073 (5)	81 ± 4	18 ± 3	98 ± 2	96 ± 2
UV15-19 (8)	50 ± 4	0 ± 5	94 ± 1	89 ± 2
UV15-27 (10)	87 ± 11	29 ± 7	98 ± 2	98 ± 1
UV15-31 (11)	69 ± 6	14 ± 4	100 ± 1	98 ± 3
UV15-54 (12)	67 ± 8	20 ± 4	99 ± 1	97 ± 1
UV15-93 (13)	61 ± 7	27 ± 10	98 ± 1	99 ± 0
UV15-303 (14)	85 ± 1	37 ± 4	99 ± 1	98 ± 1
UV15-86 (15)	80 ± 4	28 ± 3	98 ± 1	98 ± 0
UV15-345 (21)	79 ± 4	30 ± 4	99 ± 1	98 ± 2
UV15-352 (22)	79 ± 10	29 ± 4	99 ± 1	98 ± 0
EMS50-72 (23)	76 ± 7	11 ± 4	98 ± 1	89 ± 2
EMS50-53 (27)	77 ± 3	14 ± 4	99 ± 0	93 ± 1
UV5-42 (29)	54 ± 1	11 ± 3	99 ± 1	91 ± 3
UV15-404 (30)	66 ± 4	14 ± 3	99 ± 0	96 ± 1
EMS75-162 (31)	64 ± 5	12 ± 1	93 ± 1	92 ± 2
EMS50-101 (32)	89 ± 2	9 ± 5	98 ± 1	96 ± 2
UV5-71 (35)	69 ± 5	33 ± 1	93 ± 1	98 ± 0
UV5-72 (36)	61 ± 4	11 ± 1	99 ± 0	91 ± 2

Besides the six strains selected with this strategy, three other strains (UV15-31 (11), UV15-93 (13), UV15-345 (21)) were selected for the erythritol production assays. These strains did not have low

inhibition percentages, however, they had a noticeable growth, with denser spots, in the plate agar strategy, and, for this particular reason, they were included in the erythritol production experiments.

4.1.2 Erythritol production assays

To select the best erythritol producer strain, the ability to produce erythritol by the nine strains, previously selected, was evaluated. The erythritol production experiments were carried out in baffled flasks. The cellular growth, glycerol consumption and the production of erythritol were monitored (Figure 5).

Through the observation of Figure 5, in this experiment, five strains stood, UV15-31, UV15-345, W29, EMS75-162, and UV15-93, since for these strains higher erythritol concentration, cell growth, and also higher glycerol consumption was observed. The lower cell growth, glycerol consumption and consequent lower erythritol concentrations verified in the remaining strains, may be associated to the growth conditions. The low pH associated with the high osmotic pressure probably was too aggressive to these strains. Some authors described that erythritol production is associated with cellular growth [13], therefore it was expected that the strains with lower cellular growth produced lower erythritol concentrations.

The strain that produced the highest concentration of erythritol in these flasks batch cultures (Figures 5 and 6) was UV15-93 (26.5 ± 1.4 g·L^{-1}), closely followed by EMS75-162 (24.7 ± 0.6 g·L^{-1}), W29 (24.7 ± 0.9 g·L^{-1}), and UV15-31 (24.0 ± 0.6 g·L^{-1}). On the other hand, the lowest concentration of erythritol was produced by *Y. lipolytica* UV5-72 (4.8 ± 0.6 g·L^{-1}). For all strains only residual amounts of the by-product mannitol (0 - 1.5 g·L^{-1}) were detected in medium culture after 6 days (data not shown).

All strains were able to grow in the culture conditions used in this experiment (Figure 5A), however none of the strains were able to consume glycerol completely during the experiment time (Figure 5B). The nine strains produced significant amounts of erythritol that ranged from 4.8 to 26.5 g·L^{-1} (Figure 5C).
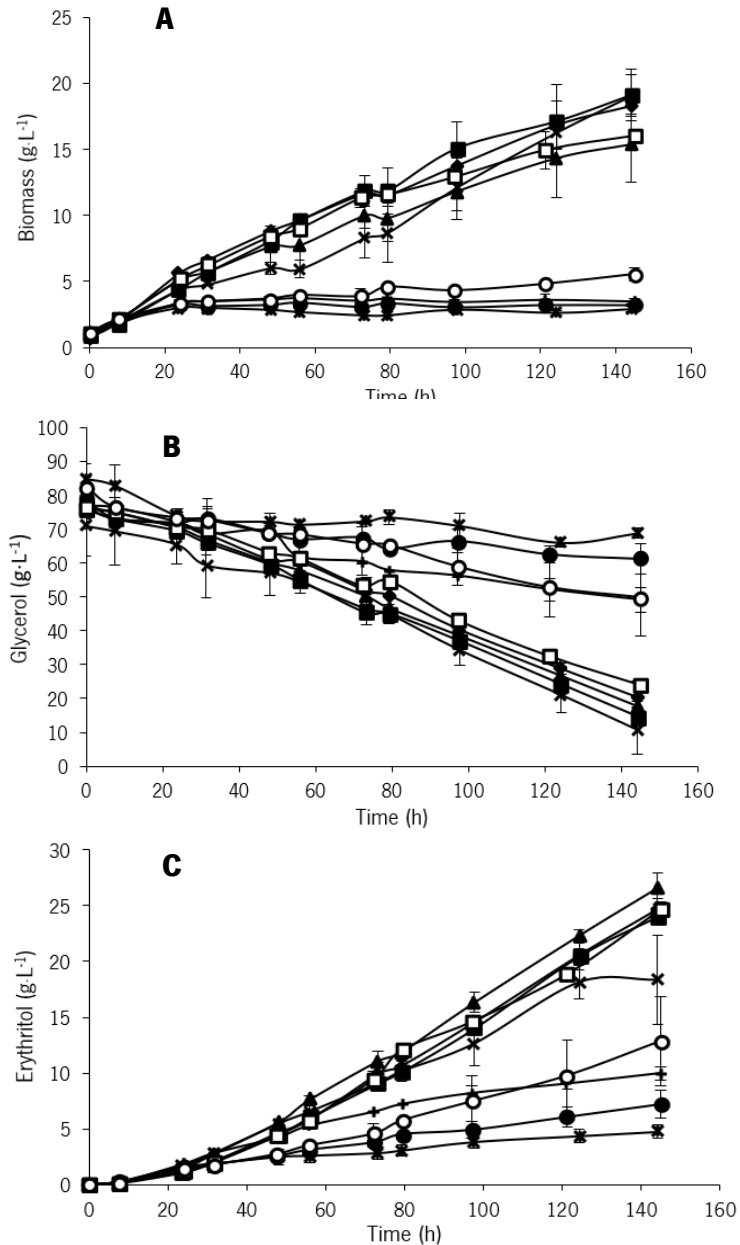
Figure 5 – Biomass growth **(A)**, glycerol consumption **(B)**, and erythritol production **(C)** profiles in batch cultures of *Yarrowia lipolytica* strains W29 (♦), UV15-345 (×), UV13-31 (■), UV15-93 (▲), UV5-72 (*), EMS50-72 (●), UV5-42 (+), EMS75-162 (□) and UV15-19 (○).

To a more accurate comparison of the erythritol produced by all the strains tested, one-way analysis of variance (one-way ANOVA) was performed (Figure 6). The erythritol concentrations produced by the *Y. lipolytica* strains W29, UV15-31, UV15-93 and EMS75-162, the highest producers of erythritol, were not statistically different (Figure 6). The erythritol concentration produced by these four strains was statistically different from the remaining and less producer strains (*Y. lipolytica* UV15-345, UV15-19, UV5-42, EMS50-72, and UV5-72).
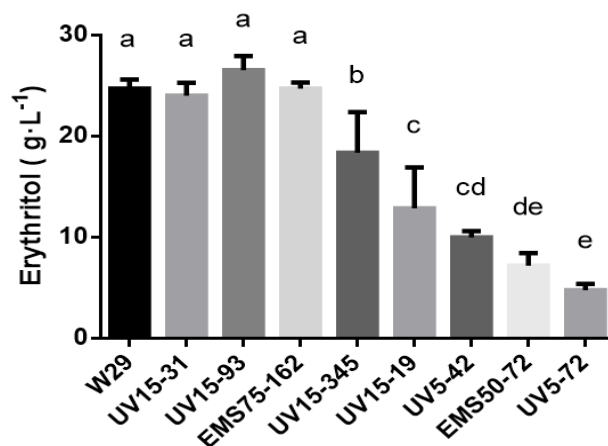
Figure 6 - Erythritol production for batch culture of *Y. lipolytica* strains. The values are presented as average and standard deviation of two independent experiments. The letters above the bars represent statistically significant differences (p<0.05), different letters indicate significant differences in mean values.

These erythritol productions corresponded to yields that varied from 0.30 to 0.49 g·g^{-1} and productivities that ranged from 0.033 to 0.184 g·L^{-1}·h^{-1} (Table 3). The conversion of glycerol into erythritol (yield) was quite similar to all strains. The highest yield was observed for the strain UV15-19. Although this strain did not produce a high amount of erythritol (12.9 g·L^{-1}), it can convert almost half of the glycerol consumed into erythritol. Despite the greater yield, the strain UV15-19 had low productivity rate, which makes it a less interesting erythritol producer, since to obtain a high erythritol concentration, the process would be very long. The strains with higher cell growth and higher erythritol production (W29, UV15-31, UV15-93 and EMS75-162) had also higher values of productivity. Similar to the erythritol concentration, the productivity values achieved by these strains were not statistically different from each other, but were different from the other five strains. The lowest biomass and erythritol concentrations were observed for the strains UV15-19, UV5-42, EMS50-72 and UV5-72 and it was reflected in low values of productivity.

Table 3 – Parameters of erythritol production by *Y. lipolytica* strains in flasks assays. The values are presented as average and standard deviation of two independent experiments.

Y. lipolytica strains	Yield (g·g^{-1})	Productivity (g·L^{-1}·h^{-1})
W29	0.436 ± 0.001[abc]	0.171 ± 0.005[a]
UV15-31	0.39 ± 0.05[bc]	0.17 ± 0.01[a]
UV15-93	0.46 ± 0.03[ab]	0.184 ± 0.008[a]
EMS75-162	0.47 ± 0.02[ab]	0.171 ± 0.004[a]
UV15-345	0.30 ± 0.06[d]	0.13 ± 0.03[b]
UV15-19	0.49 ± 0.05[bc]	0.09 ± 0.03[c]
UV5-42	0.37 ± 0.03[cd]	0.069 ± 0.004[cd]
EMS50-72	0.41 ± 0.02[abc]	0.050 ± 0.009[de]
UV5-72	0.30 ± 0.02[d]	0.033 ± 0.004[e]

The yield values obtained in these experiments were not very different from the ones described in the study of Tomaszewska *et al.* [15], where erythritol production by *Y. lipolytica* wild type strains (A-1, A-2-4, A-1, and A-15) and acetate-negative mutants (Wratislavia 1.31 and Wratislavia K1) was evaluated from pure glycerol. In that study, yields that varied between 0.34 and 0.42 g·g^{-1} for the wild-type strains, and 0.35 to 0.43 g·g^{-1} for the mutant strains were achieved. In another study published by Silva *et al.* [61], the *Y. lipolytica* strain W29 was used in erythritol production assays from pure glycerol and similar values of yield (0.42 g·g^{-1}) and productivity (0.14 g·L^{-1}·h^{-1}) were obtained. In the present study, the strains W29, UV15-31, UV15-93, and EMS75-162 achieved similar yields using crude glycerol, a cheaper carbon source.

4.2 Bioreactor experiments

According to the results of the flask experiments, the better strains to produce erythritol were W29, UV15-31, UV15-93 and EMS75-162. Since no statistical differences were observed between these strains, *Y. lipolytica* W29, a wild type strain, was the strain selected for the following assays in bioreactor.

4.2.1 Batch cultures – Effect of oxygen concentration

Since *Y. lipolytica* is a strictly aerobic yeast, oxygen availability might influence the production of this polyol. However, to our best knowledge, only one work is available in literature addressing this issue, thus, in this work, different operation strategies were tested to evaluate the effect of dissolved oxygen concentration in erythritol production, such as the use of constant aeration and stirring rates and the use of controlled constant dissolved oxygen concentration.

An experiment with low aeration and stirring rates was performed, where the culture was initiated with a stirring rate of 200 rpm and an aeration rate of 1 vvm. At these conditions, no cellular growth, no erythritol production and no glycerol consumption (Figure 7A) were observed during the first 48 hours. This lack of growth was due oxygen depletion from the medium, since the dissolved oxygen concentration (Figure 7B) was around 0 % during this period. In order to increase air supply to the cells, the stirring rate was raised to 400 rpm at this point.
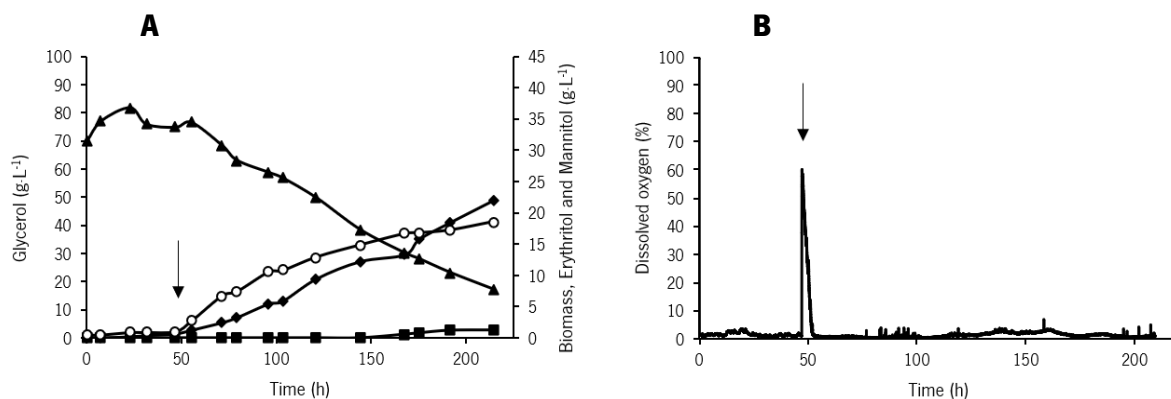
Figure 7 – **(A)** Biomass concentration (○), glycerol consumption (▲), erythritol (♦) and mannitol (■) production by *Y. lipolytica* W29 and **(B)** dissolved oxygen profile in batch culture performed with low constant aeration and stirring rates: aeration rate of 1 vvm and agitation rate of 200 rpm, in the first 48 hours, and afterwards of 400 rpm. The arrows indicate the time when the stirring rate was altered.

After the change of the stirring rate to 400 rpm, an increase in dissolved oxygen concentration was observed and, after a few hours, it decreased again to values close to zero (Figure 7B). This decrease in dissolved oxygen concentration occurred when the cells started to grow and produce erythritol (Figure 7A). After 215 hours of cultivation, the maximum biomass concentration was 18.58 g·L⁻¹, glycerol was not completely consumed and 21.98 g·L⁻¹ of erythritol was produced. In this condition, only 1.32 g·L⁻¹ of mannitol was detected.

The parameters of erythritol production were calculated considering only the time after the stirring rate had been altered, only after the first 48 hours of experiment, since it was after this time that cellular growth and erythritol production were noticed. In this batch culture, the conversion yield of glycerol to erythritol was 0.42 g·g^{-1}, very similar to the obtained in the flasks experiments for the same strain (0.44 g·g^{-1}). Also, the productivity obtained in this batch (0.13 g·L^{-1}·h^{-1}) was similar to the one achieved in the flask culture (0.17 g·L^{-1}·h^{-1}). Additionally, an identical maximum biomass concentration was obtained in the corresponding flask assay (18.4 g·L^{-1}). The similarity in these parameters may be due to the dissolved oxygen concentration that, even with higher stirring rate, remained close to zero during the experiment.

Considering the low dissolved oxygen concentration detected in these operating conditions, a batch with higher stirring and aeration rates was performed in order to augment the dissolved oxygen available in the medium. The stirring and aeration rates used were 900 rpm and 3 vvm, respectively.

In this experiment, after 8 hours of lag phase, yeast cells grew to a maximum concentration of 35.2 g·L^{-1}, registered at 72 hours (Figure 8A). The increase of stirring and aeration rate resulted in a faster glycerol consumption and in a 1.5-fold increase in erythritol produced (31.8 g·L^{-1}). Like in the last batch culture, mannitol was produced at low concentrations (1.1 g·L^{-1}). This erythritol production translated to a yield of 0.43 g·g^{-1}, and a productivity of 0.44 g·L^{-1}·h^{-1}.
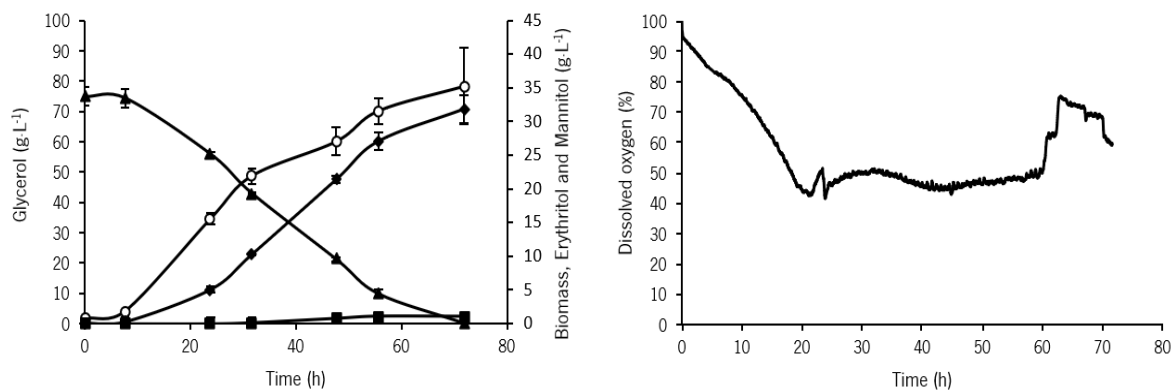
Figure 8 – (A) *Y. lipolytica* W29 cellular growth (○), glycerol consumption (▲), erythritol (♦) and mannitol (■) production and **(B)** dissolved oxygen profile in batch culture, which occurred with the following constant conditions: a stirring rate of 900 rpm and an aeration rate of 3 vvm. The error bars represent the standard deviation of two independent replicas.

With this aeration and stirring rate conditions, it was possible to avoid oxygen depletion (Figure 8B). In the first 20 hours of experiment, it was observed a gradual decrease in dissolved oxygen percentage, justified by the higher requirement for oxygen of yeasts cells over time, since they are

starting to grow exponentially. After this time, the dissolved oxygen in the production medium stabilized around 40 %. In the last hours of experiment, the dissolved oxygen percentage augmented, because there was a deceleration of cells metabolism, a decline in growth rate (end of exponential phase and beginning of stationary phase.

The increase of agitation and aeration conditions increased, as expected, the dissolved oxygen concentration in the medium. This increase in oxygen available in the medium resulted in an augment of the biomass concentration obtained and, consequently, in the erythritol produced. The erythritol produced in the batch with higher stirring and aeration rates was around 10 g·L^{-1} higher than using lower stirring and aeration rates. However, the biggest improvement observed was in the productivity, since the batch time was reduced, resulting in a 4.4-fold increase of the productivity in these conditions. With these results it is possible to state that the oxygen available in the medium is a very important parameter in the erythritol production. Rywińska *et al.* [42] studied the effect of different stirring and aeration rates in erythritol production, using crude glycerol and the mutant strain *Y. lipolytica* Wratislavia K1. Like the results obtained in the present work, the increase of the stirring and aeration rates from 600 to 800 rpm and 0.36 to 0.6 vvm, respectively, resulted in the production of an additional 16 g·L^{-1} of erythritol. Also, a 1.3-fold increase in productivity was observed with 800 rpm and 0.6 vvm conditions. However, no significant differences were observed in the biomass concentration produced.

The experiments using constant aeration and stirring rates presented very different dissolved oxygen profiles, one 0 % and the other with dissolved oxygen stabilized around 40 %. Therefore, experiments with controlled constant dissolved oxygen concentrations of 20 % and 40 % were performed. These concentrations were maintained using a cascade control mode varying the aeration and stirring rates.

Since for experiments with lower oxygen concentration more time was needed to consume all glycerol, the experiment conducted at 20 % of oxygen had the duration of 142 hours. In this experiment, like in the previous, an 8 hours of lag phase was observed and after the 142 hours of cultivation, a concentration of 38.8 g·L^{-1} of biomass was achieved (Figure 9). However, at 55 hours of cultivation, a maximum erythritol concentration (21.33 g·L^{-1}) was reached (Figure 9). After this time, since glycerol had been completely exhausted, the secondary metabolites, erythritol and mannitol, started to be consumed by the cells (Figure 9). At the end of the batch, the concentration of these polyols was null (Figure 9). Rywińska *et al.* [62] described the same behavior by the strain *Y. lipolytica* Wratislavia 1.31, where erythritol and mannitol were totally consumed after glycerol had been depleted.
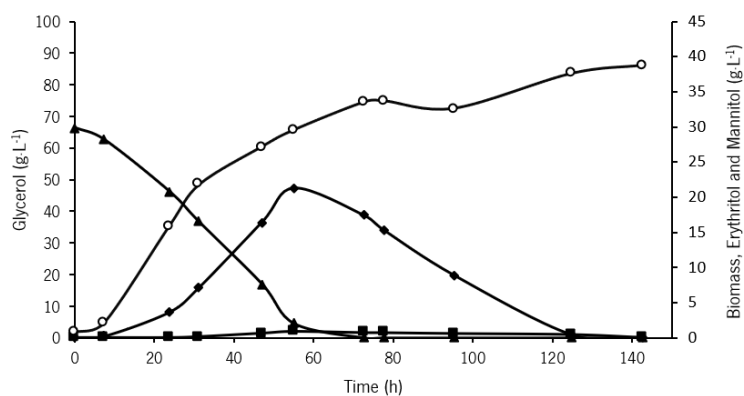
Figure 9 – *Y. lipolytica* W29 growth (○), glycerol consumption (▲), erythritol (♦) and mannitol (■) production in batch culture performed at controlled dissolved oxygen concentration of 20 %.

Since the maximum erythritol concentration was obtained at 55 hours of fermentation and afterwards it was consumed, yield and productivity were calculated at this specific time. Thus, the values for yield and productivity were 0.35 g·g⁻¹ and 0.39 g·L⁻¹·h⁻¹, respectively. Comparing these results with the batch performed at 900 rpm and 3 vvm, a lower concentration of erythritol was produced and a slightly lower yield and productivity were observed. However, comparing with the low constant stirring and aeration rates batch, the conditions of dissolved oxygen at 20 % translated in a higher productivity, and a similar yield.

Considering that the erythritol concentration was 1.5-fold lower than the one observed in the batch with high stirring and aeration rates, another batch culture was performed using the oxygen concentration observed during the batch conducted at 900 rpm and 3 vvm. In this new batch culture, the dissolved oxygen concentration was maintained at 40 %.

After a lag phase that lasted for 8 hours, yeast cells started to grow (Figure 10). And after 72 hours of cultivation, cells grew to a maximum of 30.1 g·L⁻¹. At the end of the experiment, 23.3 g·L⁻¹ of erythritol had been produced, corresponding to a 0.40 g·g⁻¹ yield and 0.33 g·L⁻¹·h⁻¹ productivity. Comparing the results with the 20 % controlled dissolved oxygen batch, similar concentrations of erythritol and biomass were produced in both batches. Nonetheless, when compared to the batch with high stirring and aeration rates, although similar yield values were achieved, an erythritol concentration 1.4-fold lower was produced and a productivity 1.3-fold lower was obtained.
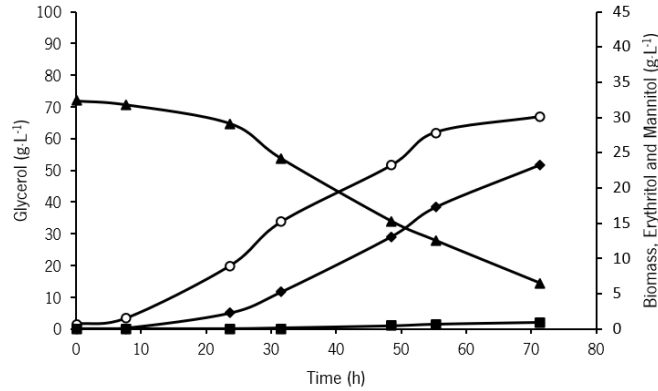
Figure 10 – *Y. lipolytica* W29 cellular growth (○), glycerol consumption (▲), erythritol (♦) and mannitol (■) production in batch culture performed at controlled dissolved oxygen of 40 %.

In Table 4, the results of all batches are summarized. Comparing all the batches performed, the higher concentration of erythritol, yield and productivity were obtained in the batch performed at high constant stirring and aeration rates (900 rpm and 3 vvm, respectively), indicating that keeping oxygen transfer constant would be more important than keeping dissolved oxygen concentration constant during the production time. The maximum erythritol concentrations produced by the other three batches were not very different from each other. The same is verified for all the yield values. However, the parameter that seemed to be more influenced by a dissolved oxygen concentration in the medium different from zero was the productivity. In other words, the lowest productivity of all four batches was achieved in the batch with low constant aeration and stirring rates, where the dissolved oxygen in the medium remained close to zero. This shows that the oxygen availability is a very important parameter in the erythritol production. On the other hand, the productivities of the batches performed with controlled dissolved oxygen were lower than the one obtained in high constant stirring and aeration rates batch. This also might indicate that the oscillations caused by the constant variations in the stirring and aeration rates observed in these experiments do not favor erythritol production.

Table 4 – Kinetic parameters of erythritol production by *Y. lipolytica* W29 in batch assays with different oxygenation strategies: constant stirring and aeration rates (400 rpm and 1 vvm; 900 rpm and 3 vvm); and controlled dissolved oxygen (20 % dissolved oxygen; 40 % dissolved oxygen).

Batch assays	Erythritol (g·L⁻¹)	Yield (g·g⁻¹)	Productivity (g·L⁻¹·h⁻¹)
400 rpm and 1 vvm	22	0.42*	0.13*
900 rpm and 3vvm	32 ± 6	0.43 ± 0.05	0.44 ± 0.03
20 %	21	0.35	0.39
40 %	23 ± 3	0.40 ± 0.02	0.33 ± 0.04

*Values were calculated for the period between 48 hours (when the stirring rate was changed) and 215 hours of experiment.

The yield values obtained in these experiments are comparable to some studies, where *Y. lipolytica* strains were used in bioreactor batch fermentations and crude glycerol was used as carbon source. The values for yield described in literature vary between 0.34 and 0.49 g·g⁻¹ [15, 34, 42]. However, in the same studies, higher productivities are reported, varying between 0.60 and 1.00 g·L⁻¹·h⁻¹ [15, 34, 42]. For instance, in a study published by Rywińska *et al.* [14], where the mutant strain *Y. lipolytica* Wratislavia K1 was used, it was achieved a yield of 0.40 g·g⁻¹, a value similar to that verified in this work, but the productivity obtained was higher (0.66 g·L⁻¹·h⁻¹). However, unlike *Y. lipolytica* W29, the strains used in these studies were mutant strains.

Considering all the results obtained, the further experiments were performed with constant stirring and aeration rates of 900 rpm and 3 vvm, respectively.

4.2.2 Fed-batch cultures

Considering that erythritol production ends when glycerol is completely consumed, experiments using a fed-batch operation mode were performed. Therefore, different fed-batch strategies were tested: fed-batch at constant feeding rate with completed and concentrated medium, fed-batch at constant feeding rate only with crude glycerol, and a step-wise fed-batch with the addition of crude glycerol. All the experiments started with a 72 hours of batch culture and then the feeding was initiated.

The first fed-batch strategy tested was at constant feeding rate, and the reactor was fed with completed and concentrated medium. The biomass, glycerol, erythritol, mannitol and dissolved oxygen concentration profiles are presented in Figure 11.
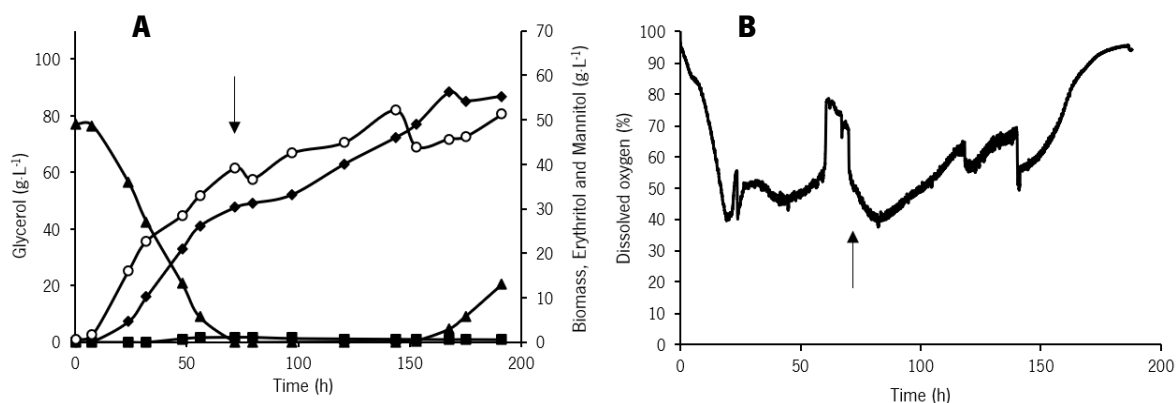
Figure 11 – (A) *Y. lipolytica* W29 cellular growth (○), glycerol consumption (▲), erythritol (♦) and mannitol (■) production and **(B)** dissolved oxygen profile in fed-batch culture performed at 900 rpm and 3 vvm with a feeding rate of 6.3 mL·h⁻¹. The dilution rate varied between 3.8×10^{-3} h⁻¹ and 2.7×10^{-3} h⁻¹. The arrows indicate the moment when the fed-batch was initiated.

The cellular growth and erythritol production profiles during the initial batch phase were similar to the batch performed with the same stirring and aeration conditions (Figure 11A). The biomass and erythritol produced during this phase were 39.2 and 30.4 g·L⁻¹, respectively, values identical to the ones obtained in the corresponding batch. Also, the dissolved oxygen concentration in the medium remained above the 40 % just like the high constant stirring and aeration rates batch (Figure 11B). And in the last hours of the batch phase, an increase of the dissolved oxygen was observed (Figure 11B), due to the low levels of glycerol detected in the medium and a consequent deceleration of growth rate.

The feeding started at 72 hours of experiment, when no glycerol was detected (Figure 11A). For nearly 81 hours of feeding, all glycerol added was consumed (Figure 11A). Although, in the last 38 hours, a glycerol accumulation (Figure 11A), a decrease in biomass concentration (Figure 11A), and also an increase in the dissolved oxygen in the medium (Figure 11B) were observed. The deceleration of cellular growth results in less oxygen requirements and, consequently, in an increase in the dissolved oxygen concentration. The decrease in biomass also results in less glycerol consumption and as a consequence, the glycerol fed to the medium was accumulated.

From the beginning of the feed addition until the end of the experiment, the production of erythritol continued, reaching a final concentration of 55.4 g·L⁻¹, demonstrating that the production can continue if the culture still has glycerol. After the addition of concentrated medium, cell growth was observed (Figure 11A) and after 120 hours of feeding, a biomass concentration of 51.3 g·L⁻¹ was achieved. As in the previous experiments, some mannitol was detected in a very low concentration (0.6 g·L⁻¹).

With the addition of concentrated medium to the bioreactor, it was possible to continue the erythritol production, and also cellular growth was observed. Thus, with a fed-batch mode it is possible

to achieve higher concentrations of erythritol and biomass at the end of the experiment than in the correspondent batch culture. However, when special attention is given to the fed-batch phase, it was possible to observe that biomass and erythritol were produced at slower rates, when compared to the batch phase of this experiment (Figure 11A), which resulted in a lower productivity (0.29 g·L⁻¹·h⁻¹). Additionally, a slight decrease in erythritol yield (0.30 g·g⁻¹) was also observed.

During most of the fed-batch phase, the glycerol concentration was equal to zero therefore, the system might have been in starvation during a period of time. Considering that, another fed-batch experiment with a higher feeding rate (11.9 mL·h⁻¹) was performed.

As in the previous fed-batch assay, the feed was initiated after 72 hours of a batch culture. The results obtained during this fed-batch culture are presented in Figure 12.
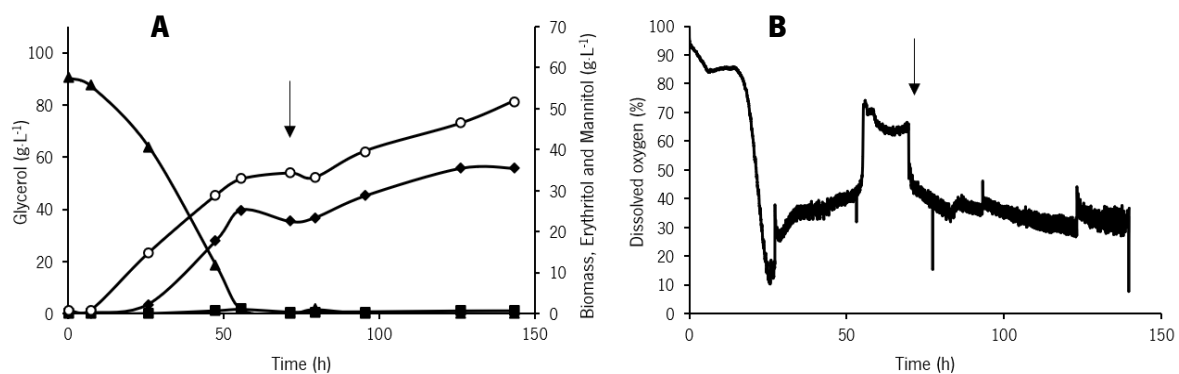
Figure 12 – **(A)** *Y. lipolytica* W29 cellular growth (○), glycerol consumption (▲), erythritol (♦) and mannitol (■) production and **(B)** dissolved oxygen profile in fed-batch culture performed at 900 rpm and 3 vvm with a feeding rate of 11.9 mL·h⁻¹. The dilution rate varied between 7.2×10⁻³ h⁻¹ and 4.8×10⁻³ h⁻¹. The arrows indicate the moment when the fed-batch was initiated.

The feeding was initiated at 72 hours of experiment, but a carbon source limitation was observed before, at 56 hours (Figure 12A). Between 56 and 72 hours of batch culture, a decrease of the cell growth rate was observed and some erythritol (2.9 g·L⁻¹) was consumed (Figure 12A). During the same period, an increase in the dissolved oxygen in the medium was detected (Figure 12B). Nevertheless, with the beginning of the fed-batch, cells started to grow again (Figure 12A) and it was observed a decrease of the dissolved oxygen to values similar to those detected during the batch period (Figure 12B). Additionally, no glycerol accumulation was observed during the fed-batch (Figure 12A).

After the addition of the concentrated feed, the production of erythritol was detected, although, at a smaller rate than in the batch phase (Figure 12A), reaching a final concentration of 35.3 g·L⁻¹. As in the previous fed-batch, cells also continued to grow at a smaller rate (Figure 12A). After 72 hours of

feeding, a maximum biomass concentration of 51.9 $g \cdot L^{-1}$ was reached. The concentration of mannitol was, as before, negligible, only 0.7 $g \cdot L^{-1}$ was produced (Figure 12A).

Like in the fed-batch with lower feeding rate, a lower productivity was observed (0.25 $g \cdot L^{-1} \cdot h^{-1}$). Taking into consideration the experience from the beginning to end, when compared to the fed-batch with lower feeding rate, similar amounts of biomass were produced, however, a smaller concentration of erythritol was achieved, and consequently lower yield (0.22 $g \cdot g^{-1}$) was observed. This low erythritol production observed in this fed-batch with higher feeding rate and completed concentrated medium can be probably related to the excess of phosphate added to the medium. In a study published by Rywińska *et al.* [14], the effect of phosphate concentration in erythritol production by Wratislavia K1 strain was assessed. In that study, phosphate concentrations higher than the optimum concentration (0.25 $g \cdot L^{-1}$) resulted in lower amounts of erythritol produced, while the biomass concentration was not affected. The same behavior had also been described in another study [52], however, for a different microorganism, *Torula* sp.. Rymowicz *et al.* [13] studied erythritol production in fed-batch cultures with constant feeding by *Y. lipolytica* A-101, a wild type strain. Higher values of erythritol yield and productivity (0.46 $g \cdot g^{-1}$ and 0.77 $g \cdot L^{-1} \cdot h^{-1}$, respectively) were obtained in that studied compared to the ones obtained in this study, nevertheless, the feeding medium used was only composed by crude glycerol. Therefore, considering that study and the possible negative effect of the addition of completed medium, in the next fed-batch strategy, the culture was only fed with crude glycerol at a constant feeding rate.

After a 72 hours batch culture, a crude glycerol solution (200 $g \cdot L^{-1}$) was added to the medium at a constant flow rate of 12.7 $mL \cdot h^{-1}$. The Figure 13 shows the profiles of biomass, erythritol, glycerol, mannitol and dissolved oxygen of the fed-batch experiment.

Figure 13 - **(A)** *Y. lipolytica* W29 cellular growth (○), glycerol consumption (▲), erythritol (♦) and mannitol (■) production and **(B)** dissolved oxygen profile in fed-batch culture performed at 900 rpm and 3 vvm with a feeding rate of 12.7 $mL \cdot h^{-1}$. Feeding medium was composed of crude glycerol only. The dilution rate varied between 7.6×10^{-3} h^{-1} and 4.8×10^{-3} h^{-1}. The arrow indicates the moment when the fed-batch was initiated.
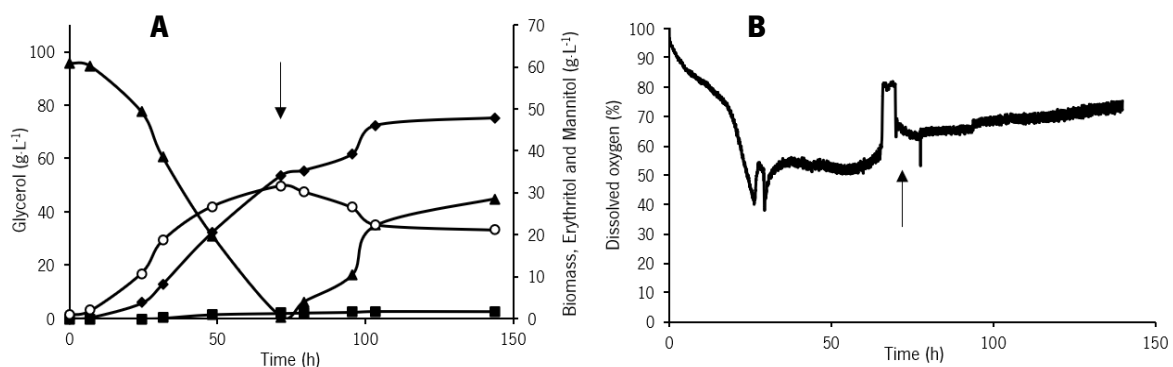
When the feeding was initiated, the glycerol concentration was 0 g·L^{-1} (Figure 13A). In the last hours of the batch culture, a deceleration of the growth rate was observed (Figure 13A) and, as consequence, an increase in the dissolved oxygen was detected (Figure 13B). However, with the beginning of the fed-batch culture, cells became active again, thus resulting in a decrease of the dissolved oxygen (Figure 13B).

During the fed-batch culture, it was possible to observe a decrease in the biomass concentration (Figure 13A), and, at the end of the fed-batch assay, a final biomass concentration of 21.1 g·L^{-1} was obtained, resulting in a decrease of 10.6 g·L^{-1}. This decrease was a consequence of dilution, since the feeding solution was composed only by crude glycerol, so no cellular growth was observed due to nitrogen and phosphate limitation. During the fed-batch phase, dissolved oxygen remained close to 70 % (Figure 13B), a value higher than the one observed in the previous experiment, where complete medium was added, which rounded 40 ± 5 %. Considering that no cellular growth was observed, the cells' oxygen requirements were lower, thus resulting in a higher oxygen concentration. As opposite to the previous fed-batch cultures, in this experiment glycerol was accumulated from the beginning of the feeding (Figure 13A). Once again, this may be due to the lack of cellular growth. In the previous fed-batch cultures, glycerol was used to produce erythritol and biomass and in this fed-batch it was only used for erythritol production. Thus, the glycerol consumption rate was lower than the feeding rate, causing the glycerol accumulation. Despite the observation of a decrease in biomass concentration and an accumulation of glycerol in the medium (Figure 13A), erythritol production was detected (Figure 13A), and at the end of the fed-batch, a concentration of 47.9 g·L^{-1} was achieved. Mannitol concentration was higher than in the other fed-batches, although the concentration was still very low (1.7 g·L^{-1}).

When compared to the fed-batch culture with similar feeding rate, at the end of the fed-batch culture, a higher erythritol concentration was produced (47.9 g·L^{-1}), while a lower biomass concentration was achieved (21.1 g·L^{-1}). The conversion yield and the productivity obtained in this fed-batch culture were 0.37 g·g^{-1} and 0.33 g·L^{-1}·h^{-1}, respectively. These values were higher than the ones achieved with previous fed-batch strategy. Thus, the fed-batch fed with only crude glycerol is a better strategy to continue the production of erythritol. Nonetheless, the productivity value was still lower comparing to those described in the study of Rymowicz *et al.* [13], where a similar feeding strategy was used. In that study, the wild-type strains *Y. lipolytica* A-101 and 1.22 reached higher productivities (0.77 and 0.51 g·L^{-1}·h^{-1}, respectively).

Since in the fed-batch culture with the addition of only crude glycerol at a feeding rate of 12.7 mL·h^{-1} a glycerol accumulation was observed, another experiment was done with one pulse addition of crude glycerol. That way the dilution of biomass would be much lower, since crude glycerol would be added directly to the culture medium. The step-wise fed-batch started after a 72 hours batch culture, when crude glycerol was added to restore the initial concentration (100 g L^{-1}). The results obtained during the step-wise fed-batch experiment are presented in Figure 14.
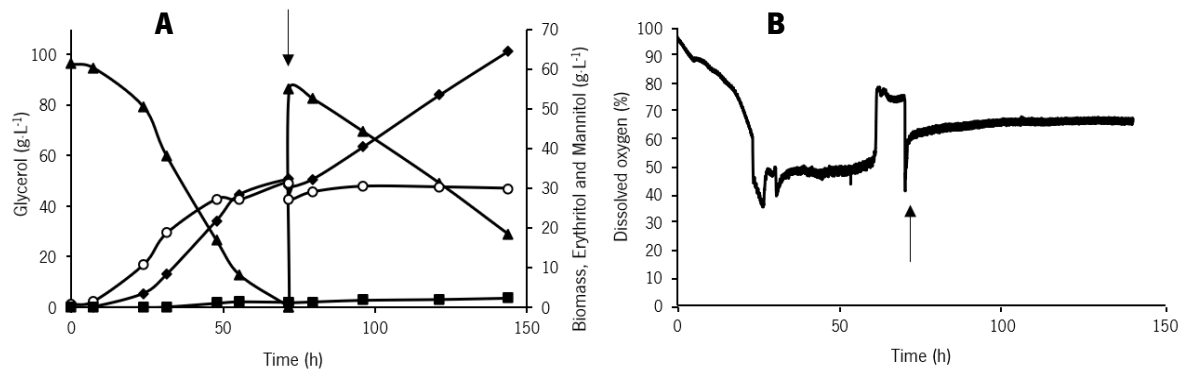
Figure 14 – **(A)** *Y. lipolytica* W29 cellular growth (○), glycerol consumption (▲), erythritol (♦) and mannitol (■) production and **(B)** dissolved oxygen profile in step-wise fed-batch culture performed at 900 rpm and 3 vvm. The arrow indicates the moment when crude glycerol was added into the bioreactor.

Such as the previous assays, after 72 hours of fermentation, the glycerol concentration was 0 g·L^{-1} (Figure 14A). In the last 16 hours of the batch phase, a deceleration on cellular growth rate (Figure 14A) was observed, followed by an increase of the dissolved oxygen in the medium (Figure 14B), due to the low glycerol concentration. At the end of the batch period, a maximum biomass of 31.35 g·L^{-1} was achieved (Table 5) and the concentrations of the polyols erythritol and mannitol produced were 32.30 g·L^{-1} and 1.23 g·L^{-1}, respectively (Figure 14A, Table 5). With the addition of crude glycerol to the medium, the dissolved oxygen concentration decreased and like in the fed-batch fed only with crude glycerol, dissolved oxygen remained at 60 - 70 % (Figure 14B).

The step-wise fed-batch was initiated after 72 hours of batch culture with the addition of crude glycerol into the culture medium, which resulted in a shift in the concentration of glycerol from zero to 86.42 g·L^{-1} (Figure 14A). The biomass concentration remained almost the same throughout the step-wise fed-batch experiment (Figure 14A). Once again, this absence on cellular growth may be explained by the fact that no nitrogen and phosphate were added to the culture. In opposite, after glycerol addition, the erythritol production continued, reaching a final concentration of 64.4 g·L^{-1} (Figure 14A, Table 5). At the end of the experiment, a total of 2.2 g·L^{-1} of mannitol was produced.

Table 5 – Erythritol and biomass concentration, erythritol yield and erythritol productivity calculated for the step-wise fed-batch experiment and for each phase (batch phase and fed-batch phase), using *Y. lipolytica* W29.

	Batch phase (until 72 hours)	Fed-batch phase (after 72 hours)	Step-wise fed-batch (144 h)
Biomass (g·L^{-1})	31.4	0	29.9
Erythritol (g·L^{-1})	32.3	32.1	64.4
Yield (g·g^{-1})	0.34	0.56	0.42
Productivity (g·L^{-1}·h^{-1})	0.45	0.45	0.45

Comparing with the fed-batch only with glycerol, a smaller decrease in the biomass concentration was observed, this is due to the smaller volume added, and therefore, the smaller dilution detected in this experiment. Regarding the erythritol produced at the end of this experiment, 64.4 g·L^{-1}, this was the highest concentration achieved in all the fed-batch experiments performed. After adding the glycerol, no growth was observed, thus more glycerol was available to be converted into erythritol, resulting in a higher yield (0.56 g·g^{-1}) during this phase (Table 5). Even though a higher yield being observed in the fed-batch phase, the values of productivity were equal is both phases, 0.45 g·L^{-1}·h^{-1} (Table 5), a phenomenon that was not observed in the previous fed-batch experiments, where a smaller productivity was observed in the fed-batch phase. Mirończuk *et al.* [8] also observed similar results. In that study, they evaluated the erythritol production by *Y. lipolytica* MK1, a mutant strain, in repeated batch cultures, where a percentage (40 % or 30 %) of culture medium was punctually replaced by the same quantity of fresh medium [8]. During the fed-batch phase, they described an augment in yield comparing to the batch phase, while the productivity remained similar in both operation modes [8]. Also, Tomaszewska *et al.* [58] studied erythritol production by *Y. lipolytica* using a fed-batch experiment with pulsed additions of crude glycerol. For the same biomass concentration produced (29.0 g·L^{-1}), higher erythritol concentration (180 g·L^{-1}), yield (0.55 g·L^{-1}) and productivity (1.2 g·L^{-1}·h^{-1}) were obtained, although the strain used, *Y. lipolytica* Wratislavia K1, is a mutant strain.

In a general way, all the fed-batch experiments demonstrated that is possible to continue erythritol production with the addition of glycerol. However, the step-wise fed-batch might be the best strategy to continue erythritol production at the same rate as the one observed in the batch phase. The Table 6 summarizes the results obtained for all fed-batch strategies performed.

Table 6 - Parameters of erythritol production by *Y. lipolytica* W29 in fed-batch assays with different strategies of operation: feeding rate of 6.3 mL·h⁻¹, feeding rate of 11.9 mL·h⁻¹, feeding rate of 12.7 mL·h⁻¹ with feeding medium composed only by crude glycerol and step-wise fed-batch with crude glycerol, and in batch culture performed at constant 900 rpm and 3 vvm.

Fed-batch assays	Yield $(g·g^{-1})$	Productivity $(g·L^{-1}·h^{-1})$
Feed rate 6.3 mL·min⁻¹	0.30	0.29
Feed rate 11.9 mL·min⁻¹	0.22	0.25
Feed rate 12.7 mL·min⁻¹ (only crude glycerol)	0.37	0.33
Step-wise with crude glycerol	0.42	0.45
Batch with constant 900 rpm and 3 vvm	0.43	0.44

In the fed-batch strategies performed, different factors were tested: the feeding rate (6 and 12 mL·h⁻¹), the composition of the feeding solution (concentrated medium or crude glycerol solution) and also adding all glycerol at once. The factor that had more influence was the composition of feeding solution. The use of complete feeding medium resulted in lower productivity and yield, while feeding composed only by crude glycerol did not favor the cellular growth, but better yield and productivity were observed (Table 6). The low cellular growth in the fed-batches fed only with crude glycerol was caused by the lack of nitrogen and phosphate sources and, with no glycerol being used for cellular growth, more glycerol could be used for erythritol production. Comparing both strategies that used only crude glycerol, the step-wise fed-batch presented better results (Table 6). In the step wise fed-batch, the dilution of cells was not significant, and the productivity was 1.4-fold higher. The step-wise fed-batch was the strategy with better results, and with this strategy it was possible to continue the erythritol production with yields and productivities similar to the batch performed at constant 900 rpm and 3 vvm (Table 6). Therefore, with this operation mode, it was possible to extend the production of erythritol without losing performance.

5. CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

In this chapter, the overall conclusions of the work are presented, as well as some suggestions for future work related to this field of research.

5.1 Final conclusions

The growing demand for healthier options to refined sugars leads to the search for alternatives, such as erythritol. This sugar alcohol is produced in response to osmotic stress by several microorganisms, including *Y. lipolytica*.

In the present work, a screening of wild-type and mutant strains of *Y. lipolytica* was performed, and their salt resistance was evaluated. From the 43 strains tested, 9 proved to be more resistant to osmotic stress. Erythritol production was assessed for the 9 strains and the strains W29, UV15-31, UV15-93 and EMS75-162 produced the highest concentrations of erythritol. The wild-type strain *Y. lipolytica* W29 was selected to perform the scale-up of the bioprocess, to optimize the production of erythritol and to evaluate the effect of dissolved oxygen concentration on erythritol production.

In the batch bioreactor assays, the effect of oxygen in the production of erythritol was evaluated, using two different operation strategies. It was demonstrated that the dissolved oxygen concentration in the medium affects erythritol productivity, i. e., higher dissolved oxygen percentage favors the erythritol production. The optimal oxygenation conditions were: high constant stirring and aeration conditions (900 rpm and 3 vvm).

To prolong erythritol production, some fed-batch strategies were performed. The composition of the feeding medium was found to affect the erythritol production performance on this operation mode, and better results were obtained when only crude glycerol was added to the production medium. The step-wise fed-batch strategy had the best results, and with this strategy it was possible to extend erythritol production without losing performance during the process.

5.2 Suggestions for future work

The present work brings new perspectives on the erythritol production by *Y. lipolytica* W29, contributing for the optimization of some relevant parameters. Nonetheless, there are still some new ideas for future studies and developments.

In the present work, to assess if erythritol production could continue with the addition of glycerol, several fed-batch strategies were tested and step-wise fed-batch proved to be the most promising. Therefore, it would be interesting to perform step-wise fed-batch studies with more punctual additions of crude glycerol, in order to assess if erythritol production could be extended and higher titers could be attained.

Several authors reported erythritol production by *Y. lipolytica* on batch, fed-batch and repeated batch cultures. However, few studies have focused on the use of this yeast in a continuous culture for erythritol biosynthesis. The possibility of combining this operation mode with immobilized cells presents several advantages. Thus, studies of erythritol production with continuous operation mode, using immobilized cells could be carried out in bioreactor.

Finally, the erythritol production by the strains *Y. lipolytica* W29 and *Y. lipolytica* UV15-93 was not statistically different although, the *Y. lipolytica* UV15-93 presented a slightly higher erythritol concentration, yield and productivity. Therefore, it would be interesting to evaluate the erythritol production by this strain in stirred tank bioreactor.

6. REFERENCES

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

1.   Drewnowski, A., et al., *Sweetness and food preference.* J Nutr, 2012. **142**(6): p. 1142s-8s.

2.   Grembecka, M., *Sugar Alcohols as Sugar Substitutes in Food Industry*, in *Sweeteners: Pharmacology, Biotechnology, and Applications*, J.-M. Merillon and K.G. Ramawat, Editors. 2017, Springer International Publishing: Cham. p. 1-27.

3.   Goran, M.I., L. Tappy, and K.A. Lê, *Dietary Sugars and Health.* 2014: Taylor & Francis.

4.   Johnson, R.J., et al., *Potential role of sugar (fructose) in the epidemic of hypertension, obesity and the metabolic syndrome, diabetes, kidney disease, and cardiovascular disease.* Am J Clin Nutr, 2007. **86**(4): p. 899-906.

5.   Grabitske, H.A. and J.L. Slavin, *Low-digestible carbohydrates in practice.* J Am Diet Assoc, 2008. **108**(10): p. 1677-81.

6.   Grembecka, M., *Sugar alcohols—their role in the modern world of sweeteners: a review.* European Food Research and Technology, 2015. **241**(1): p. 1-14.

7.   Grembecka, M., *Natural sweeteners in a human diet.* Rocz Panstw Zakl Hig, 2015. **66**(3): p. 195-202.

8.   Mirończuk, A.M., et al., *Newly isolated mutant of Yarrowia lipolytica MK1 as a proper host for efficient erythritol biosynthesis from glycerol.* Process Biochemistry, 2015. **50**(1): p. 61-68.

9.   Rzechonek, D.A., et al., *Recent advances in biological production of erythritol.* Critical Reviews in Biotechnology, 2018. **38**(4): p. 620-633.

10. Lin, S.-J., et al., *Screening and production of erythritol by newly isolated osmophilic yeast-like fungi.* Process Biochemistry, 2001. **36**(12): p. 1249-1258.

11. Moon, H.J., et al., *Biotechnological production of erythritol and its applications.* Appl Microbiol Biotechnol, 2010. **86**(4): p. 1017-25.

12. Guo, J., et al., *Improving Erythritol Production of Aureobasidium pullulans from Xylose by Mutagenesis and Medium Optimization.* Applied Biochemistry and Biotechnology, 2016. **180**(4): p. 717-727.

13. Rymowicz, W., A. Rywinska, and M. Marcinkiewicz, *High-yield production of erythritol from raw glycerol in fed-batch cultures of Yarrowia lipolytica.* Biotechnol Lett, 2009. **31**(3): p. 377-80.

14. Rywinska, A., et al., *Optimization of medium composition for erythritol production from glycerol by Yarrowia lipolytica using response surface methodology.* Prep Biochem Biotechnol, 2015. **45**(6): p. 515-29.

15. Tomaszewska, L., A. Rywinska, and W. Gladkowski, *Production of erythritol and mannitol by Yarrowia lipolytica yeast in media containing glycerol.* J Ind Microbiol Biotechnol, 2012. **39**(9): p. 1333-43.

16. Kobayashi, Y., et al., *Erythritol production by Moniliella megachiliensis using nonrefined glycerol waste as carbon source.* Lett Appl Microbiol, 2015. **60**(5): p. 475-80.

17. Coelho, M.A., P. Amaral, and I. Belo, *Yarrowia lipolytica: an industrial workhorse*, in *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology.* 2010, Formatex Research Center. p. 930-940.

18. A G Gonçalves, F., G. Colen, and J. Takahashi, *Yarrowia lipolytica and Its Multiple Applications in the Biotechnological Industry.* Vol. 2014. 2014, The Scientific World Journal. 476207.

19. Margesin, R., et al., *Hydrocarbon degradation and enzyme activities of cold-adapted bacteria and yeasts.* Extremophiles, 2003. **7**(6): p. 451-8.

20. Papanikolaou, S. and G. Aggelis, *Selective uptake of fatty acids by the yeast Yarrowia lipolytica.* European Journal of Lipid Science and Technology, 2003. **105**(11): p. 651-655.

21. Chernyavskaya, O.G., et al., *Synthesis of alpha-ketoglutaric acid by Yarrowia lipolytica yeast grown on ethanol.* Appl Microbiol Biotechnol, 2000. **53**(2): p. 152-8.

22. Mironczuk, A.M., et al., *Enhanced production of erythritol by Yarrowia lipolytica on glycerol in repeated batch cultures.* J Ind Microbiol Biotechnol, 2014. **41**(1): p. 57-64.

23. Antonucci, S., et al., *Selectivity in citric acid production by Yarrowia lipolytica.* Enzyme and Microbial Technology, 2001. **28**(2): p. 189-195.

24. Lazar, Z., et al., *Optimized invertase expression and secretion cassette for improving Yarrowia lipolytica growth on sucrose for industrial applications.* Journal of Industrial Microbiology & Biotechnology, 2013. **40**(11): p. 1273-1283.

25. Rodrigues, G. and C. Pais, *The Influence of Acetic and Other Weak Carboxylic Acids on Growth and Cellular Death of the Yeast Yarrowia lipolytica.* Food Technology and Biotechnology, 2000. **12**(1): p. 27-32.

26. Lanciotti, R., et al., *Use of Yarrowia lipolytica strains for the treatment of olive mill wastewater.* Bioresource Technology, 2005. **96**(3): p. 317-322.

27. Rufino, R.D., L.A. Sarubbo, and G.M. Campos-Takaki, *Enhancement of stability of biosurfactant produced by Candida lipolytica using industrial residue as substrate.* World Journal of Microbiology and Biotechnology, 2007. **23**(5): p. 729-734.

28.   Papanikolaou, S., et al., *Single cell oil production by Yarrowia lipolytica growing on an industrial derivative of animal fat in batch cultures.* Appl Microbiol Biotechnol, 2002. **58**(3): p. 308-12.

29.   Fickers, P., J. Destain, and P. Thonart, *Improvement of Yarrowia lipolytica lipase production by fed-batch fermentation.* J Basic Microbiol, 2009. **49**(2): p. 212-5.

30.   Braga, A. and I. Belo, *Production of γ-decalactone by Yarrowia lipolytica: insights into experimental conditions and operating mode optimization.* Journal of Chemical Technology & Biotechnology, 2015. **90**(3): p. 559-565.

31.   Ferreira, P., et al., *Oxygen mass transfer impact on citric acid production by Yarrowia lipolytica from crude glycerol.* Biochemical Engineering Journal, 2016. **110**: p. 35-42.

32.   Kamzolova, S.V., et al., *Biosynthesis of isocitric acid by the yeast yarrowia lipolytica and its regulation.* Prikl Biokhim Mikrobiol, 2015. **51**(2): p. 251-7.

33.   Morgunov, I.G., et al., *Pyruvic acid production by a thiamine auxotroph of Yarrowia lipolytica.* Process Biochemistry, 2004. **39**(11): p. 1469-1474.

34.   Tomaszewska, L., et al., *A comparative study on glycerol metabolism to erythritol and citric acid in Yarrowia lipolytica yeast cells.* FEMS Yeast Res, 2014. **14**(6): p. 966-76.

35.   Andreishcheva, E.N., et al., *Adaptation to salt stress in a salt-tolerant strain of the yeast Yarrowia lipolytica.* Biochemistry (Mosc), 1999. **64**(9): p. 1061-7.

36.   Rakicka, M., et al., *Efficient utilization of inulin and glycerol as fermentation substrates in erythritol and citric acid production using Yarrowia lipolytica expressing inulinase.* Chemical Papers, 2016. **70**(11): p. 1452-1459.

37.   Xiaoyan, L., et al., *A cost-effective process for the coproduction of erythritol and lipase with Yarrowia lipolytica M53 from waste cooking oil.* Food and Bioproducts Processing, 2017. **103**: p. 86-94.

38.   Rymowicz, W., A. Rywińska, and W. Gładkowski, *Simultaneous production of citric acid and erythritol from crude glycerol by Yarrowia lipolytica Wratislavia K1.* Chemical Papers, 2008. **62**(3): p. 239.

39.   Ghezelbash, G.R., I. Nahvi, and R. Emamzadeh, *Improvement of erythrose reductase activity, deletion of by-products and statistical media optimization for enhanced erythritol production from Yarrowia lipolytica mutant 49.* Curr Microbiol, 2014. **69**(2): p. 149-57.

40.   Tomaszewska-Hetman, L. and A. Rywiʹnska, *Erythritol biosynthesis from glycerol by Yarrowia lipolytica yeast: effect of osmotic pressure.* Chemical Papers, 2016. **70**(3): p. 272-283.

41.   Carly, F., et al., *Enhancing erythritol productivity in Yarrowia lipolytica using metabolic engineering.* Metab Eng, 2017. **42**: p. 19-24.

42.   Rywińska, A., L. Tomaszewska-Hetman, and W. Rymowicz, *Erythritol biosynthesis by Yarrowia lipolytica yeast under various culture conditions.* African journal of microbiology research, 2013. **7**: p. 3511-3516.

43.   Yang, L.B., et al., *A novel osmotic pressure control fed-batch fermentation strategy for improvement of erythritol production by Yarrowia lipolytica from glycerol.* Bioresour Technol, 2014. **151**: p. 120-7.

44.   Rakicka, M., et al., *An Effective Method of Continuous Production of Erythritol from Glycerol by Yarrowia lipolytica MK1.* Food Technol Biotechnol, 2017. **55**(1): p. 125-130.

45.   Rakicka, M., et al., *Technology of efficient continuous erythritol production from glycerol.* Journal of Cleaner Production, 2016. **139**: p. 905-913.

46.   Nelson, D.L.L., Albert L., Cox, Michael M., *Lehninger Principles of Biochemistry.* 6th ed. 2012: Macmillan Learning.

47.   Murray, R.K., et al., *Harper's Illustrated Biochemistry.* 26th ed. 2003: Mcgraw-hill.

48.   Fakas, S., et al., *Pathways to aerobic glycerol catabolism and their regulation*, in *Microbial Conversions of Raw Glycerol*. 2009, Nova Science Publishers. p. 9-18.

49.   Yang, L.B., et al., *Optimization of a low-cost hyperosmotic medium and establishing the fermentation kinetics of erythritol production by Yarrowia lipolytica from crude glycerol.* Prep Biochem Biotechnol, 2016. **46**(4): p. 376-83.

50.   Liu, X., et al., *Effects of osmotic pressure and pH on citric acid and erythritol production from waste cooking oil by Yarrowia lipolytica.* Engineering in Life Sciences, 2018. **18**(6): p. 344-352.

51.   Mironczuk, A.M., et al., *A two-stage fermentation process of erythritol production by yeast Y. lipolytica from molasses and glycerol.* Bioresour Technol, 2015. **198**: p. 445-55.

52.   Oh, D.K., et al., *Increased erythritol production in fed-batch cultures of Torula sp. by controlling glucose concentration.* J Ind Microbiol Biotechnol, 2001. **26**(4): p. 248-52.

53.   Braga, A., et al., *Aroma production by Yarrowia lipolytica in airlift and stirred tank bioreactors: Differences in yeast metabolism and morphology.* Biochemical Engineering Journal, 2015. **93**: p. 55-62.

54.   Lopes, M., et al., *Yarrowia lipolytica lipase production enhanced by increased air pressure.* Lett Appl Microbiol, 2008. **46**(2): p. 255-60.

55. Ferreira, P., et al., *Oxygen transfer rate and pH as major operating parameters of citric acid production from glycerol by Yarrowia lipolytica W29 and CBS 2073.* Chemical Papers, 2016. **70**(7): p. 869-876.

56. Macauley-Patrick, S.a.F., B. , *Modes of Fermenter Operation*, in *Practical Fermentation Technology*, L.H. Brian McNeil, Editor. 2008, John Wiley & Sons. p. 69-95.

57. Carly, F. and P. Fickers, *Erythritol production by yeasts: a snapshot of current knowledge.* Yeast, 2018. **35**(7): p. 455-463.

58. Tomaszewska, L., A. Rywińska, and W. Rymowicz, *High selectivity of erythritol production from glycerol by Yarrowia lipolytica.* Biomass and Bioenergy, 2014. **64**: p. 309-320.

59. Ferreira, P., *Biotechnological approaches of crude glycerol use: optimization of citric acid production by Yarrowia lipolytica*, in *Center of Biological Engineering*. 2015, University of Minho.

60. Frédéric, C. and F. Patrick, *Erythritol production by yeasts: a snapshot of current knowledge.* Yeast. **0**(0).

61. Silva, L.V., et al., *A novel osmotic pressure strategy to improve erythritol production by Yarrowia lipolytica from glycerol.* Bioprocess Biosyst Eng, 2018.

62. Rywińska, A. and W. Rymowicz, *Citric acid production from raw glycerol by Yarrowia lipolytica Wratislavia 1.31*, in *Microbial Conversions of Raw Glycerol*. 2009, Nova Science Publishers. p. 19-30.