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ABSTRACT

Yarrowia lipolytica whole cells immobilization: impact on citric acid production

Cell immobilization has been used as a strategy to solve the limitations of using suspended free cells in the medium. Whole cells immobilization has been used to improve yields and volumetric productivity of bioprocesses.

The central aim of this work was to select and optimize immobilization systems for *Y. lipolytica* W29 that could be used in the citric acid production from glycerol. Whole-cells immobilization by entrapment into calcium alginate gel matrix and by adsorption on plastic material was studied, with particular focus on the entrapment method.

Firstly, the effect of calcium alginate gel matrix composition was evaluated and the best conditions for cells retention were 3.3 % (w/v) of sodium alginate and 0.5 M of calcium chloride. Also, different sizes of gel spheres were tested (2 mm, 3 mm and 4 mm) and no significant differences on citric acid production were found, in the range of diameter tested. The influence of dry cell mass per mass of wet support in citric acid production was also analyzed and it was observed that, in the range of 5 to 16 mg of dry cell mass per gram of wet gel matrix, citric acid production kinetics was unchanged. However, in the experiments with free cells, citric acid production decreased with cell concentration increase (from 1.5 $g{\cdot}L^{-1}$ to 3 $g{\cdot}L^{-1}$ and 4.5 $g{\cdot}L^{-1}$), due to citric acid consumption. The results demonstrated an improvement in citric acid production (5 $g{\cdot}L^{-1}$) with immobilized cells, highlighting the advantages of immobilized cells instead of free cells suspended.

After selecting the best conditions for cell immobilization, the influence of initial glycerol concentration on citric acid batch production was analyzed and for the range of values studied (40 $g{\cdot}L^{-1}$ to 80 $g{\cdot}L^{-1}$) no cellular inhibition by the substrate was observed. Furthermore, the use of crude glycerol as an alternative substrate was also analyzed and a similar citric acid production was obtained with this low-cost substrate as with pure glycerol. pH adjustment during the production of citric acid, revealed to be very important to increase the final acid concentration.

The immobilization by adsorption of *Y. lipolytica* W29 in DupUm® was studied, however high level of cells desorption was observed during citric acid production that make difficult to discrimanete the experiments with free and immobilized cells. In fact, similar profiles of citric acid production were obtained in both systems.

RESUMO

Imobilização de células de *Yarrowia lipolytica*: impacto na produção de ácido cítrico

A imobilização de células tem sido utilizada para promover o aumento dos rendimentos e da produtividade de bioprocessos.

O objetivo central deste trabalho reside na seleção e otimização de um sistema de imobilização de *Y. lipolytica* W29 para ser utilizado na produção de ácido cítrico a partir de glicerol. Neste contexto, foi estudada a imobilização de células por inclusão numa matriz de alginato de cálcio e por adsorção num material de plástico, com particular ênfase na imobilização por inclusão.

Primeiramente avaliou-se o efeito da concentração da matriz de alginato de cálcio foi e as melhores condições de retenção celular encontradas foram 3,3 % (m/V) de alginato de sódio e 0,5 M de cloreto de cálcio. Adicionalmente testaram-se diferentes tamanhos de esferas de alginato de cálcio (2 mm, 3 mm e 4 mm), não se tendo observado diferenças significativas na produção de ácido cítrico, na gama de diâmetros testados. A influência da razão de massa seca de células por massa húmida de suporte na produção de ácido cítrico foi também analisada, tendo-se observado que na gama de 5 a 16 mg de massa seca de células por grama húmida de gel, a cinética de produção de ácido cítrico manteve-se praticamente inalterada. No entanto, nas experiências em que foram utilizadas células livres, a produção de ácido cítrico diminuiu com o aumento da concentração celular (de 1,5 $g \cdot L^{-1}$ para 3 $g \cdot L^{-1}$ e para 4,5 $g \cdot L^{-1}$), como resultado do consumo do ácido cítrico. Os resultados demonstraram um aumento na produção de ácido cítrico (5 $g \cdot L^{-1}$) com células imobilizadas, salientando as vantagens na utilização de um sistema com células imobilizadas em vez de células livres.

Após a seleção das melhores condições de imobilização, foi estudada a influência da concentração de glicerol (de 40 $g \cdot L^{-1}$ a 80 $g \cdot L^{-1}$) e não foi observada inibição celular pelo substrato. Além disso, comprovou-se a possibilidade de utilização de glicerol bruto como uma alternativa ao glicerol puro, tendo-se observado uma produção de ácido cítrico similar nas duas situações. O efeito do ajuste de pH no meio de produção foi também avaliado, revelando-se importante no aumento da produção de ácido cítrico.

Finalmente foi estudada a imobilização por adsorção de *Y. lipolytica* W29 a DupUM®, tendo-se observado uma elevada dessorção de células durante a produção de ácido cítrico, o que dificulta a discriminação entre os ensaios com células livres e células imobilizadas. De facto, os perfis de produção de ácido cítrico foram semelhantes nos dois sistemas.

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LIST OF ABBREVIATIONS

[ ]	Concentration of compound
3-P-GDH	3-P-glycerol dehydrogenase
a	Radius of the spheres
A	Peaks area detected by HPLC
Ac	cis-Aconitate
b	Ordinate at the origin
Biom	Biomass (dry mass) concentration grown
C	Solute concentration in the liquid phase
C0	Initial solute concentration in the liquid phase
CA	Citric acid concentration in the culture liquid at the end of cultivation
CDW	Biomass (dry mass) concentration
CS	Citrate synthase
De	Effective diffusion coefficient
FDA	Food and Drug Administration
G residue	α-L-guluronic
GK	Glycerol kinase
Glu	Glucose concentration
Glyc	Total amount of glycerol consumed
GRAS	Generally Recognized As Safe
HPLC	High-performance liquid chromatography
ICDH	Iso-citrate dehydrogenase
ICL	Iso-citrate lyase
IUPAC	International Union of Pure and Applied Chemistry
m	Slope of the calibration curve
M residue	1,4-linked β-D-mannuronic
MD_m	Malate dehydrogenase (mitochondrial)
NTG	N-methyl-NT-nitro-N-nitrosoguanidine
OD_{540}	Optical density at 540 nm
OD_{600}	Optical density at 600 nm
P_{CA}	Maximum volumetric productivity of citric acid

PD	Pyruvate dehydrogenase
PUF	Oil-absorbent polyurethane
PVA	Poly-vinyl alcohol
Q_{Glyc}	Glycerol consumption rate
qn	Nonzero roots of $\tan(qn) = 3qn * (3 + \alpha qn^2)^{-1}$
Rpm	Revolutions per minute
S_b	Standard error associated with the ordinate at the origin
SCO	Single cell oil
S_m	Standard error associated with the slope
t	Time of fermentation duration
T1	Systems transporting pyruvic acid from cytosol to mitochondrion and inversely
T2	Systems transporting pyruvic acid from cytosol to mitochondrion and inversely
T3	Systems transporting pyruvic acid from cytosol to mitochondrion and inversely
T4	System transporting citric and malic acid from cytosol to mitochondrion and inversely
TCA	Tricarboxylic acid cycle
vvm	Volume per volume per minute
$Y_{Biom/Glyc}$	Biomass yield per glycerol
$Y_{CA/Glyc}$	Citric acid yield by glycerol

Greek letters

α	Volume ratio between volume of the free liquid and volume of spheres

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1. INTRODUCTION

This chapter introduces the background information about the theme of this work and its main objectives.

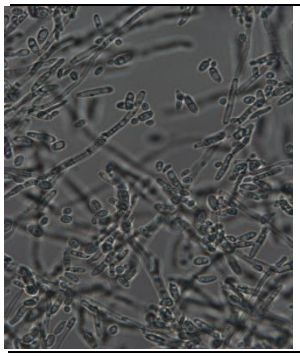
Cell immobilization is a strategy used in the last decades as a solution to the limitations of the use of suspended free cells in the medium. This technology promotes the protection of cells against stress environmental conditions, prevent cells washout on continuous processes, increases reusability of the biocatalyst, improve yields and volumetric productivity of bioprocesses (Léonard *et al.*, 2011; Park and Chang, 2000; Trelles *et al.*, 2010).

Generally, cell immobilization methods are based on surface attachment (by physical adsorption or covalent biding), entrapment within porous matrix, containment behind a barrier (by microencapsulation or two-phase emulsion) and self-aggregation (by cross-linking or flocculation) (Pilkington *et al.*, 1998).

Since 1991, *Y. lipolytica* whole cells immobilization started to be investigated when Kautola experimented several supports for citric acid biosynthesis using glucose as substrate (Kautola *et al.*, 1991). Further, immobilization was applied in order to optimize processes productivity by this yeast, including citric acid biosynthesis using glucose and fructose as substrate (Mansfeld *et al.*, 1995), removal of oil film on surface waters (Oh *et al.*, 2000), crude oil degradation (Zinjarde and Pant, 2000), oil wastewater degradation (Wu *et al.*, 2009) and biosynthesis of γ-decalactone (peach-aroma) (Braga and Belo, 2013).

Yarrowia lipolytica is an obligate aerobic, dimorphic and ''non-conventional'' yeast, known by its capabilities to use hydrophobic and non-hydrophobic substrates, to secrete aromas, organic acids and enzymes and to accumulate large amounts of lipids (Ageitos *et al.*, 2011; Nicaud, 2012; Zhao *et al.*, 2010). These features increased the biotechnological and industrial interest of *Y. lipolytica* (Barth and Gaillardin, 1997; Fickers *et al.*, 2005; Hamme *et al.*, 2003). *Yarrowia lipolytica* W29 (ATCC 20460) has been proven to be a very robust strain for many of the applications previously described, thus its application for citric acid production from crude glycerol (from biodiesel) has a great interest of development and optimization.

The central aim of this work is to select immobilization methods and supports for *Y. lipolytica* W29 whole cells immobilization in order to improve citric acid production by this strain. At first, calcium alginate gel matrix and DupUM® were tested for cell immobilization by entrapment and adsorption, respectively, in order to select the best carrier and immobilization conditions. After that, the influence of gel pieces size, cell concentration and environmental conditions were studied. Biosynthesis of citric acid with immobilized cells was monitored and compared with freely suspended cells.



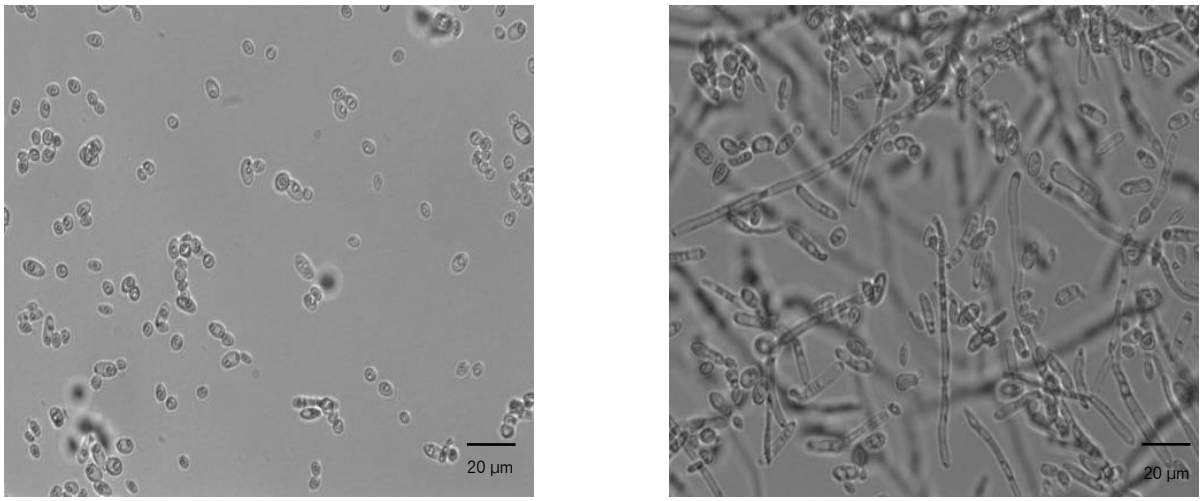
2. LITERATURE REVIEW

This chapter covers the characterization of *Yarrowia lipolytica*, highlighting the metabolism of this yeast. It is given special attention to the biotechnological production of citric acid, with reference to its properties and consequent importance to the industry, producer microorganisms, substrates used, required conditions in this bioprocess to obtain the best productivity and yields (effect of mineral components of the medium, pH, temperature and aeration), fermentation type, strains improvement strategies and citric acid recovery. Further, the potentialities of cells immobilization technology are described with particular focus on the application to *Y. lipolytica* and citric acid production.

2.1. *YARROWIA LIPOLYTICA*

Yarrowia lipolytica is an eukaryotic microorganism, belonging to the Fungi kingdom. It is taxonomically assigned to the subclass *Hemiascomycetes*, the order *Saccharomycetales* and to the family *Dipodascacea* (Kurtzman and Fell, 2000). In an earlier stage, *Y. lipolytica* was named as *Candida lipolytica* and was included in the class *Deuteromycetous*. Further observation of ascospores enabled the rename to *Endomycopsis, Saccharomycopsis* and finally to *Yarrowia lipolytica* (Barth and Gaillardin, 1996, 1997; Walt and Arx, 1980; Yarrow, 1972).

Yarrowia lipolytica is an obligate aerobic microorganism and one of the most extensively studied ''non-conventional'' yeast species. This classification of "non-conventional" was adopted to distinguish *Y. lipolytica* and other species from other well studied yeasts, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, with respect to its phylogenetic evolution, physiology, genetics and molecular biology (Barth and Gaillardin, 1997). *Y. lipolytica* is a dimorphic yeast (Figure 1), since it is capable to grow under two distinct morphological forms, single oval cells and pseudo-hyphae. The form that the yeast presents is the result of a defense mechanism from adverse growth conditions (aeration, carbon and nitrogen conditions, pH, among others) or conditioned by genetic characteristics of strain (Barth and Gaillardin, 1997; Kawasse *et al.*, 2003). The most important regulator on dimorphic transition is pH, occurring maximal hyphae formation at pH near neutrality (Ruiz-Herrera and Sentandreu, 2002). With respect to the other conditions, glucose and nitrogen salts favor hyphae form (Pérez-Campo and Dominguez, 2001; Ruiz-Herrera and Sentandreu, 2002), as well semi-anoxic conditions provided in liquid or solid media (Ruiz-Herrera and Sentandreu, 2002).



(A) (B)

Figure 1 - Morphological forms exhibited by *Y. lipolytica* as dimorphic yeast. (A) Single oval cells; (B) Pseudo-hyphae.

Y. lipolytica is able to grow in stressful environments such as hypersaline environments, and therefore, it has been used as a model in salt tolerant studies (Thevenieau *et al.*, 2009). Additionally, *Y. lipolytica* can be found in sewage, oil polluted media, marine and food environments (Thevenieau *et al.*, 2009), suggesting the versatility of its metabolism, essentially related to the development of very efficient mechanisms for breaking down and use of hydrophobic substrates (n-alkanes, oils, fats, fatty acids (Barth and Gaillardin, 1997; Fickers *et al.*, 2005)), as well as non-hydrophobic subtracts (sugars, alcohols and organic acids). In fact, genome sequencing of *Y. lipolytica* (Casaregola *et al.*, 2000; Sherman *et al.*, 2004) brought knowledge, among others, about the genes encoding enzymes involved in n-alkanes, fats and fatty acid metabolism (Thevenieau *et al.*, 2009). This feature made *Y. lipolytica* a microorganism with biotechnological interest for bioremediation processes, to solve many ecological and environmental damages (Barth and Gaillardin, 1997; Fickers *et al.*, 2005; Hamme *et al.*, 2003).

Yarrowia lipolytica is a nonpathogenic yeast and classified as "Generally Recognized As Safe" (GRAS) by the Food and Drug Administration (FDA, USA) (Beckerich *et al.*, 1998; Tsugawa *et al.*, 1969), with capability to secrete a wide range of compounds like organic acids (Mauersberger *et al.*, 2003), lactones (Aguedo *et al.*, 2005) and enzymes (mainly lipases (Fickers *et al.*, 2003), but also proteases (Kulkarni and Gadre, 1999), RNases, phosphatases, esterases (Barth and Gaillardin, 1997), among others. The organic acids produced by *Y. lipolytica* are citric acid, isocitric acid, α-ketoglutaric acid, fumaric acid, malic acid and piruvic acid, which are intermediates of tricarboxylic acids (TCA) (Kamzolova *et al.*, 2003; Otto *et al.*, 2013; Rymowicz *et al.*, 2008). Additionally, *Y. lipolytica* can accumulate large amounts of lipids, about 40 % of its cellular dry weight, with industrial interest (Ageitos *et al.*, 2011; Nicaud, 2012; Zhao *et al.*, 2010).

2.2. CITRIC ACID PRODUCTION

Citric acid properties as acidifier flavoring agent, preservative, pH adjuster and antioxidant, made this metabolite attractive for metal cleaning, food, beverage, chemical, pharmaceutical, agriculture, detergents, cosmetic and toiletries industries (Kamzolova *et al.*, 2005; Rymowicz *et al.*, 2008; Soccol *et al.*, 2006).

Global production of citric acid exceed 1.7 million tons per year and the volume of citric production is continually rising at an high annual rate of 5 % (Anastassiadis *et al.*, 2008; Vendruscolo *et al.*, 2008), being 99 % of its production through biotechnological methods, the simple, stable, easiest and economically most successful strategy at industrial scale (Kuforiji *et al.*, 2010; Rywińska *et al.*, 2012;

Soccol *et al.*, 2006). A wide range of microorganisms has the ability to secret citric acid, including bacteria, fungi and yeast (Crolla and Kennedy, 2001; Kuforiji *et al.*, 2010; Papagianni, 2007). Among these, *Aspergillus niger* is known as the greater producer of citric acid, being the traditional fungus used in this process at industrial scale, due to its ease of handling, capability to use a variety of raw materials and high yield (Vandenberghe *et al.*, 1999). However, in the last years, *Y. lipolytica* has been gained importance in this bioprocess due to the several substrates that can use and the high product yields (Förster *et al.*, 2007). Beyond the microorganisms and strains used, citric acid bioprocess yields are strongly affected by subtracts, mineral composition of medium, aeration, pH, temperature, fermentation type, strains improvement strategies and recovery techniques used (Antonucci *et al.*, 2001; Lotfy *et al.*, 2007b; Papagianni *et al.*, 1999).

2.2.1. Industrial process

About 80 % of world citric acid production is performed by *A. niger* under submerged fermentation (Kumar *et al.*, 2003; Leangon *et al.*, 2000; Vandenberghe *et al.*, 1999). The substrates contain glucose or sucrose, being reported the use of date syrup (Roukas and Kotzekidou, 1997), black strap molasses (Haq *et al.*, 2001), corn cobs (Hang and Woodams, 2001), potato starch (Lesniak *et al.*, 2002), cane molasses (Haq *et al.*, 2004), orange peel (Rivas *et al.*, 2008) and sphagnum peat moss (supplemented with glucose) (Suzelle *et al.*, 2009). Although the batch system is the most used on submerged fermentation, fed batch and continuous system can be also applied (Dhillon *et al.*, 2011; Soccol *et al.*, 2006). Citric acid fermentation can be completed in 5 to 12 days, according to the process conditions.

The advantages of submerged fermentation include the higher productivity and yield, lower labor cost, lower contamination risk and labour consumption (Soccol *et al.*, 2006; Vandenberghe *et al.*, 1999). Nevertheless, it is important to attend the sophisticated installations and rigorous control mechanisms required (Soccol *et al.*, 2006).

2.2.2. Substrates

The quality and quantity of substrate directly influence it conversion into citric acid by microorganisms, as well the nature of substrate.

Industrial citric acid biosynthesis can be carried out under consumption of sugar industry by-products, hydrocarbons, agro-industrial waste residues and starchy materials (Ambati and Ayyanna, 2001; Hang and Woodams, 1998; Jianlong *et al.*, 2000; Kumar *et al.*, 2003; Leangon *et al.*, 2000;

Mourya and Jauhri, 2000; Vandenberghe *et al.*, 2000). For *A. niger*, the most suitable substrate are molasses (Ikram-ul *et al.*, 2004; Lotfy *et al.*, 2007a), which contain sugars in form of sucrose, glucose and fructose (40 % to 55 %) (Grewal and Kalra, 1995). Despite the low cost of this substrate, the yield of citric acid produced in media containing cane molasses can be compromised due to the high content of trace metals (calcium, magnesium, manganese, iron, zinc) and other inhibitory substances (Chaudhary and Pirt, 1996; Chaudhary *et al.*, 1978; Dasgupta *et al.*, 1981). Therefore, the use of molasses by *A. niger* requires a pretreatment for removal of these compounds (Grewal and Kalra, 1995).

The use of yeasts, such as *Y. lipolytica*, brings advantages with respect to *A. niger*, due to it insensibility to trace metals. Therefore, this yeast can use crude substrates without any treatment. Moreover, *Y. lipolytica* was highlight by the efficient grow in n-alkanes, vegetable oils, ethanol, molasses, hydrolyzed starch, yielding high citric acid yields (Crolla and Kennedy, 2004a; Finogenova *et al.*, 2005; Förster *et al.*, 2007; Papanikolaou *et al.*, 2006). However, in the last years the use of glycerol as substrate has gained importance.

2.2.2.1. Glycerol

Glycerol, or propan-1,2,3-triol (IUPAC name), is a trihydric alcohol with chemical formula $OH\text{-}CH_2\text{-}CH(OH)\text{-}CH_2\text{-}OH$ (Perry and Green, 1997). It is a colorless, odorless and viscous liquid with sweet-taste, nontoxic and hygroscopic properties (Perry and Green, 1997). Glycerol is mainly obtained from biodiesel production, but also from alcoholic beverage, textile, food and soap manufacture, industrial processes which present glycerol as by-product (Papanikolaou and Aggelis, 2003). The concerns about carbon dioxide emission and global warming as a result of fossil fuels use, the decrease and future exhaustion of oil supplies and increase of crude oil price, leaded to increased biodiesel exploration with the concomitant glycerol accumulation (Kemp, 2006; Soriano *et al.*, 2006). Biodiesel is a renewable, biodegradable and nontoxic fuel, having its worldwide production increased exponentially in last years (Kulkarni and Dalai, 2006). Biodiesel manufacturing process involves (i) hydrolysis of triglycerides to form glycerol and fatty acids, and (ii) esterification of fatty acids with formation of methylated fatty acids which are just used as biodiesel. About 10 kg of the biodiesel produced origin 1 kg of glycerol. In fact, Posada and Cardona (2010) refers as a typical composition of crude glycerol stream obtained from the biodiesel production process 60.05 wt % glycerol, 32.59 wt % methanol, 2.8 wt % ash, 2.62 wt % $NaOCH_3$ and 1.94 wt % fats. This composition is dependent of the

catalyst used for biodiesel production, transesterification efficiency, recovery efficiency, feedstock impurities and the methanol and catalysts were recovered (Hansen *et al.*, 2009). Further, according to the purification method (Leung *et al.*, 2010; Singhabhandhu and Tezuka, 2010; Van-Gerpen, 2005), crude glycerol composition can include from 40 % to 99 % of glycerol, residual oil, free fatty acids (about 1 %), sodium and potassium salts and water (Rymowicz *et al.*, 2010). The accumulated glycerol (874 m^3 of biodiesel produced per day in Portugal in 2011 (EIA, 2014)) could be a future environmental problem, despite its application in food, pharmaceutical and cosmetic industries, among others (Pachauri and He, 2006). Nowadays, it is known the capabilities of several microorganisms to use crude glycerol as substrate, being involved in biotechnological processes to convert this low cost by-product into a value-added product (Table 1).

Table 1 – Products obtained from different microorganism using crude glycerol as substrate

Products	Microorganisms	References
Citric acid	*Clostridium butyricum* *Yarrowia lipolytica*	(Finogenova *et al.*, 1986; Imandi *et al.*, 2007; Levinson *et al.*, 2007; Papanikolaou and Aggelis, 2003; Papanikolaou *et al.*, 2008a; Rymowicz *et al.*, 2006; Rywinbska and Rymowicz, 2010; Rywinbska *et al.*, 2009)
Erythritol	*Yarrowia lipolytica*	(Rywinbska *et al.*, 2009)
Food-grade pigments	*Blakeslea trispora*	(Mantzouridou *et al.*, 2008)
Lipids	*Cryptococcus curvatus* *Yarrowia lipolytica*	(Chi *et al.*, 2007; Johnson and Taconi, 2007; Meesters *et al.*, 1996; Papanikolaou and Aggelis, 2002)
L-Lysine	*Corynebacterium glutamicum*	(Rittmann *et al.*, 2008)
Mannitol	*Yarrowia lipolytica*	(André *et al.*, 2009)
Microbial biomass	*Cryptococcus curvatus* *Yarrowia lipolytica*	(Chi *et al.*, 2007; Johnson and Taconi, 2007; Meesters *et al.*, 1996; Papanikolaou and Aggelis, 2002)
1,3-Propanediol	*Clostridium butyricum*	(Papanikolaou and Aggelis, 2003; Papanikolaou *et al.*, 2008a)

The purification degree of the crude glycerol can influence the biochemical pathways of microorganisms, leading to different yields of metabolites produced when crude glycerol, without purification, is used. This can be explained by the crude glycerol composition (Samul *et al.*, 2014).

The use of glycerol by *Y. lipolytica* as substrate has been explored in the last years for the biosynthesis of organic acids (Papanikolaou and Aggelis, 2009; Rywinbska *et al.*, 2009) and single cell oil (SCO) (Papanikolaou and Aggelis, 2002).

2.2.3 CITRIC ACID PRODUCTION BY *Y. LIPOLYTICA*

Citric acid production by *Y. lipolytica* was reported by several authors, regarding to the fermentation type and substrate. In the Table 2 some of these studies are summarized, with the information of the maximum citric acid concentration achieved.

Table 2 - Citric acid production by several strains of *Y. lipolytica* at different fermentation conditions (adapted from (Yalcin *et al.*, 2010))

Strain	Fermentation type	Substrate	Initial concentration of substrate ($g \cdot L^{-1}$)	Maximum citric acid concentration ($g \cdot L^{-1}$)	References
C. lipolytica Y 1095	Batch	Glucose	50-150	13.6 - 78.5	(Rane and Sims, 1993)
Y. lipolytica A-101	Repeated batch	Glucose	92	34.3	(Rymowicz *et al.*, 1993)
C. lipolytica Y 1095	Batch	*n*-paraffin	100 - 150	9.8	(Crolla and Kennedy, 2001)
Y. lipolytica N1	Continuous	Ethanol	0.01 - 1.0	14.4 - 19.2	(Finogenova *et al.*, 2002)
Y. lipolytica LGAM S(7)1	Batch	Raw glycerol	80 - 120	33 - 35	(Papanikolaou *et al.*, 2002)
Candida lipolytica	Fed batch	*n*-paraffin	157	42	(Crolla and Kennedy, 2004a)
Y. lipolytica UOFS Y-1701	Batch	Sunflower oil	30	18.7	(Venter *et al.*, 2004)
Y. lipolytica 187/1	Batch	Rapeseed oil	> 5	135	(Kamzolova *et al.*, 2005)
Y. lipolytica 1.31	Batch	Raw glycerol	200	124.5	(Rymowicz *et al.*, 2006)
Y. lipolytica NCIM 3589	Batch	Raw glycerol	54.4	77.39	(Imandi *et al.*, 2007)
Y. lipolytica NRRL YB-423	Batch	Pure glycerol	40	21.6	(Levinson *et al.*, 2007)

(Continue)

Table 2 – Continuation

Strain	Fermentation type	Substrate	Initial concentration of substrate ($g \cdot L^{-1}$)	Maximum citric acid concentration ($g \cdot L^{-1}$)	References
Y. lipolytica ACA-DC 50109	Batch	Glucose in OMW-based medium	65	28.9	(Papanikolaou *et al.*, 2008b)
Y. lipolytica NBRC 1658	Batch	Mannitol	120	20.25	(Karasu *et al.*, 2009a)
Y.lipolytica 57	Batch	Pure glycerol	160	32.80	(Karasu *et al.*, 2009a)
Y. lipolytica NBRC 1658	Batch	Glucose in whey-based medium	100	38.88	(Karasu *et al.*, 2009b)
Y. lipolytica 57	Batch	Fructose in whey-based medium	150	49.23	(Karasu *et al.*, 2009b)
Y. lipolytica ACA-DC 50109	Repeated batch	Glycerol	27.8	13.95	(Makri *et al.*, 2010)
			104.9	33.55	
Y. lipolytica Wratislavia AWG7	Fed batch	Raw glycerol	200 (total glycerol concentration	-	(Rywińska *et al.*, 2010)
			300 (total glycerol concentration)	-	
Yarrowia lipolytica A-101-1.22	Cell recycle	Raw glycerol	250	96–107	(Rymowicz *et al.*, 2010)
Y. lipolytica Wratislavia AWG7	Continuous	Glycerol	50	97.8	(Rywinska *et al.*, 2011)
Y. lipolytica Wratislavia 1.31				76.0	
Y. lipolytica Wratislavia AWG7	Batch	Glycerol	150	85.7	(Tomaszewska *et al.*, 2014)
Y. lipolytica Wratislavia K1				65.0	

2.2.4. Fermentation conditions for yeasts

Nitrogen source

Levels of nitrogen available in the medium have effect on citric acid accumulation by yeasts (Soccol *et al.*, 2006), being yeast extract and ammonium chloride the most suitable organic and inorganic nitrogen sources, respectively, for citric acid production by *Y. lipolytica* among other yeasts (Rane and Sims, 1996). Under excess of the carbon source, limitation of mineral components of the medium, mainly nitrogen, are required to induce and obtain high levels of citric acid production (Aiba and Matsouka, 1978; Klasson *et al.*, 1989; Lozinov *et al.*, 1974; Papanikolaou *et al.*, 2008b; Ratledge and Wynn, 2002). In the presence of high nitrogen concentrations, biomass growth is promoted (Vandenberghe *et al.*, 1999). However, at low concentrations of nitrogen, after nitrogen exhaustion, intracellular AMP concentration rapidly decreases due to its cleavage by AMP-desaminase. Therefore, NAD^+ or $NAPD^+$ iso-citrate dehydrogenase, the enzyme responsible for the transformation of isocitric to α-ketoglutaric acid and allosterically activated by intracellular AMP, loses its activity (Papanikolaou and Aggelis, 2009). It results in accumulation of citric acid inside the mitochondrion (Papanikolaou and Aggelis, 2009), which is secreted into cytosol when its concentration overcomes a critical value (Figure 2).

pH

The pH parameter is reported as the most critical on citric acid production by yeasts (Yalcin *et al.*, 2010). Therefore, initial pH must be defined and optimized according to the microorganism, the nature of the substrate and the fermentation type used (Yokoya, 1992). For yeasts, it was recommended to use an initial pH above 5. Bellow this pH value, citric acid production is impaired due to the accumulation of polyalcohols (*i.e.* erythritol, arabitol and mannitol) (Mattey, 1992; Roehr *et al.*, 1993), the inhibition of citrate production in the cell and the transport of citrate from cell membrane (Anastassiadis and Rehm, 2005). Studies of Karasu *et al.* (2010) reported that for *Y. lipolytica* 57 and *Y. lipolytica* NBRC 1658 the maximum citric acid concentrations and citric acid yields were obtained in range of pH 5.2 to 7.0 (fermentation medium containing glucose). Moreover, the maximum specific growth rates were obtained at pH 5.2 and 6.0 for the strain 57 and NBRC 1658, respectively.

Temperature

The optimum temperature for citric acid production is dependent of microorganism and medium conditions used. For yeasts, the temperatures used for citric acid biosynthesis can range between 22 °C and 35 °C (Yalcin *et al.*, 2010). Anastassiadis and Rehm (2006) observed that *C. lipolytica* growth and produce citric acid at 35 °C, using a medium containing glucose.

Aeration

Citric acid production is an aerobic processes and therefore yields and process time are dependent of aeration (Cartledge, 1987; Kubicek, 2001; Soccol *et al.*, 2006). The dissolved oxygen directly influences the citric acid formation, whereby oxygen concentration must be maintained above 25 % of saturation (Antonucci *et al.*, 2001). Therefore, efficient aeration devices must be available, particularly when viscous fermentation media are used (Soccol *et al.*, 2006). Both, aeration and agitation affect the dissolved oxygen and substrate available in the medium, by dispersing air and insoluble substrate in the medium in the form of fine bubbles (Crolla and Kennedy, 2004a, b). However, it was necessary to establish an optimum agitation in order to prevent shear stress on cell walls (Braga *et al.*, 2015; Crolla and Kennedy, 2004a). Kamzolova *et al.* (2003) observed that citric acid production was inhibited by decreasing dissolved oxygen concentration from 60 to 95 % to 28 to 30 % saturation, for *Y. lipolytica* 704. However, for *Y. lipolytica* N1 it was reported an accumulation of 120 $g \cdot L^{-1}$ of this metabolite at low dissolved oxygen concentrations and at high iron concentration.

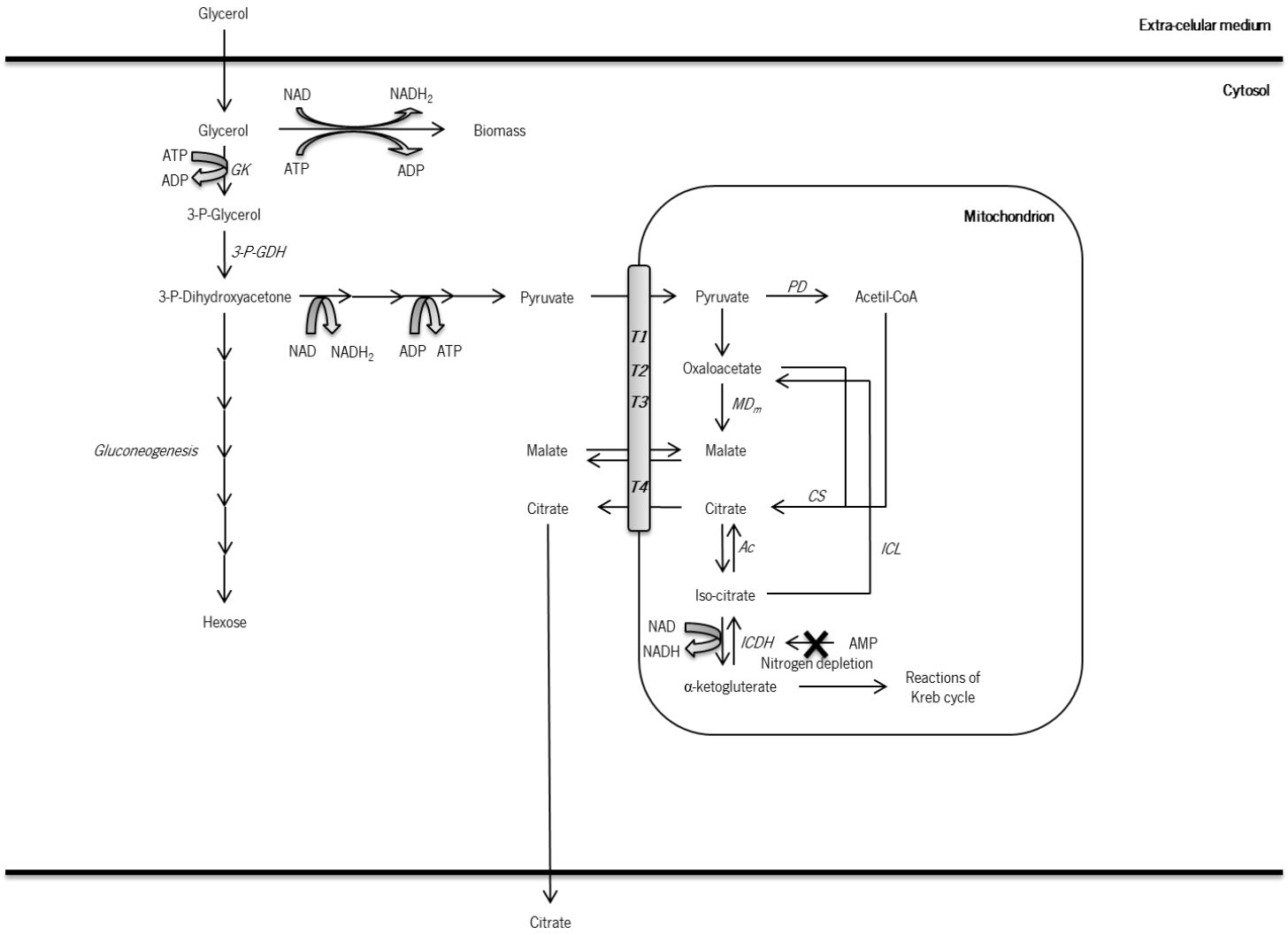


Figure 2 - Pathway for the glycerol catabolism from *Y. lipolytica* and other yeasts (adapted from (Papanikolaou *et al.*, 2008a; Ratledge and Wynn, 2002)). Abbreviations: GK - glycerol kinase; 3-P-GDH - 3-P-glycerol dehydrogenase; PD - pyruvate dehydrogenase; MD_m - malate dehydrogenase (mitochondrial); CS - citrate synthase; Ac – cis-Aconitate; ICDH - iso-citrate dehydrogenase; ICL - iso-citrate lyase; T1, T2, T3 - systems transporting pyruvic acid from cytosol to mitochondrion and inversely; T4 - system transporting citric and malic acid from cytosol to mitochondrion and inversely.

2.2.5. CITRIC ACID IMPROVEMENT STRATEGIES

Nowadays, the practices used to improve biotechnological production of citric acid overcome the optimization of fermentation operational conditions, since citric acid production are highly dependent of strain diversity (Kamzolova *et al.*, 2005; Levinson *et al.*, 2007). Therefore, a new approach about mutagenesis had been experimented in wild strains, using γ -irradiation, UV irradiation and chemical mutagens (Soccol *et al.*, 2006). In some cases, UV irradiation was combined with some chemical mutagens. In the final, the selected mutants must be stable without physiological or biochemical degeneration upon subculture for mass propagation, non-utilization of the acid formed and non-formation of gluconic, oxalic and malic acid (Sahasrabudhe and Sankpal, 2001). Hamissa *et al.* (1982) reported for *C. lipolytica* Y-1095 that the UV-irradiation was better than N-methyl-NT-nitro-N-nitrosoguanidine (NTG) in inducing more productive isolates. Mutants with a citric acid yield 75 % to 80 % higher than the original parent were obtained. Moreover, Finogenova *et al.* (2008) reported that mutants of *Y. lipolytica* VKM Y-2373 increased the ability to synthesize citric acid by using UV-irradiation and NTG. Also, three mutants were obtained from the combined action of UV and NTG, with a biosynthetic activity that exceeded of the initial strain by 43.9 %.

Citric acid improvement has been also conducted using genetic engineering. Fickers *et al.* (2004) introduced in different strains of *Y. lipolytica* the *S. cerevisiae* SUC2 gene, expressed with the XPR2 promoter and signal sequence, in order to obtain Suc+ transformants capable to grow on sucrose and promote citric acid production from molasses. Moreover, Liu *et al.* (2013) deleted some of the ATP-citrate lyase genes and increased the copy number of the iso-citrate lyase gene in the marine-derived yeast *Yarrowia lipolytica* SWJ-1b displaying the recombinant inulinase. The transformant 30 was the selected, and showed an highly increase of citric acid production (84 $g \cdot L^{-1}$).

2.2.6. CITRIC ACID RECOVERY

The citric acid recovery from fermentation broth involves *in situ* product recovery, precipitation, solvent extraction or adsorption. For the last three methods, fermentation both is first filtrated and concentrated, to obtain clear fermented liquor.

Precipitation is one of the most conventional methods for citric acid recovery. In this method, citric acid is precipitated as calcium salt by addition of calcium carbonate to the fermented liquor, followed by heating up to 50 °C during 20 min, and final addition of sulfuric acid (Pazouki and Panda, 1998).

During this process, pollutants as carbon dioxide, wastewater and gypsum are accumulated (Jinglan *et al.*, 2009), which promoted the exploration of separation technique for citric acid recovery.

Solvent extraction has been explored for citric acid recovery using high-molecular-weight-aliphatic amines (Pazouki and Panda, 1998), however the use of ion-exchange resins for organic acids recovery and purification have been largely applied by several authors (Kulprathipanja, 1990; Peng, 2002).

In situ product recovery arises as a solution for low productivity and yield of the process, which may be due to product inhibition or hydrolysis of product by catalytic reactions (Lye, 1999). Therefore, this method can be used to improve yield and productivity of citric acid.

2.3. WHOLE CELLS IMMOBILIZATION

On biotechnological processes, submerged fermentations are traditionally carried out with suspended free cells in the medium. Despite its wide use, this strategy leads to some problems which influences the process yield, namely low cell density, nutritional limitations, potential toxicity of products and difficulties in the recovery of final products (Léonard *et al.*, 2011). Therefore, in the last decades, cells immobilization technology has been explored arising as a solution for those constraints.

Ramakrishna and Prakasham (1999) define cells immobilization as the attachment or inclusion of cells in a solid phase. Beside all the advantages of this technology, it is crucial to attend at immobilizations costs, mass transport limitations, applicability of final product, ensure about cell activity and viability, among others, when a particular immobilization method is selected (Zacheus *et al.*, 2000).

2.3.1. METHODS AND SUPPORTS

In nature, cells can be found immobilized as biofilms, microbial flocs and mycellial pellets (Pradella *et al.*, 2001). Artificially, the cells immobilization methods are based on surface attachment, entrapment within porous matrices, containment behind a barrier and self-aggregation (Figure 3) (Pilkington *et al.*, 1998).

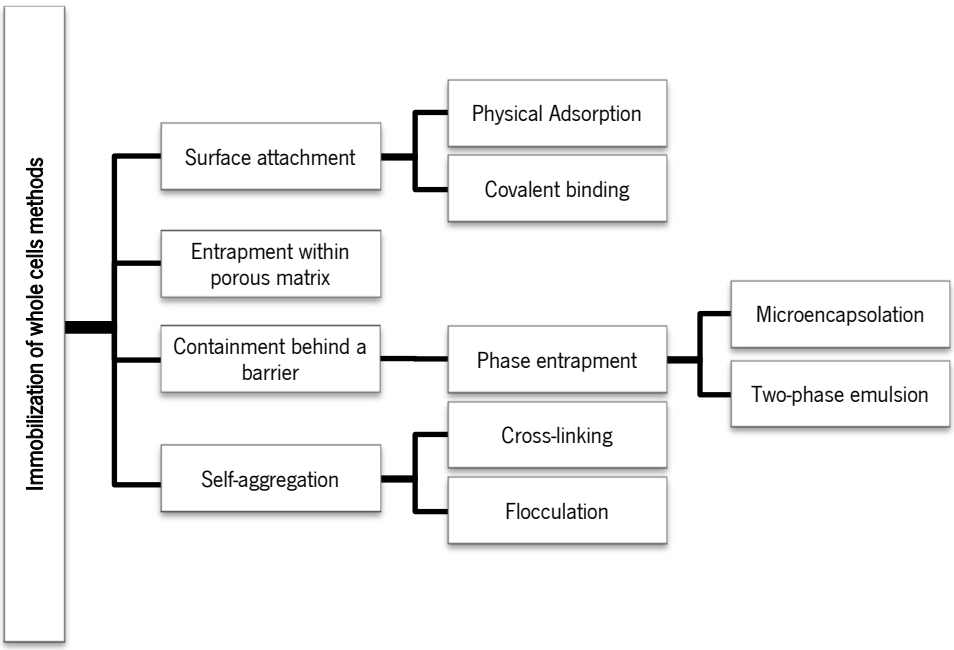


Figure 3 - Cells immobilization methods (adapted from (Pilkington *et al.*, 1998)).

2.3.1.1. Physical adsorption

At physical adsorption, interaction between microbial cells and the solid carriers occurs by electrostatic, ionic and hydrophobic interactions (Oliveira, 1997). Since cells are not confined into a support, cells detachment and relocation is possible. Thus, the establishment of equilibrium between adsorbed and freely suspended cells, influenced by cell membrane and carrier properties, such as hydrophobicity, charge, electron-donor and electron-acceptor, plays an important role in the success of the method (Klein and Ziehr, 1990; Kolot, 1981).

Physical adsorption is used to immobilize microorganisms with natural abilities to adhere and multiply under some surfaces. The first method used for whole cells immobilization was reported by Hattori and Furusaka (1960) by binding of *Escherichia coli* cells onto an ion exchange resin. Beyond this carrier, a wide range of them have been explored and used including microcarriers (small-diameter spheres, 100 μm to 200 μm) manufactured from different synthetic polymers (polystyrene, polyamide and polyacrylamide), inorganic carriers (clay materials, silica, activated charcoal and metal oxides), biopolymers (cellulose and derivates, dextran, starch, collagen and gelatin), ionic exchangers (amberlite, Dowex DEAE-Sephadex and DEAE-cellulose), glass, wood, glass ceramic, plastic materials and ceramics (Ramakrishna and Prakasham, 1999; Villeneuve *et al.*, 2000).

The advantages on using physical adsorption include the simplicity of the method, easy manipulation and the diversity of carriers. However, it is dependent of pH, ionic strength, surface

charge, cell age and composition of the carrier surface. Moreover, this method deal with difficult diffusion of nutrients and products for and from immobilized cells (Klein and Ziehr, 1990; Xavier *et al.*, 2013).

2.3.1.2. Covalent binding

The covalent binding method involves the covalent bind formation between the inorganic carrier and the whole cell, in the presence of a binding agent (Ramakrishna and Prakasham, 1999). This binding agent (*i.e.* glutaraldehyde or isocyanate) is responsible by chemical modification of the carrier, making it active by the presence of a specific functional group: amino, carboxyl or phenolic groups of tyrosine (Silva, 2011). The carriers that can be used in this method are cellulose, agarose, silica and derivatives and glass.

Immobilization by covalent binding is usually used for enzymes immobilization, but is not a good option for whole cells immobilization, since most of the binding agents are toxic for the cells, which often results in loss of cell viability (Faber, 1995).

2.3.1.3. Entrapment within porous matrices

The entrapment methods are based on the inclusion of whole cells within a rigid network (with any connection established) in order to prevent the cells diffusion into the medium, while is still possible to transfer nutrients and metabolites. The most extensively studied method for cells immobilization is the entrapment of microbial cells in polymer matrices, called entrapment within porous matrices (Pradella *et al.*, 2001).

Entrapment within porous matrices can be divided into two methods: in the first case, whole cells are enabled to diffuse into the pre formed porous matrix; in the second, the porous matrix is synthesized *in situ* around the whole cells (Verbelen *et al.*, 2006). In both cases, cellular growth is one of the most important parameter on selection of best carrier, since one of the problems of this kind of immobilization is the ability of whole cells located on the outer surface of the spheres to multiply and release from the spheres (Kourkoutas *et al.*, 2004).

Considering the first method, once inside the matrix, cells start to growth until the mobility become committed because the presence of new cells and the matrix. In that situation, cells are effectively entrapped (Baron and Willaert, 2004). The pre-formed matrices that can be used include porous glass,

sponge, ceramics, silicon carbide, polyurethane foam and stainless steel fibres (Masschelein, 1994; Scott and O'Reilly, 1995; Shen *et al.*, 2003; Tata *et al.*, 1999).

Considering the second method, several natural and synthetic materials can be used as carrier for entrapment. Polyester, polyurethane, polystyrene, chitosan, alginate, k-carrageenan, agar, collagen, agarose, pectin, cellulose and it derivates, gelatin, epoxy resin, poly-vinyl alcohol (PVA) and photo-crosslinkable resin gels (Ramakrishna and Prakasham, 1999) are the most used and described carriers.

2.3.1.3.1. Entrapment of cells in alginate gel

Entrapment of cells in alginate gel is one of the most frequently described, because of simplicity, quickly and low cost of process, requiring of mild conditions (pH, temperature and osmolality) and non-toxicity of method (Fraser and Bickerstaff, 1997; Kierstan and Bucke, 1977; Zhou *et al.*, 2010).

Alginates are natural polysaccharides produced by bacteria (including *Azotobacter* and *Pseudomonas*) (Remminghorst and Rehm, 2006) and present at cellular wall from brown algae from class *Phaeophyceae* (Stephen *et al.*, 2006). Commercial alginates are commonly extracted from algae from species *Laminaria hyperborea*, *Laminaria digitata*, *Laminaria japonica*, *Ascophyllum nodosum*, and *Macrocystis pyrifer* (Smidsrod and Skjak-Bræk, 1990).

Alginates are a family of unbranched binary copolymers composed by 1,4-linked β-D-mannuronic (M residue) and α-L-guluronic (G residue), which vary in proportions and sequential arrangements (Haug, 1959). Blocks can be composed of consecutive M residues, consecutive G residues and alternating M and G residues. According to the source, alginates can differ about M-blocks and G-block content, existing more than 200 different alginates being produced (Tonnesen and Karlsen, 2002).

The main characteristic of alginates is its ability to form gels in the presence of a solution with divalent cations, such as calcium. It is the result of a specific, strong and coordinated binding of calcium ions between aligned G-blocks of two alginate chains. The G-blocks of one polymer form junctions with the G-blocks of adjacent polymer chains, in what is named the egg-box model of cross-linking, resulting in a gel structure matrix (Grant *et al.*, 1973) (Figure 4).

Therefore, the gelling properties of the calcium alginate matrix are strongly related to M and G residues composition, sequential arrangements of blocks, the lengths of the G-block in the polymeric chains, concentration of copolymer and calcium ions. Other aspects which influence the properties of a gel matrix is the molecular weight of alginate and the gelation rate (George and Abraham, 2006;

MartInsen *et al.*, 1989; Ouwerx *et al.*, 1998; Velings and Mestdagh, 1995). Regarding to the molecular weight, the increasing of the molecular weight of alginate can improve the physical properties of resultant gel, although the use of alginates with higher molecular weight lead with high viscosity (LeRoux *et al.*, 1999). About gelation rate, it must be slower, in order to obtain a uniform and strength gel (Kuo and Ma, 2001). The use of lower temperatures can be used as controller of this parameter, since the calcium reactivity is reduced and, therefore, the cross-linking becomes slower. It results in a network structure of gel matrix with high order level and enhanced mechanical properties (Augst *et al.*, 2006).

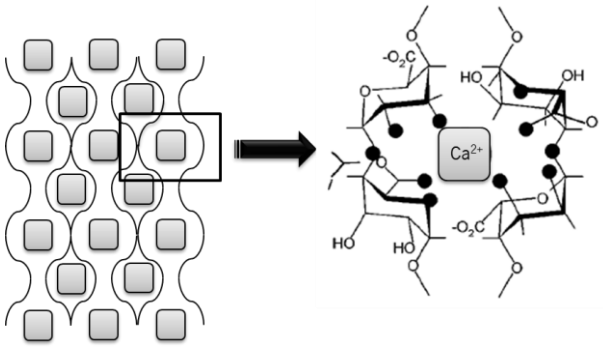


Figure 4 - Schematic drawing of the egg-box model. Dark circles represent the oxygen atoms involved in the coordination of the calcium ion (adapted from (Grant *et al.*, 1973)).

The disadvantage about alginate gels is the limited stability in physiological conditions, besides the difficult diffusion of nutrients and products for and from immobilized cells (Klein and Ziehr, 1990; Xavier *et al.*, 2013). The exchange of monovalent cations between calcium alginate gel and the surrounding media can result in dissolution of the matrix (Leea and Mooneya, 2012).

2.3.2. *Y. LIPOLYTICA* IMMOBILIZATION

Dimorphism feature makes *Y. lipolytica* a yeast species which exhibits different cellular structures at each stage of it lifecycle. Immobilization of cells arises as a solution to preserve the desirable biocatalytic activity of this yeast, decreasing the influence of *Y. lipolytica* morphology in the process yield (Prasad *et al.*, 2005).

Currently it is known many techniques applied in *Y. lipolytica* immobilization, for degradation of some oils, as well for production of citric acid and γ-decalactone.

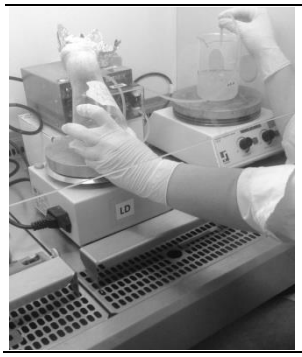
Kautola *et al.* (1991) reported *Y. lipolytica* immobilization for citric acid production, using glucose as substrate. Several carriers were evaluated, including alginate, k-carrageenan, polyurethane gel, nylon

web and polyurethane foam. A productivity of 0.155 $g \cdot L^{-1} \cdot h^{-1}$ was obtained with alginate in a batch fermentation. Mansfeld *et al.* (1995) tested for *Y. lipolytica* EH 59 microencapsulation in polyelectrolyte complex with (co-immobilization) and without invertase, in continuous fermentation for acid citric production. The production rate of citric acid achieved 0.125 $g \cdot L^{-1} \cdot h^{-1}$ when microencapsulated cells without invertase were used, being registered a lower value (0.014 $g \cdot L^{-1} \cdot h^{-1}$) with co-immobilized invertase/cells. Also, Oh *et al.* (2000) tested *Y. lipolytica* 180 immobilized onto oil-absorbent polyurethane (PUF) for extraction of oil films on surface water. The results revealed the success of method. Beyond the excellent floatability of PUF, PUF absorbed 7 to 9 times its own weight of crude oil. Also, Wu *et al.* (2009) immobilized *Y. lipolytica* W29 cells in calcium alginate for oil wastewater degradation. The ability of *Y. lipolytica* NCIM 3589 immobilized into agar-alginate composite spheres, to degrade aliphatic compounds present in crude oil was evaluated by Zinjarde and Pant (2000). The immobilized cells were active for thirty days with no loss of activity, in a continuous flow reactor. Braga and Belo (2013) studied γ-decalactone production with *Y. lipolytica* W29 immobilized by adsorption in two different carriers, methyl polymethacrylate and DupUM®, using castor oil as substrate. The highest productivity of the process was obtained with free cells (0.007±1 $g \cdot L^{-1} \cdot h^{-1}$), although the maximum aroma concentration was obtained with adsorbed cells in DupUM®.

In Table 3 the different carriers applied for *Y. lipolytica* immobilization, and respective bioprocess are compiled.

Table 3 - Immobilization methods and carriers applied in *Y. lipolytica*

Strain	Bioprocess	Immobilization method/Carriers	Reference
Y. lipolytica A-101	Citric acid biosynthesis using glucose as substrate	Alginate k –Carrageenan Polyurethane gel Nylon web Polyurethane foam	(Kautola *et al.*, 1991)
Y. lipolytica EH 59	Citric acid biosynthesis using glucose and fructose as substrate	Co-immobilization Microencapsulation in polyelectrolyte complex	(Mansfeld *et al.*, 1995)
Y. lipolytica 180	Removal of oil films on surface waters	Oil-absorbent polyurethane	(Oh *et al.*, 2000)
Y. lipolytica NCIM 3589	Crude oil degradation	Agar-alginate composite spheres	(Zinjarde and Pant, 2000)
Y. lipolytica W29	Oil wastewater degradation	Calcium alginate	(Wu *et al.*, 2009)
Y. lipolytica W29	γ-Decalactone biosynthesis using castor oil as substrate	Methyl polymethacrylate DupUM®	(Braga and Belo, 2013)



3. MATERIALS AND METHODS

This chapter covers the general methodologies, experimental conditions, equipment and analytical methods used in experiments of citric acid biosynthesis.

3.1. MICROORGANISM

The strain used in this work was *Y. lipolytica* W29 (ATCC 20460) that was preserved in glycerol (30 % (w/v)) and kept at - 80 °C (stock culture). After thawing, yeast cells was cultured into yeast-peptone-dextrose-agar (YPDA) medium, composed of 10 $g{\cdot}L^{-1}$ yeast extract, 20 $g{\cdot}L^{-1}$ peptone, 20 $g{\cdot}L^{-1}$ glucose and 20 $g{\cdot}L^{-1}$ agar, and placed to growth at 27 °C during 48 hours, after what was maintained at 4 °C.

3.2. MEDIUM

The growth medium for inoculum was yeast-peptone-dextrose (YPD) medium, composed by 10 $g{\cdot}L^{-1}$ yeast extract, 20 $g{\cdot}L^{-1}$ peptone and 20 $g{\cdot}L^{-1}$ glucose. Sterilization was made at 121 °C during 20 minutes in the autoclave.

The basic composition of citric acid production medium included 40 $g{\cdot}L^{-1}$ pure glycerol (minimum assay: 99.5 %), 0.5 $g{\cdot}L^{-1}$ yeast extract, 6 $g{\cdot}L^{-1}$ KH_2PO_4, 0.5 $g{\cdot}L^{-1}$ Na_2HPO_4, 1.5 $g{\cdot}L^{-1}$ $MgSO_4.7H_20$, 0.075 $g{\cdot}L^{-1}$ $CaCl_2$, 0.001 $g{\cdot}L^{-1}$ $ZnSO_4.7H_20$, 0.075 $g{\cdot}L^{-1}$ $FeCl_3.6H_20$ and 0.030 $g{\cdot}L^{-1}$ $MnSO_4.H_20$ (adapted from (Makri *et al.*, 2010; Papanikolaou *et al.*, 2008b; Rywinska *et al.*, 2011)). Sterilization was made at 110 °C during 30 minutes in the autoclave (to prevent salts precipitation) and without $CaCl_2$, $ZnSO_4.7H_20$, $FeCl_3.6H_20$ and $MnSO_4.H_20$. A stock solution of these salts was prepared, filtrated (0.2 µm) under sterile conditions and added to the sterilized medium.

3.3. OPERATIONAL CONDITIONS

In this work, cell growth and production experiments were performed at 27 °C and 170 rpm. Exceptionally, immobilization of *Y. lipolytica* onto DupUM® and production carried out by this immobilization system were carried out at 140 rpm.

Experiments were performed in baffled Erlenmeyer flasks and samples were taken each 24 hours, during 7 days.

3.4. EXPERIMENTS WITH FREE CELLS

Cells were pre-grown in 500 mL Erlenmeyer flask with 200 mL of YPD medium, overnight. Cells from this pre-grown culture were used to inoculate 700 mL of YPD medium in 2000 mL Erlenmeyer

flask to give an optical density at 600 nm (OD_{600}) of 0.5, which grew for 24 h. After that, cells were separated from the medium by centrifugation (5000 rpm, 10 min), washed in sodium chloride (0.9 %) and ressuspended in the citric acid production medium. The influence of cell concentration on citric acid production was analysed and different cellular suspensions were used, 0.5 $g \cdot L^{-1}$, 1.5 $g \cdot L^{-1}$, 3 $g \cdot L^{-1}$ and 4.5 $g \cdot L^{-1}$. Sampling was conducted twice a day, over 7 days, for biomass growth, pH variation, glycerol and citric acid concentration monitoring.

3.5. IMMOBILIZATION BY ENTRAPMENT INTO CALCIUM ALGINATE

Pre-grown cells in 250 mL Erlenmeyer flask with 100 mL of YPD medium were used to inoculate a 500 mL Erlenmeyer flask with 200 mL of YPD medium (OD_{600}=0.5). After 24 hours, grown cells were recovered, washed, ressuspended in NaCl (0.9 % (w/v)) and added to the sodium alginate (Acros organics) solution (after a brief cooling). The mixture, after homogenized, was pumped through a tube of defined diameter, using a peristaltic pump, into the calcium chloride solution. For harden, spheres were maintained in solution with agitation during 6 hours at room temperature. Then, they were kept at 4 °C overnight and, after that, washed in sterile water with magnet agitation and transferred to the production medium.

3.5.1. GEL MATRIX

The effect of calcium alginate gel matrix composition on citric acid production was studied, using 2.5 % (w/v) and 3.3 % (w/v) of sodium alginate and 0.05 M, 0.1 M and 0. 5 M of calcium chloride. Spheres with 3 mm of diameter and 5 mg of dry cell mass per gram of wet support (1.5 $g \cdot L^{-1}$ of cells in the medium) were used. Cellular release to the medium, pH variation, glycerol and citric acid concentration was monitored.

Afterwards, the influence of spheres diameter was also analysed, between 2 mm, 3 mm and 4 mm, using the gel matrix composition optimised. The glycerol and citric acid concentration along experiment was measured.

After selecting the best calcium alginate gel matrix, cellular viability of immobilized cells was estimated by an indirect method, comparing the glucose consumption rate by free and immobilized cells in 500 mL Erlenmeyer flasks with 200 mL of YPD medium. Samples were taken hourly over 6 hours and the concentration of glucose was evaluated by DNS method (Miller, 1959).

3.5.2. CELL CONCENTRATION

Pre-grown cells were used to inoculate a 500 mL Erlenmeyer flask with 200 mL of YPD medium (OD_{600}=0.5) and spheres with a dry cell mass per mass of wet support of 5 $mg \cdot g^{-1}$ (1.5 $g \cdot L^{-1}$ of cells in the medium), 10 $mg \cdot g^{-1}$ (3 $g \cdot L^{-1}$ of cells in the medium) and 16 $mg \cdot g^{-1}$ (4.5 $g \cdot L^{-1}$ of cells in the medium) were prepared, in order to evaluate the effect of immobilized cell concentration on citric acid production. The glycerol consumption and citric acid concentration along experiment was determined.

3.5.3. SUBSTRATE

The substrate used for citric acid production was varied regarding to its concentration and the use of pure and crude glycerol. The range of glycerol concentrations evaluated were 40 $g \cdot L^{-1}$, 60 $g \cdot L^{-1}$ and 80 $g \cdot L^{-1}$ pure glycerol. For experiments with crude glycerol, the concentration of substrate used was the best identified for pure glycerol. Crude glycerol was obtained from Prio Combustíveis, S.A. (Aveiro, Portugal) and its characteristics are presented in Table 4 (analysis report from the supplier company). The glycerol and citric acid concentration along experiment was monitored.

Table 4 - Crude glycerol characteristics

Characteristics	Results
Density (Kg/m^3) at 20 °C - water solutions	1264.0
pH	6.5
Water content [% wt]	9.0
Sodium chloride [% wt]	4.9
Glycerol content [% wt]	90.4
Methanol content [% wt]	< 0.001
Matter Organic Non-Glycerol [% wt]	< 0.5

3.5.4. DIFFUSIVITY

Glycerol diffusivity into calcium alginate gel matrix was assessed in two different matrices composed by 2.5 % (w/v) of sodium alginate and 0.1 M of calcium chloride and 3.3 % (w/v) of sodium alginate and 0.5 M of calcium chloride. A solution with 40 $g \cdot L^{-1}$ of pure glycerol was prepared in 100 mL of distilled water, added to 28.84 g of spheres without cells in a 250 mL Erlenmeyer flask, and kept in a heating bath at 27 °C and 140 rpm. Samples were taken each 0.5 minutes until 2 minutes and then for each minute until 10 minutes.

The diffusion coefficients were obtained from the equation (1) (Crank, 1975), where the ideality of the mixture is assumed.

$$C = \frac{\alpha C0}{1+\alpha}\left[1 + \sum_{n=1}^{x} \frac{6(1+\alpha)exp\frac{(-Deqn^2 t)}{a^2}}{9+9\alpha+\alpha^2 qn^2}\right] \qquad (1)$$

where, *C* is solute concentration in the liquid phase (g·L^{-1}), α is volume ratio between volume of the free liquid and volume of spheres, *C0* is initial solute concentration in the liquid phase (g·L^{-1}), *De* is effective diffusion coefficient (cm^2·s^{-1}), *qn* is nonzero roots of $\tan(qn) = 3qn * (3 + \alpha qn^2)^{-1}$, *t* is time (h) and *a* is radius of the spheres (mm).

A routine in Matlab was performed in order to calculate glycerol diffusion coefficients parameters and to simulate diffusion behavior of this substrate in the gel matrices.

3.5.5. REACTOR FERMENTATION OPTIMIZATION

Experiments were conducted in 300 mL glass bioreactor (Figure 5). Temperature was controlled using a heated bath and the agitation was carried by a magnetic agitator. Additionally, it was implemented an aeration system which operated at 0.5 vvm.

Figure 5 - Glass bioreactor used in this work.

Citric acid production with immobilized cells was performed with and without nitrogen source (yeast extract). This procedure was first applied in Erlenmeyer flasks in order to validate the results obtained in this system. Cellular release to the medium, glycerol and citric acid concentration were measured.

3.6. IMMOBILIZATION BY ADSORPTION INTO DupUM®

Immobilization by adsorption was carried out using pieces of DupUM® (a thermoplastic support

developed at University of Minho (Matos *et al.*, 2011)), considering the experiments with *Y. lipolytica* W29 already performed with this immobilization system (Braga and Belo, 2013).

Yeast cells were pre-grown in 2000 mL Erlenmeyer flask with 1000 mL of YPD medium for 24 h. After that, cells were recovered, washed twice in sodium chloride (10 mM, pH=9) and ressuspended in 90 mL of this solution. Cell suspension (10 $g \cdot L^{-1}$) was transferred into 500 mL baffled Erlenmeyer flask with 30 pieces of DupUM®. The contact of cells with the carrier was maintaining during 50 h. Then, carrier pieces were transferred to production medium in order to give a cellular concentration of 1.5 $g \cdot L^{-1}$ in the medium. Experiments with free cells in the same operational conditions were performed and used as a control. Cellular detachment to the medium, glycerol and citric acid concentration were monitored.

3.7. ANALYTICAL METHODS

Biomass concentration was monitored by reading optical density of samples using a microplate reader (Sunrise, Tecan), at 600 nm. The obtaining values were converted to cellular concentration (dry mass) thought the calibration curve previously obtained (Attachment). Additionally cell growth was assessed by direct counting to the microscope, using the Neubauer chamber. This procedure was implemented in order to evaluate cell growth inside of calcium alginate gel matrix spheres. For this, a sample of 10 spheres was collected in the beginning and in the final of each experiment and disrupted thought contact with 20 mL of sodium citrate solution (1 % (w/v)) overnight (Estapé *et al.*, 1992).

The medium pH values were read by a bench pH meter (pH 320, WTW).

Glucose concentration was determined by DNS method (Miller, 1959) (Attachment). In this method, 100 μL of DNS and 100 μL of sample were mixed and immersed in a water bath at 100 °C for 5 minutes. A control was also prepared with distilled water instead of sample. After that, the mixture was cooled in a cold water bath, being added 1 mL of distilled water. Once homogenized, the optical density of mixture was analyzed in a microplate reader (Sunrise, Tecan) at 540 nm.

Glycerol and citric acid concentration was evaluated by HPLC (High Performance Liquid Chromatography). For glycerol concentration, the system used (Jasco) was provided with a Metacab 87 H (300 mm x 7.80 mm) column (Varian) and linked to a RI detector (1530, Jasco). The column was eluted with H_2SO_4 (0.005 M) at 60 °C, pump flow of 0.5 mL·minute^{-1} and injection volume of 20 μL. For citric acid concentration, the system used (Jasco) was provide with a Pack ODS-AQ (250 mm x 4.6 mm) column (YMC) and linked to a UV detector (2075-Plus, Jasco). The column was

eluted with KH_2PO_4 (20 mM; pH 2.8) at room temperature, pump flow of 0.7 mL·min^{-1} and injection volume of 20 µL. Citric acid concentration and glycerol concentration were calculated according to calibrations in Attachment.

3.8. CALCULATION OF FERMENTATION PARAMETERS

The fermentation parameters were calculated according to the equations presented in Table 5.

Table 5 – Equations used to calculate the fermentation parameters. *CA* - citric acid concentration in the culture liquid at the end of cultivation (g·L^{-1}); *Glyc* - total amount of glycerol consumed (g·L^{-1}); *t* - time of fermentation duration (h); *Biom* - Biomass (dry mass) concentration (g·L^{-1}) grown

Parameters	Equations		Units
Massic citric acid yield per glycerol ($Y_{CA/Glyc}$)	$Y_{CA/Glyc} = \frac{CA}{Glyc}$	(2)	g·g^{-1}
Massic biomass yield per glycerol ($Y_{Biom/Glyc}$)	$Y_{Biom/Glyc} = \frac{Biom}{Glyc}$	(3)	g·g^{-1}
Maximum volumetric productivity of citric acid (P_{CA})	$P_{CA} = \frac{CA}{t}$	(4)	g·L^{-1}·h^{-1}
Glycerol consumption rate (Q_{Glyc})	$Q_{Glyc} = \frac{Glyc}{t}$	(5)	g·L^{-1}·h^{-1}

4. RESULTS AND DISCUSSION

This chapter covers the results obtained concerning the effect of initial cell concentration on citric acid production with free *Y. lipolytica* cells. Also, the influences of different parameters in cell immobilization (gel composition, sphere diameter, ratio between dry cell mass per mass of wet support, production medium composition and pH) were investigated in order to achieve a suitable method for *Y. lipolytica* immobilization for citric acid production from glycerol. The performance of immobilized cells by adsorption into DupUM® for citric acid production was assessed. Moreover, results obtained in bioreactor, are shown.

4.1. EFFECT OF THE INITIAL YEAST CELL CONCENTRATION ON CITRIC ACID PRODUTION

In order to better understand the effect of initial cell concentration on citric acid production and select the best condition to use in the immobilized cell system, experiments with different cell concentrations (0.5 $g \cdot L^{-1}$; 1.5 $g \cdot L^{-1}$, 3 $g \cdot L^{-1}$ and 4.5 $g \cdot L^{-1}$) were carried out.

The increase of initial cell concentration favored cellular growth (Figure 6-A) and biomass yield per glycerol which varied from 0.214 to 0.803 $g \cdot g^{-1}$ (Table 6). Concerning the glycerol consumption rates, it increased with an increase of initial cell concentration (Table 6 and Figure 6-B). Regarding to citric acid concentration, the increase of initial cell concentration from 0.5 $g \cdot L^{-1}$ to 1.5 $g \cdot L^{-1}$ slightly increased the maximum volumetric productivity of citric acid from 0.013 $g \cdot L^{-1} \cdot h^{-1}$ to 0.018 $g \cdot L^{-1} \cdot h^{-1}$ (Table 6), as well as the maximum citric acid concentration (Figure 6-C). However, for the initial cell density of 3 $g \cdot L^{-1}$ and 4.5 $g \cdot L^{-1}$, the citric acid production increased up to maximum (around 96 hours and 72 hours, respectively) and after that a decrease was observed. In fact, a quickly glycerol consumption was observed due to the high amount of energy spent for biomass growth and yields. Therefore, without any substrate available, the yeast started to use the citric acid for cell maintenance and growth, which conducted to the decline of citric acid concentration. The concentration of citric acid in the medium arises as the balance between what is produced and consumed. Rodrigues and Pais (1997) earlier reported the capability of *Y. lipolytica* to uptake and use citric acid for cell growth, when citric acid is the only substrate in the medium. The effect of initial cell concentration on citric acid production was also observed in Rane and Sims (1996) experiments, with *Y. lipolytica* NRRL Y-I095. These authors conducted batch fermentations using glucose as substrate, in two phases: the growth phase and the production phase, initialized after 40 hours of growth and with the cells recovered from the growth medium. In the production phase, a decrease on citric acid yield from 0.77 $g \cdot g^{-1}$ to 0.58 $g \cdot g^{-1}$ was observed when 26 $g \cdot L^{-1}$ to 56 $g \cdot L^{-1}$ of biomass were used, respectively.

Concerning the maximum volumetric productivity of citric acid, similar values were obtained between 1.5 $g \cdot L^{-1}$ and 3 $g \cdot L^{-1}$, decreasing to 0.010 $g \cdot L^{-1}$ when 4.5 $g \cdot L^{-1}$ were used (Table 6). Moreover, citric acid yield per glycerol decreased with an increase in initial cell concentration from 0.083 $g \cdot g^{-1}$ to 0.019 $g \cdot g^{-1}$ (Table 6), occurring a marked accumulation of this metabolite during stationary growth phase (Figure 6-A and Figure 6-C).

In fact, the increase of initial cell concentration from 0.5 $g \cdot L^{-1}$ to 1.5 $g \cdot L^{-1}$ seems to be advantageous for citric acid production, since the use of 1.5 $g \cdot L^{-1}$ of cells enabled to improve the maximum volumetric productivity of citric acid and to obtain the highest concentration of this metabolite.

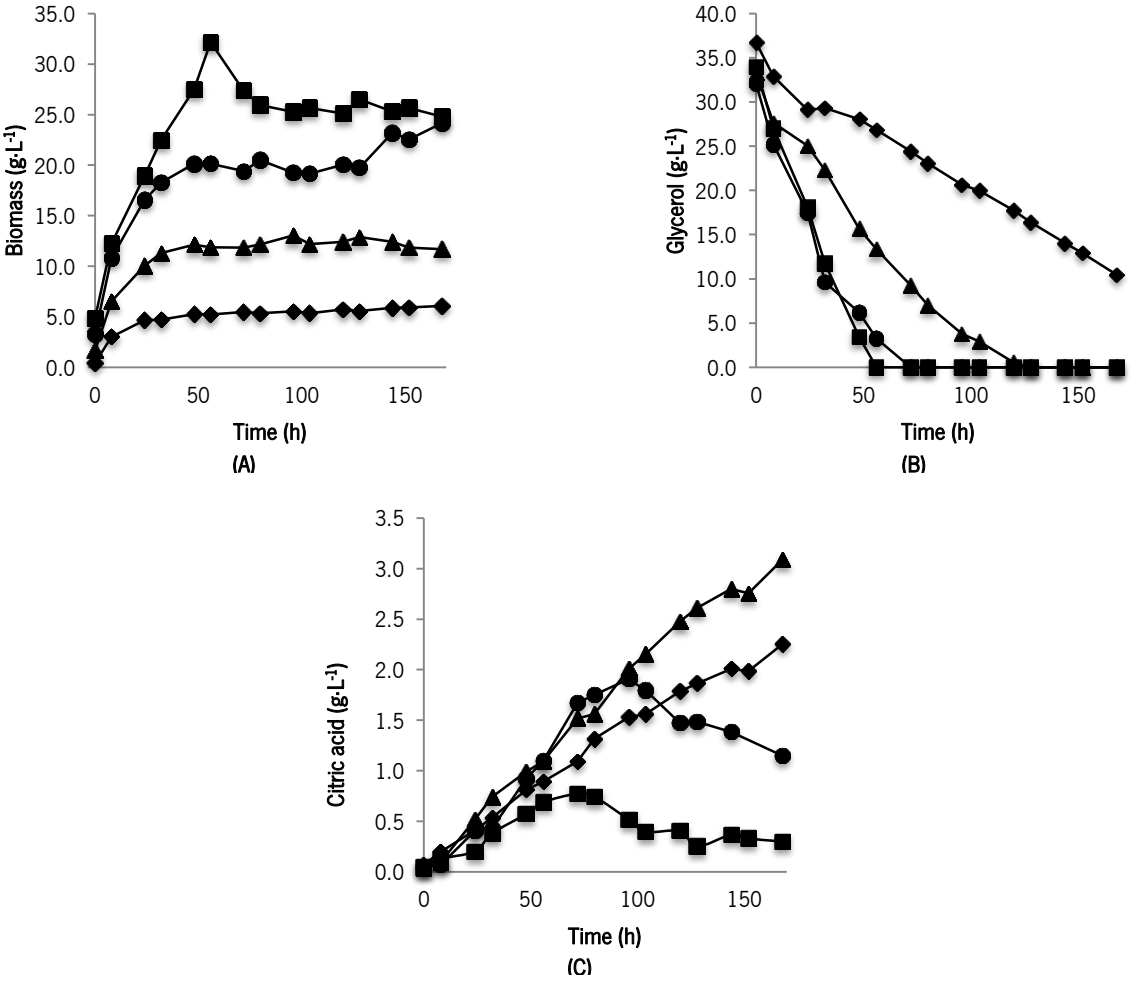


Figure 6 – Cellular growth (dry mass) (A), glycerol consumption (B) and citric acid production (C) obtained, using 0.5 g·L^{-1} (♦), 1.5 g·L^{-1} (▲), 3 g·L^{-1} (●) and 4.5 g·L^{-1} (■) of cells (dry mass). Standard errors with 95 % of confidence are lower than 23.0 % of the average.

A marked accumulation of citric acid was detected in the stationary growth phase, probably due to the nitrogen source exhaustion (not measured), available in the medium in limiting concentrations. According to the literature, the nitrogen exhaustion leads to the decrease of intracellular AMP concentration and, therefore, to the NAD^+ or $NAPD^+$ iso-citrate dehydrogenase loss of activity, promoting citric acid accumulation until total glycerol consumption (Papanikolaou and Aggelis, 2009; Papanikolaou *et al.*, 2002). In experiments with a cellular concentration of 0.5 g·L^{-1}, it would be expected to obtain higher citric acid concentrations if the fermentation time has been prolonged (low volumetric citric acid production rate) (Table 6).

Thus, the best initial cell concentration for citric acid production was 1.5 g·L^{-1} and it was selected to start the experiments with immobilized cells by entrapment.

Table 6 – Global results of *Y. lipolytica* batch cultures on glycerol medium, for different initial cells concentrations. Q_{Glyc} - glycerol consumption rate; $Y_{Biom/Glyc}$ - biomass yield per glycerol; $Y_{CA/Glyc}$ - citric acid yield per glycerol; P_{CA} – maximum volumetric productivity of citric acid

	Initial cell concentration (dry mass) ($g \cdot L^{-1}$)			
	0.5	**1.5**	**3**	**4.5**
Q_{Glyc} ($g \cdot L^{-1} \cdot h^{-1}$)	0.156	0.260	0.445	0.607
$Y_{Biom/Glyc}$ ($g \cdot g^{-1}$)	0.214	0.337	0.502	0.803
$Y_{CA/Glyc}$ ($g \cdot g^{-1}$)	0.083	0.077	0.051	0.019
P_{CA} ($g \cdot L^{-1} \cdot h^{-1}$)	0.013	0.018	0.019	0.010

4.2. ENTRAPMENT OF WHOLE *Y. LIPOLYTICA* CELLS

Entrapment of *Y. lipolytica* W29 whole cells was performed into calcium alginate gel matrix. Aiming to attain the best conditions for citric acid production using immobilized cells, different parameters were optimized, namely the gel matrix (regarding the concentration of sodium alginate and calcium chloride and the diameter of gel matrix spheres), the ratio between dry cell mass per gram of wet support, the production medium composition and the pH. The obtained results are described and discussed in the following sections.

4.2.1. OPTIMIZATION OF GEL MATRIX

Gel matrix composition

One of the most important parameters for cell immobilization by entrapment is the gel matrix composition. The sodium alginate and calcium chloride concentrations will determine the method success by regulating the porosity of the core and gel carrier matrix (Salam *et al.*, 2011). In this work two percentages of sodium alginate (2.5 % (w/v) and 3.3 % (w/v)) and three calcium chloride molarities (0.05 M, 0.1 M and 0.5 M) were investigated, using 1.5 $g \cdot L^{-1}$ of cells (the best condition for experiments with free cells) grown on pure glycerol.

For the lowest molarity of calcium chloride used, the increase of sodium alginate percentage resulted in a similar leakage of cells to the medium (Figure 7-A). However, when 0.1 M or 0.5 M of calcium chloride were used, a better immobilization system was obtained (Figure 7-A). Additionally, concerning to the percentage of sodium alginate used, the increase of calcium chloride molarity resulted in an improvement of immobilization efficiency (Figure 7-A). On the other hand, for each

percentage of sodium alginate evaluated, the increase of calcium chloride used enable to reduce the biomass leakage.

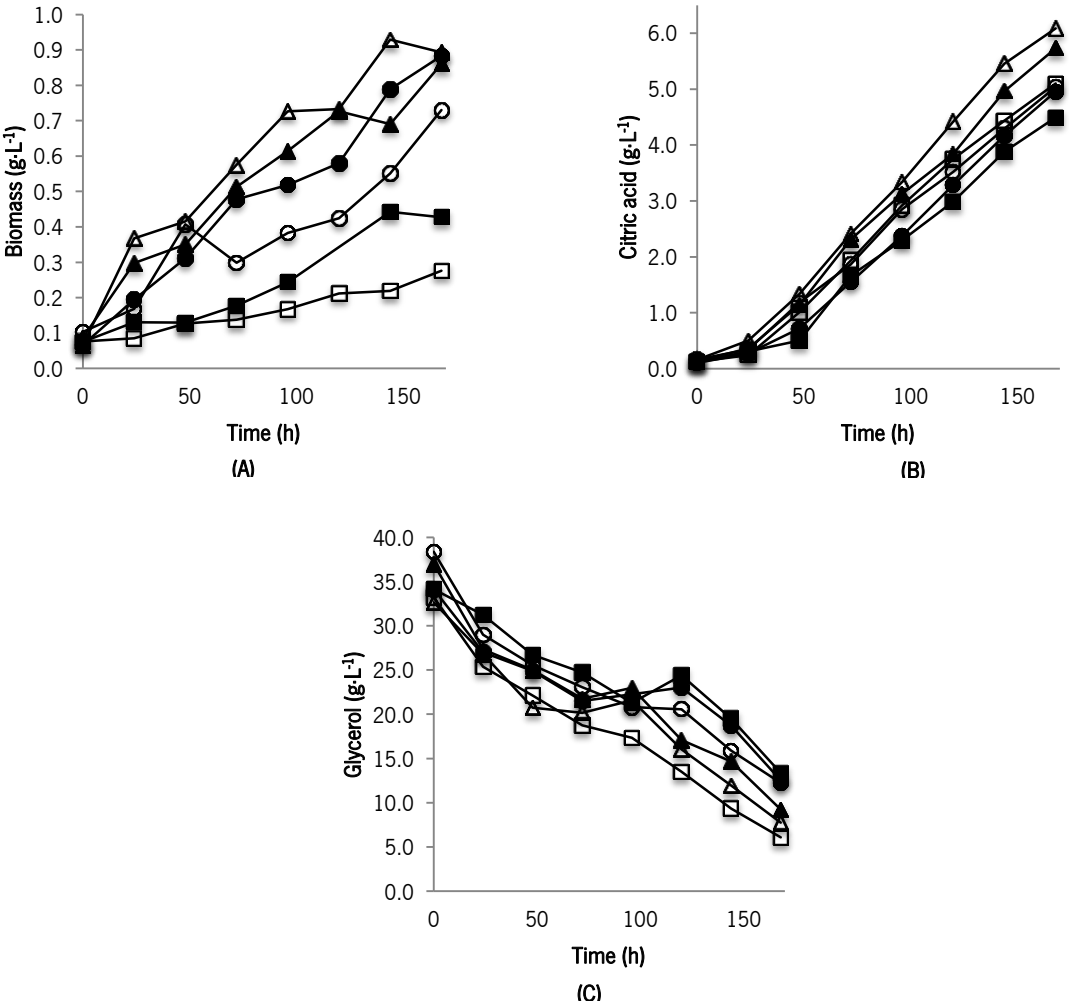


Figure 7 – Biomass leakage (dry mass) to the medium (A), citric acid concentration (B) and glycerol concentration (C) obtained using 2.5 % (w/v) (closed symbols) and 3.3 % (w/v) (open symbols) of sodium alginate and 0.05 M (▲, Δ), 0.1 M (●, ○) and 0.5 M (■, □) of calcium chloride. Conditions: 3 mm of spheres diameter and 1.5 $g \cdot L^{-1}$ (5 $mg \cdot g^{-1}$). Standard errors with 95 % of confidence are lower than 14.0 %.of the average.

According to the results obtained, the calcium chloride seems to be the most relevant parameter for immobilization efficiency. In fact, as described in the literature, the gelation process was only finished when the calcium ions contained within the core capsule is exhausted (Blandino *et al.*, 2000). Thus, for 0.05 M of calcium chloride, the quantity of calcium ions released was not probably enough to complete gelation, taking into account the percentages of sodium alginate used, compromising the immobilization efficiency. The increase of calcium chloride concentration improved the immobilization success due to the increase in the number of calcium ions in the medium enable to attain a small pore

size of more tightly crossed linked spheres (Anwar *et al.*, 2009). With respect to the sodium alginate, with a higher number of copolymers available close to the capsule core, the number of binding sites for calcium ions increases, promoting a more strong and dense cross-linked matrix (Blandino *et al.*, 2000). Therefore, the best gel matrix was reached with 3.3 % (w/v) of sodium alginate and 0.5 M of calcium chloride, which conferred the mechanical rigidity and stability necessary to maintain their structure under the agitation rate used and mechanical perturbations present in the baffled Erlenmeyer flasks. In fact, most of works in literature for *Y. lipolytica* reported a similar gel matrix composition, 4 % of sodium alginate and 0.1 M of calcium chloride for biodegradation of oil wastewater (Wu *et al.*, 2009) and 4 % of sodium alginate and 0.5 M of calcium chloride for citric acid production (Kautola *et al.*, 1991).

The effect of gel matrix composition on citric acid production was highly detected when 0.05 M of calcium chloride was used, being achieved 5.7 $g \cdot L^{-1}$ and 6.1 $g \cdot L^{-1}$ of maximum citric acid concentration with 2.5 % (w/v) and 3.3 (w/v) of sodium alginate, respectively (Figure 7-B). Among the other gel matrix composition the concentration of citric acid obtained was very similar, ranged between 4.5 $g \cdot L^{-1}$ and 5.1 $g \cdot L^{-1}$. The same behavior was observed for citric acid yield per glycerol, ranged from 0.183 $g \cdot g^{-1}$ to 0.238 $g \cdot g^{-1}$, and for the maximum volumetric productivity of citric acid, varied between 0.026 $g \cdot L^{-1} \cdot h^{-1}$ and 0.035 $g \cdot L^{-1} \cdot h^{-1}$ (Table 7). Additionally, the glycerol consumption rate ranged from 0.124 $g \cdot L^{-1} \cdot h^{-1}$ to 0.165 $g \cdot L^{-1} \cdot h^{-1}$ (Table 7), being obtained similar profiles of glycerol consumption (Figure 7-C). Finally, the effective diffusion coefficient of glycerol in gel matrix composed by 2.5 % (w/v) of sodium alginate and 0.1 M of calcium chloride and 3.3 % (w/v) of sodium alginate and 0.5 M of calcium chloride were, respectively, 2.12×10^{-4} $cm^2 \cdot s^{-1}$ and 2.99×10^{-4} $cm^2 \cdot s^{-1}$.

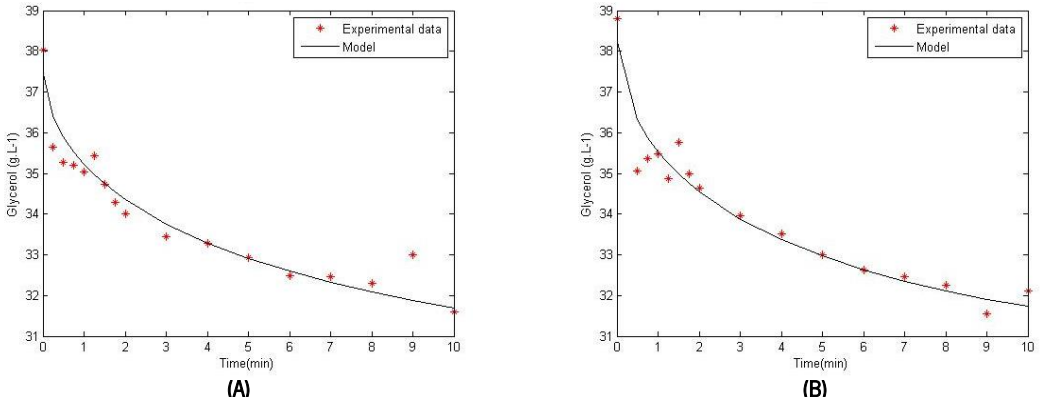


Figure 8 – Glycerol diffusivity into two gel matrices used: 2.5 % (w/v) of sodium alginate and 0.1 M of calcium chloride (A) and 3.3 % (w/v) of sodium alginate and 0.5 M of calcium chloride (B). Standard errors with 95 % of confidence are lower than 4.4 % of the average.

The similarity of glycerol consumption rates detected among the calcium alginate matrixes used, suggests that slightly differences can exist in glycerol diffusion. The residence time of gel matrix

spheres in calcium chloride solution (during gelation process) and the sodium alginate concentration used are factors which may affect the molecule diffusion through the gel matrix (Garbayo *et al.*, 2002). Therefore, the presence of hydrophobic polymeric chains, that makes molecule diffusion more difficult, and the reduction of useful sphere volume for molecule diffusion, will depend on these factors. The increase of sodium alginate percentage from 2.5 % (w/v) to 3.3 % (w/v) slightly increase the glycerol effective diffusion coefficient, showing the interference (although small) of sodium alginate percentage on glycerol diffusion, as previously described. However, it was reported in the literature, for entrapment of other microorganisms, the construction of a rigid gel matrix can lead to some diffusional limitations that compromises the process yield (Anisha and Prema, 2008). The glycerol effective diffusion coefficient values obtained is this work were higher than ones obtained by Garbayo *et al.* (2002) with 3 % (w/v) and 0.1 M of calcium chloride (6.71×10^{-6} $cm^2 \cdot s^{-1}$).

Table 7 – Global results of *Y. lipolytica* batch cultures on glycerol medium, using different gel matrix composition. Q_{Glyc} - glycerol consumption rate; $Y_{CA/Glyc}$ - citric acid yield per glycerol; P_{CA} – maximum volumetric productivity of citric acid

Sodium alginate (%) (w/v)	Calcium chloride (M)	Q_{Glyc} ($g \cdot L^{-1} \cdot h^{-1}$)	$Y_{CA/Glyc}$ ($g \cdot g^{-1}$)	P_{CA} ($g \cdot L^{-1} \cdot h^{-1}$)
2.5	0.05	0.165	0.202	0.033
	0.1	0.128	0.225	0.029
	0.5	0.124	0.210	0.026
3.3	0.05	0.148	0.238	0.035
	0.1	0.156	0.186	0.029
	0.5	0.162	0.183	0.030

The glycerol concentrations detected in the medium were modeled according to the equation 1 (Figure 8). For both gel matrices, the glycerol concentration profiles were very similar, with an initial and a final glycerol concentration around 39 $g \cdot L^{-1}$ and 31 $g \cdot L^{-1}$, respectively.

The similarity of diffusional limitations among the gel matrix used, explain the similarity of maximum volumetric productivity obtained. Moreover, the citric acid yield per glycerol and the profile of citric acid concentrations obtained was also similar. The exception is the higher amount of citric acid obtained when 0.05 M of calcium chloride were used. In this case, a more fragile gel matrix spheres were constructed and, consequently, higher free cell concentration were detected in the medium, which also contributed for citric acid production.

Comparing profiles of citric acid concentration when free cells suspended in the medium (section 4.1.) and immobilized cells by entrapment were used, it was possible to observe that the maximum

citric acid concentration were obtained with immobilized cells (Figure 6 and Figure 7), behind the higher maximum volumetric productivity of citric acid and acid yield per glycerol (Table 6 and Table 7). Therefore, the advantages of the second system are proven. Nevertheless, the glycerol consumption rate was lower in the experiments with immobilized cells (Table 6 and Table 7), that can be attributed to diffusional limitations caused by calcium alginate gel matrix, which interfered with the access of glycerol into the cells. On the other hand, in immobilized system, cells were confined into a space and cell growth was restricted and occurred mostly inside the spheres, centring the metabolism on citric acid production.

The construction of a strong gel matrix allowed the possibility to select the best conditions for cell retention that can be used for citric acid production.

Gel matrix spheres diameter

Kautola *et al.* (1991) reported for *Y. lipolytica* A-101, on repeated-batch fermentations, that an increase in calcium alginate gel matrix spheres diameter from 2-3 mm to 5-6 mm lead to a 3-fold decreased in the volumetric citric acid productivity. Therefore, in order to evaluate the effect of calcium alginate spheres built, experiments with 2 mm, 3 mm and 4 mm were performed.

For the range of diameters used, the citric acid production followed a similar profile, with a maximum concentration range from 5.0 $g \cdot L^{-1}$ to 5.5 $g \cdot L^{-1}$ (Figure 9). Additionally, glycerol consumption rate varied from 0.158 $g \cdot L^{-1} \cdot h^{-1}$ to 0.173 $g \cdot L^{-1} \cdot h^{-1}$, the citric acid yield per glycerol from 0.183 $g \cdot g^{-1}$ to 0.186 $g \cdot g^{-1}$ and maximum volumetric productivity of citric acid also varied from 0.029 $g \cdot L^{-1} \cdot h^{-1}$ to 0.032 $g \cdot L^{-1} \cdot h^{-1}$ (Table 8).

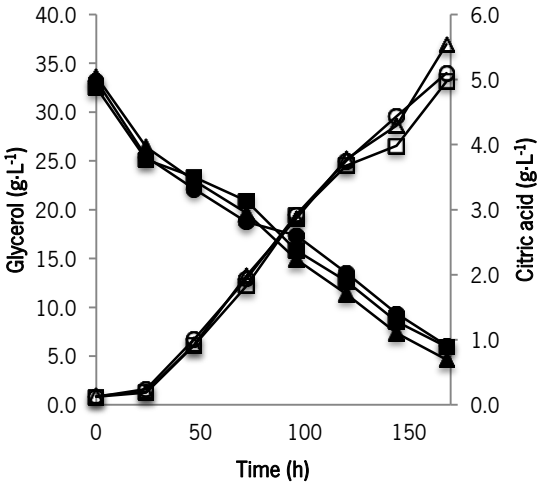


Figure 9 – Glycerol (closed symbols) and citric acid concentration (open symbols) obtained, using 2 mm (▲, Δ), 3 mm (●,○) and 4 mm (■, □) of spheres diameter. Conditions: 3.3 % (w/v) of sodium alginate and 0.5 M of calcium chloride and 1.5 g·L^{-1} (5 mg·g^{-1}). Standard errors with 95 % of confidence are lower than 14.0 % of the average.

Table 8 – Global results of *Y. lipolytica* batch cultures on glycerol medium, using different calcium alginate spheres diameter. Q_{Glyc} - glycerol consumption rate; $Y_{CA/Glyc}$ - citric acid yield per glycerol; P_{CA} – maximum volumetric productivity of citric acid

	Calcium alginate spheres diameter (mm)		
	2	3	4
Q_{Glyc} (g·L^{-1}·h^{-1})	0.173	0.162	0.158
$Y_{CA/Glyc}$ (g·g^{-1})	0.186	0.183	0.183
P_{CA} (g·L^{-1}·h^{-1})	0.032	0.030	0.029

These results showed the possibility to use, in further experiments, calcium alginate spheres with 3 ± 1 mm, without any considerably influence in the system performance.

Cells viability

The success of immobilization methodology was related to the immobilization efficiency (exchange of substrates, products and inhibitors) (Léonard *et al.*, 2011) and also to the ability to maintain cells viability through the gel preparation process (Kong *et al.*, 2003).

Viability of immobilized cell by entrapment into calcium alginate gel matrix was evaluated by an indirect method, comparing glucose consumption with free cells during 6 hours. Glucose consumption rate on both systems were 0.231 g·L^{-1}·h^{-1} (for free cells) and 0.227 g·L^{-1}·h^{-1} (for immobilized cells). The similarity of results suggested that viscosity and molecular weight of gel matrix used in these experiments, did not affect loss of *Y. lipolytica* cell viability. In fact, the higher viscosity and molecular

weight of gel matrix increase the shear forces required to mix cells with these solutions, favoring death of cells. Kong *et al.* (2003) reported that decreasing the molecular weight of alginate using irradiation, decreased the low shear viscosity (while maintaining high elastic moduli) of gel matrix, which resulted on increased cell viability from 40 % to 70 %.

In order to offset the possible loss of cell viability, Behera *et al.* (2010) reported a cellular enrichment inside the calcium alginate spheres after its formation, during 24 hours, using malt MYGP medium composed by malt extract (0.3 % (w/v)), yeast extract (0.3 % (w/v)), glucose (1 % (w/v)) and peptone (0.5 % (w/v)).

4.2.2. Effect of ratio between dry cell mass per mass of wet support on citric acid production

As previously shown in the section 4.1, citric acid production was affected by the initial cell concentration. In order to evaluate if a similar behavior is detected in the implemented immobilization system, experiments with 5 $mg \cdot g^{-1}$ (1.5 $g \cdot L^{-1}$ of cells in the production medium), 10 $mg \cdot g^{-1}$ (3 $g \cdot L^{-1}$ of cells in the production medium) and 16 $mg \cdot g^{-1}$ (4.5 $g \cdot L^{-1}$ of cells in the production medium) were performed.

The increase of ratio between dry cell mass per gram of wet support resulted in similar values of maximum citric acid concentrations obtained (varied from 4.8 $g \cdot L^{-1}$ to 5.1 $g \cdot L^{-1}$) (Figure 10) and of maximum volumetric productivity (varied from 0.028 $g \cdot L^{-1} \cdot h^{-1}$ to 0.030 $g \cdot L^{-1} \cdot h^{-1}$) (Table 9), contrarily to the results obtained with free cells. In fact, in the experiments with free cells, the increased of glycerol consumption rate associated with an increase of the initial cell concentration, was mainly directed for cell growth. However, when cells were entrapped into calcium alginate gel matrix, the cell growth was mostly confined into the support.

The citric acid yield per glycerol slightly decreased from 0.183 $g \cdot g^{-1}$ to 0.150 $g \cdot g^{-1}$ and glycerol consumption rate slightly increased from 0.162 $g \cdot L^{-1} \cdot h^{-1}$ to 0.196 $g \cdot L^{-1} \cdot h^{-1}$ (Table 9). Therefore, the glycerol was consumed for maintenance and cell growth inside of gel matrix, which explain the slightly increase of glycerol consumption rate observed with an increase of ratio between dry cell mass per unit mass of wet support. For the condition 16 $mg \cdot g^{-1}$, the increase of glycerol consumption rate was more pronounced due to the achievement of 1.7 $g \cdot L^{-1}$ free cells in the medium (data not shown). This shows that cellular concentration is an important parameter to consider in this kind of immobilization, once the whole cells located on the outer surface of the spheres can multiply and be release from the sphere (Kourkoutas *et al.*, 2004).

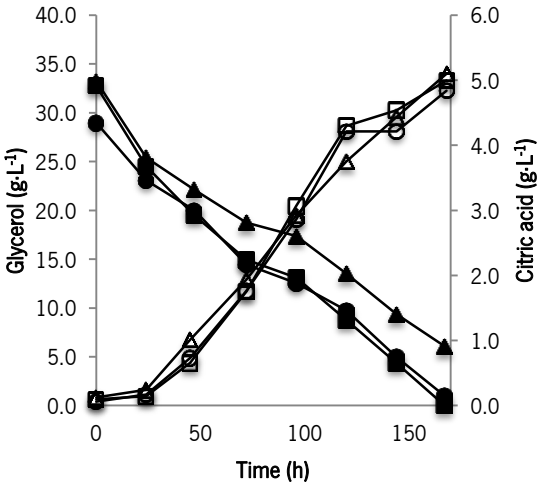


Figure 10 – Glycerol (closed symbols) and citric acid concentration (open symbols) obtained, using 1.5 g·L^{-1} (5 mg·g^{-1}) (▲, Δ), 3 g·L^{-1} (10 mg·g^{-1}) (●, ○) and 4.5 g·L^{-1} (16 mg·g^{-1}) (■, □) of cells. Conditions: 3.3 % (w/v) of sodium alginate and 0.5 M of calcium chloride and 3 mm of spheres diameter. Standard errors with 95 % of confidence are lower than 14.0 % of the average.

Table 9 – Global results of *Y. lipolytica* batch cultures on glycerol medium, using different ratio between dry cell mass per unit mass of wet support. Q_{Glyc} - glycerol consumption rate; $Y_{CA/Glyc}$ - citric acid yield per glycerol; P_{CA} – maximum volumetric productivity of citric acid

	Ratio between dry cell mass per unit mass of wet support (mg·g^{-1})		
	5	10	16
Q_{Glyc} (g·L^{-1}·h^{-1})	0.162	0.167	0.196
$Y_{CA/Glyc}$ (g·g^{-1})	0.183	0.171	0.150
P_{CA} (g·L^{-1}·h^{-1})	0.030	0.028	0.029

The higher quantity of glycerol available for citric acid production in experiments with entrapped cells into the gel matrix, comparing with free cells experiments, enabled to improve citric acid production bioprocess in this implemented system for all the evaluated conditions. Therefore, it was attained a better citric acid yield by glycerol, maximum volumetric productivity of citric acid and maximum citric acid concentration, without consumption of this metabolite.

According to the results obtained, no advantages were found with an increase of ratio between dry cell mass per mass of wet support, being selected for subsequent experiments the use of 5 mg·g^{-1}.

4.2.3. EFFECT OF GLYCEROL ON CITRIC ACID PRODUCTION

Glycerol concentration

The effect of glycerol concentration on citric acid production was studied, using 40 $g \cdot L^{-1}$, 60 $g \cdot L^{-1}$ and 80 $g \cdot L^{-1}$ of this substrate.

The maximum volumetric productivity of citric acid slightly decrease with increase of glycerol concentration used (varied from 0.030 $g \cdot L^{-1} \cdot h^{-1}$ to 0.024 $g \cdot L^{-1} \cdot h^{-1}$) (Table 10), achieving a citric acid concentration of 5.1 $g \cdot L^{-1}$ when 40 $g \cdot L^{-1}$ of pure glycerol were used after 168 h of fermentation (Figure 11). The extension of experiments for more than 168 hours, with higher glycerol concentrations, increased citric acid concentration, having attained a maximum production of 9.1 $g \cdot L^{-1}$ for 60 g L^{-1} of glycerol, without total substrate consumption for the higher concentration tested (Figure 11). The results showed that no repression by substrate was observed, suggesting the possibility to use higher glycerol concentration in order to raise citric acid concentrations. However, the use of higher glycerol concentration obliges to have longer experiments (until the total exhaustion of glycerol), raising the contamination risk. The glycerol consumption rate and citric acid yield registered similar values, varying from 0.142 $g \cdot L^{-1} \cdot h^{-1}$ and 0.162 $g \cdot L^{-1} \cdot h^{-1}$ and from 0.169 $g \cdot g^{-1}$ to 0.189 $g \cdot g^{-1}$, respectively (Table 10).

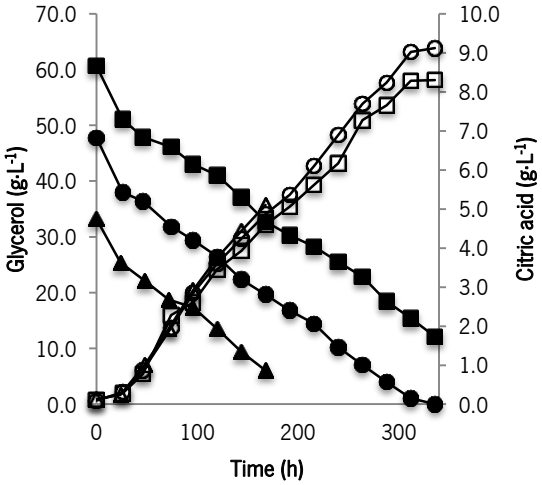


Figure 11 – Glycerol (closed symbols) and citric acid concentration (open symbols) obtained, using 40 $g \cdot L^{-1}$ (▲, Δ), 60 $g \cdot L^{-1}$ (●, ○) and 80 $g \cdot L^{-1}$ (■, □) of pure glycerol. Conditions: 3.3 % (w/v) of sodium alginate and 0.5 M of calcium chloride, 3 mm of spheres diameter and 1.5 $g \cdot L^{-1}$ (5 $mg \cdot g^{-1}$) of cells. Standard errors with 95 % of confidence are lower than 14.0 % of the average.

Table 10 – Global results of *Y. lipolytica* batch cultures on glycerol medium, for assays with different concentrations of pure glycerol. Q_{Glyc} - glycerol consumption rate; $Y_{CA/Glyc}$ - citric acid yield per glycerol; P_{CA} – maximum volumetric productivity of citric acid

	Pure glycerol ($g \cdot L^{-1}$)		
	40	60	80
Q_{Glyc} ($g \cdot L^{-1} \cdot h^{-1}$)	0.162	0.142	0.144
$Y_{CA/Glyc}$ ($g \cdot g^{-1}$)	0.183	0.189	0.169
P_{CA} ($g \cdot L^{-1} \cdot h^{-1}$)	0.030	0.027	0.024

Papanikolaou *et al.* (2008a) reported for *Y. lipolytica* ACA-DC 50109 (experiments with free cells suspended in the medium) a marked accumulation of citric acid when glycerol concentration increased from 20.5 $g \cdot L^{-1}$ to 164 $g \cdot L^{-1}$, being obtained 62.5 $g \cdot L^{-1}$ of citric acid for the higher substrate concentration. Also, citric acid yield per glycerol registered higher values in this condition due to a metabolic overflow under excess of the carbon source conditions (Anastassiadis *et al.*, 2002; Soccol *et al.*, 2006), while maximum volumetric productivity kept similar values. However, the repression of cell growth resulted in glycerol accumulation in the medium. On the other hand, Kim *et al.* (2000) reported for recombinant *Y. lipolytica* CX161-1B that the use of an initial glycerol concentration of 150 $g \cdot L^{-1}$ did not lead to consumption limitations, occurring substrate total consumption. From these results, Papanikolaou *et al.* (2008a) suggests *Y. lipolytica* strain-dependence with respect to substrate uptake and it total consumption from the medium.

Crude Glycerol

The hard accumulation of crude glycerol as a by-product from several industries, mainly from the stronger explored biodiesel process (Papanikolaou and Aggelis, 2003), became crude glycerol a profitable substrate (low cost) to use in several biotechnological process. Therefore, it was investigated the possibility to use crude glycerol, instead of pure glycerol for citric acid production with immobilized cells.

The maximum volumetric productivity of citric acid was similar in pure and crude glycerol, ranging between 0.030 $g \cdot L^{-1} \cdot h^{-1}$ and 0.033 $g \cdot L^{-1} \cdot h^{-1}$, respectively. However, citric acid yield per glycerol was higher when crude glycerol was used, achieving 0.218 $g \cdot g^{-1}$ (Figure 12 and Table 11).

Finally, glycerol consumption rate registered similar values for both tested conditions, varying between 0.162 $g \cdot L^{-1} \cdot h^{-1}$ and 0.153 $g \cdot L^{-1} \cdot h^{-1}$ with pure and crude glycerol, respectively (Table 11).

Rywińska *et al.* (2009) reported for *Yarrowia lipolytica* Wratislavia AWG7 19 and *Yarrowia lipolytica* Wratislavia K1, acetate negative mutants, close values of product concentration and yield with pure and crude glycerol. Moreover, crude glycerol was successful used for several authors for citric acid production with free cells (Papanikolaou and Aggelis, 2003, 2009; Papanikolaou *et al.*, 2008a; Papanikolaou *et al.*, 2002; Rymowicz *et al.*, 2010).

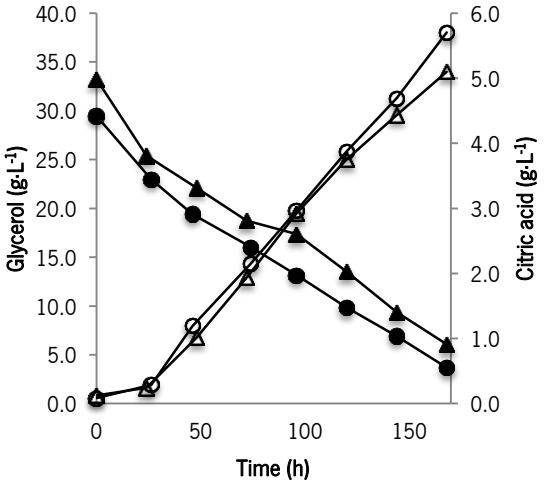


Figure 12 – Glycerol (closed symbols) and citric acid concentration (open symbols) obtained, using pure (▲, Δ) and crude (●, ○) glycerol. Conditions: 3.3 % (w/v) of sodium alginate and 0.5 M of calcium chloride, 3 mm of spheres diameter and 1.5 g·L^{-1} (5 mg·g^{-1}) of cells and 40 g·L^{-1} of glycerol. Standard errors with 95 % of confidence are lower than 17.8 % of the average.

Table 11 – Global results of *Y. lipolytica* batch cultures on glycerol medium, when crude and pure glycerol were used. Q_{Glyc} - glycerol consumption rate; $Y_{CA/Glyc}$ - citric acid yield per glycerol; P_{CA} – maximum volumetric productivity of citric acid

	Glycerol	
	Pure	**Crude**
Q_{Glyc} (g·L^{-1}·h^{-1})	0.162	0.153
$Y_{CA/Glyc}$ (g·g^{-1})	0.183	0.218
P_{CA} (g·L^{-1}·h^{-1})	0.030	0.033

The valorization of crude glycerol for citric acid production in immobilization experiments with *Y. lipolytica* W29 is, according to the obtained results, satisfactory, suggesting that impurities of industrial feedstock do not interfere on metabolism of this strain. Therefore, crude glycerol was selected to apply in further experiments.

4.2.4. EFFECT OF PH ON CITRIC ACID PRODUCTION

According to the literature, the pH was reported as the most critical parameter on citric acid production by yeasts (Yalcin *et al.*, 2010). In order to better understand the impact of pH on citric acid biosynthesis by *Y. lipolytica* immobilized cells, experiments with and without pH adjustment were carried out.

The maximum citric acid concentration, maximum volumetric productivity of citric acid and citric acid yield per glycerol increased from 5.7 $g \cdot L^{-1}$ to 9.4 $g \cdot L^{-1}$, from 0.033 $g \cdot L^{-1} \cdot h^{-1}$ to 0.064 $g \cdot L^{-1} \cdot h^{-1}$ and from 0.218 $g \cdot g^{-1}$ to 0.321 $g \cdot g^{-1}$ respectively, without and with pH adjustment (Figure 13 and Table 12). Without pH adjustment, pH varied from 5.62 to 2.82. Rymowicz *et al.* (2006) observed an increase of citric acid production by acetate mutants of *Y. lipolytica* when pH was maintained above 4.5. These results are in concordance with other authors which concerned about pH stabilization within certain values throughout the fermentation, when free (Levinson *et al.*, 2007) and immobilized cells experiments (Kautola *et al.*, 1991) were performed. The glycerol consumption rate slightly increased from 0.153 $g \cdot L^{-1} \cdot h^{-1}$ to 0.169 $g \cdot L^{-1} \cdot h^{-1}$ with pH adjustment (Table 12).

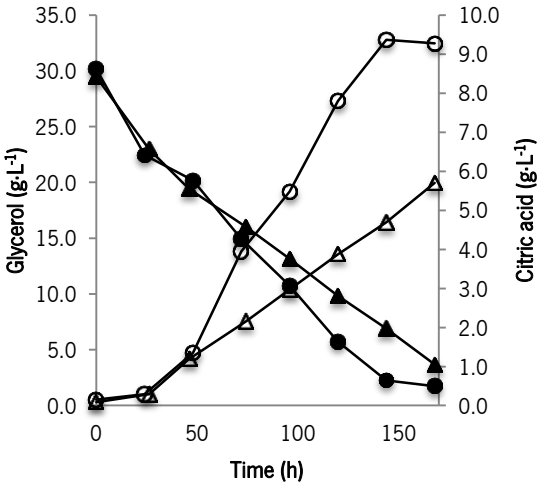


Figure 13 – Glycerol (closed symbols) and citric acid concentration (open symbols) obtained, without (▲, Δ) and with (●, ○) pH adjustment. Conditions: 3.3 % (w/v) of sodium alginate and 0.5 M of calcium chloride, 3 mm of spheres diameter and 1.5 $g \cdot L^{-1}$ (5 $mg \cdot g^{-1}$) of cells and 40 $g \cdot L^{-1}$ crude glycerol. pH adjustment (pH=5) was performed with periodic additions of KOH (2 M). Standard errors with 95 % of confidence are lower than 17.8 % of the average.

Table 12– Global results of *Y. lipolytica* batch cultures on glycerol medium, in a production medium without and with pH adjustment. Q_{Glyc} - glycerol consumption rate; $Y_{CA/Glyc}$ - citric acid yield per glycerol; P_{CA} – maximum volumetric productivity of citric acid

	Immobilized cells	
	Without pH adjustment	With pH adjustment
Q_{Glyc} ($g \cdot L^{-1} \cdot h^{-1}$)	0.153	0.169
$Y_{CA/Glyc}$ ($g \cdot g^{-1}$)	0.218	0.321
P_{CA} ($g \cdot L^{-1} \cdot h^{-1}$)	0.033	0.064

In this work, the pH adjustment resulted in an increase of maximum citric acid concentration, maximum volumetric productivity of citric acid and citric acid yield per glycerol. However, this procedure also leaded a high cell growth outside of calcium alginate spheres (1.6 $g \cdot L^{-1}$) (data not shown) which contributed to citric acid production. Therefore, pH adjustment is suitable to be implemented in the system with immobilized cells.

4.2.5. Reusability of catalyst

Among the innumerous advantages of immobilization technology, the reusability of biocatalyst is reported (Léonard *et al.*, 2011; Park and Chang, 2000; Trelles *et al.*, 2010). In order to evaluate the feasibility of this procedure for *Y. lipolytica* W29, regarding to cells stability and capability to produce citric acid, two consecutive fermentation cycles were performed, using entrapped cells into calcium alginate gel matrix.

Between the first and the second fermentation cycles, the maximum citric acid concentration, citric acid yield per glycerol and maximum volumetric productivity of citric acid increased in the second fermentation cycle, from 5.7 $g \cdot L^{-1}$ to 7.0 $g \cdot L^{-1}$, from 0.218 $g \cdot g^{-1}$ to 0.292 $g \cdot g^{-1}$ and from 0.033 $g \cdot L^{-1} \cdot h^{-1}$ to 0.043 $g \cdot L^{-1} \cdot h^{-1}$, respectively (Figure 14 and Table 13), suggesting the stability of cells. These results are similar with obtained by Kautola *et al.* (1991), that reported *Y. lipolytica* A-101 stability during five repeated-batch (similar volumetric productivity values of citric acid obtained).

Concerning the glycerol consumption rate it was observed a slightly decreased from 0.153 $g \cdot L^{-1} \cdot h^{-1}$ to 0.148 $g \cdot L^{-1} \cdot h^{-1}$, for first and second batches. Along first fermentation cycle, the citric acid concentrations achieved higher values obtained in the second fermentation cycle (Figure 14). However, in the last 17 hours, the citric acid concentration largely increased in the second cycle, which justifies the improvement on citric acid yield per glycerol and of maximum volumetric productivity of citric acid (Table 13).

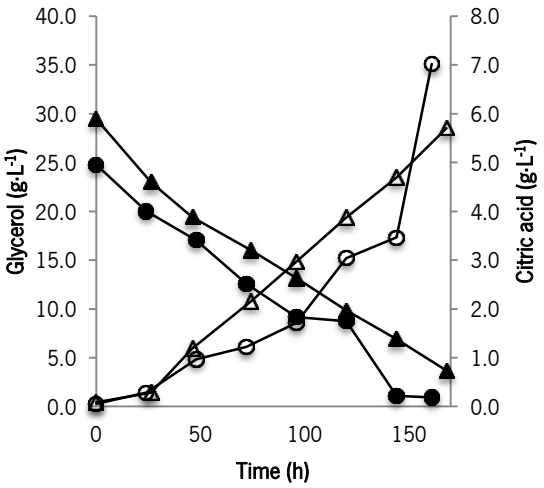


Figure 14 – Glycerol (closed symbols) and citric acid concentration (open symbols) obtained, in the first (▲, Δ) and the second (●,○) fermentation using the same cells entrapped into calcium alginate gel matrix. Conditions: 3.3 % (w/v) of sodium alginate and 0.5 M of calcium chloride, 3 mm of spheres diameter, 1.5 g·L^{-1} (5 mg·g^{-1}) of cells and 40 g·L^{-1} crude glycerol. Standard errors with 95 % of confidence are lower than 17.8 % of the average.

Table 13 – Global results of *Y. lipolytica* batch cultures on glycerol medium, in the first and second fermentation cycles, using the same yeast cells entrapped into calcium alginate. Q_{Glyc} - glycerol consumption rate; $Y_{CA/Glyc}$ - citric acid yield per glycerol; P_{CA} – maximum volumetric productivity of citric acid

	First fermentation cycle	Second fermentation cycle
Q_{Glyc} (g·L^{-1}·h^{-1})	0.153	0.148
$Y_{CA/Glyc}$ (g·g^{-1})	0.218	0.292
P_{CA} (g·L^{-1}·h^{-1})	0.033	0.043

4.3. ADSORPTION OF WHOLE *Y. LIPOLYTICA* CELLS

Immobilization of *Y. lipolytica* W29 whole cells by adsorption into DupUM®, a thermoplastic support developed at University of Minho (Matos *et al.*, 2011), was previously reported by Braga and Belo (2013), revealing successful for aroma production from castor oil. These authors optimized adsorption procedure regarding to time, cells concentration, pH of the medium and total area of support (comparing DupUM® and C-PMMA). The best conditions for cell adhesion were 10 g·L^{-1} of initial cell concentration, pH 9 for the adsorption solution (NaCl, 10 mM) and 407 m^2·m^{-3} of surface area of support (DupUM®). According with this, the adsorption profile obtained in this work for *Y. lipolytica* W29 is presented in Figure 15.

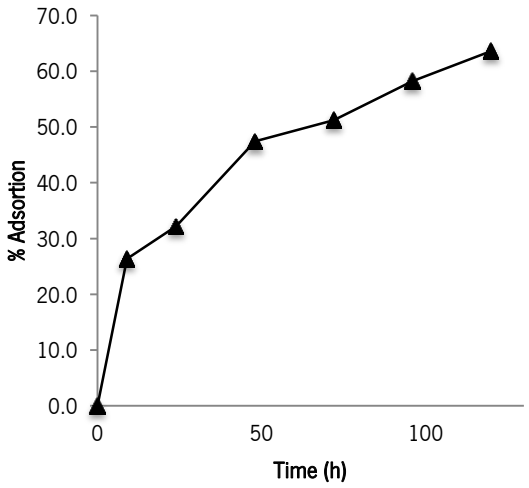


Figure 15 – Adhesion of *Y. lipolytica* W29 into DupUM® using NaCl solution (10 mM, pH 9). Standard errors with 95 % of confidence are lower than 14.8 % of the average.

Along time, the increased number of cells collisions with the support surface (stirring promoted) promoted the attachment (Bellon-Fontaine and Cerf, 1991; Fletcher, 1977), resulting on linearly increased of cells adhered with time until 9 hours (Figure 15). After that, the quantity of cells adhered decreased until achieved saturation point. After 45 hours of contact time, the number of cells adhered into DupUM® was the necessary to obtain the desirable concentration and start citric acid production experiments (1.5 $g{\cdot}L^{-1}$, the best condition determined in previous experiments by entrapment into calcium alginate matrix).

Citric acid production by yeast cells adhered into DupUM® was performed at 140 rpm instead of 170 rpm, since this was the best conditions required for an efficient immobilization by adsorption (Braga and Belo, 2013).

Comparing experiments with free and immobilized cells, citric acid concentrations followed a similar profile in both systems, being obtained a maximum concentration of 2.6 $g{\cdot}L^{-1}$ and 2.9 $g{\cdot}L^{-1}$, respectively (Figure 16-A). This may be related with the high cell concentration detected in the medium that can also contributed for citric acid production. Immediately after the transference of adhered cells into production medium, the cell density achieved 0.4 $g{\cdot}L^{-1}$ which suggests cell detachment probably due to pH decrease from 9 (the optimum for adhesion determined) to 5 (production medium pH). Experiments of Braga and Belo (2013) reported that at pH 7, the repulsive forces due to the negative charge of the yeasts surface start to have a lower effect on the adhesion process, decreasing the number of adhered cells. Therefore, at pH 5 the repulsive forces were higher, conducting to the obtained results.

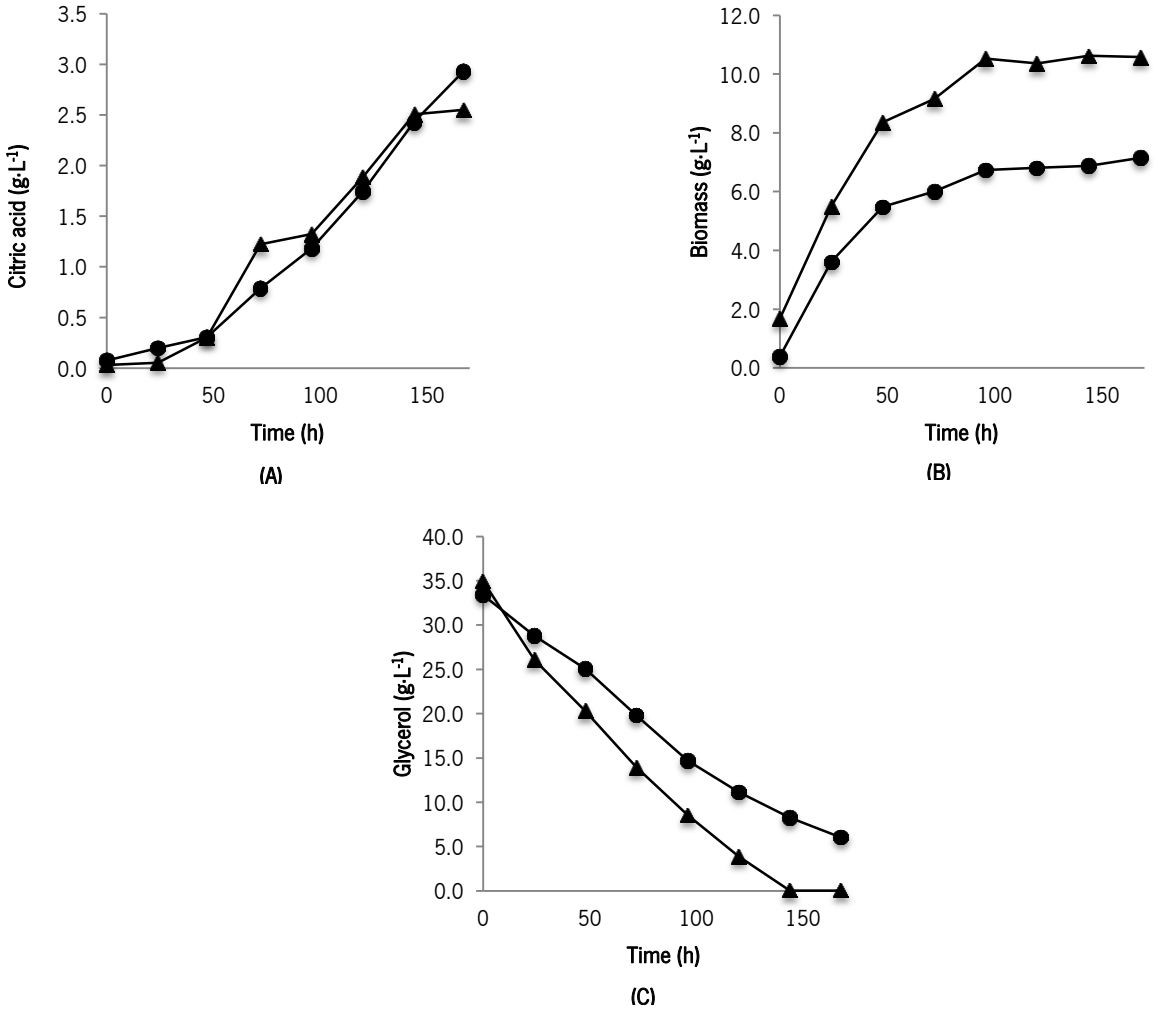


Figure 16 – Citric acid concentration (A), biomass (dry mass) (B) and glycerol concentration (C), using free (▲) and immobilized (●) cells. Conditions: 1.5 $g \cdot L^{-1}$ of cells and 40 $g \cdot L^{-1}$ crude glycerol. Standard errors with 95 % of confidence are lower than 6.6 % of the average.

The citric acid yield per glycerol and maximum volumetric productivity slightly decreased with free cells, varying from 0.086 $g \cdot g^{-1}$ to 0.071 $g \cdot g^{-1}$ and from 0.017 $g \cdot L^{-1} \cdot h^{-1}$ to 0.015 $g \cdot L^{-1} \cdot h^{-1}$, respectively (Table 14). Regarding to biomass, when free cells were used, a maximum of cell growth of 10.6 $g \cdot L^{-1}$ (Figure 16-B) and biomass yield per glycerol of 0.255 $g \cdot g^{-1}$ was achieved (Table 14). For immobilized cells, the leakage of cells from support, and freely cells growth was very high, resulting in 7.2 $g \cdot L^{-1}$ of final cell concentration in the production medium (Figure 16-B). Comparing the glycerol consumption rate for free and immobilized cells, higher values were obtained for experiments with free cells, 0.242 $g \cdot L^{-1} \cdot h^{-1}$ and 0.163 $g \cdot L^{-1} \cdot h^{-1}$ (Table 14), since a complete depletion of crude glycerol occurred after 144 hours of fermentation (Figure 16-C), due to the high energy necessary for the higher biomass growth observed.

Table 14– Global results of *Y. lipolytica* batch cultures on glycerol medium, when free and immobilized cells into DupUM® were used. Q_{Glyc} - glycerol consumption rate; $Y_{Biom/Glyc}$ - biomass yield per glycerol; $Y_{CA/Glyc}$ - citric acid yield per glycerol; P_{CA} – maximum volumetric productivity of citric acid

	Free cells	Immobilized cells
Q_{Glyc} ($g \cdot L^{-1} \cdot h^{-1}$)	0.242	0.163
$Y_{Biom/Glyc}$ ($g \cdot g^{-1}$)	0.255	-
$Y_{CA/Glyc}$ ($g \cdot g^{-1}$)	0.071	0.086
P_{CA} ($g \cdot L^{-1} \cdot h^{-1}$)	0.015	0.017

Comparing *Y. lipolytica* immobilization by adsorption and entrapment, the first one does not provide an efficient retention of cells and also enable a lower citric acid concentration (similar to the system with free cells used). Therefore, immobilization of *Y. lipolytica* whole cells by entrapment into calcium alginate gel matrix was selected to perform experiments in bioreactor.

4.4. BIOREACTOR EXPERIMENTS

Experiments in bioreactor were performed using cells entrapped into calcium alginate gel matrix, the most suitable immobilization system obtained in baffled Erlenmeyer flask.

In bioreactor experiments with yeast extract under limiting conditions, the maximum citric acid concentration obtained was 2.2 $g \cdot L^{-1}$, a lower value compared with results obtained in baffled Erlenmeyer flasks (Figure 17). In fact, citric acid yield per glycerol and maximum volumetric productivity of citric acid also registered lower values when bioreactor experiments were carried out (Table 15). Additionally, it was observed in bioreactor (data not shown) a high cell density in the production medium. In bioreactor, the presence of tubes and the use of a magnetic stirring system may had promoted the calcium alginate spheres lysis due to the constant friction that spheres were subjected. Therefore, the entrapped cells detached from gel matrix and grew in the medium as free cells. This growth was probably favored due to the continuous aeration system implemented, since *Y. lipolytica* is an obligate aerobic microorganism. In fact, a final cell density of 6.7 $g \cdot L^{-1}$ was achieved.

In order to reduce the growth of free cells in the production medium, and attempting to improve citric acid production in the bioreactor, experiments with a production medium without yeast extract were performed. As previously referred, until the total consumption of nitrogen source, the yeast metabolism was divided between cellular growth and a reduced citric acid accumulation. After nitrogen source exhaustion, occurs a decrease of intracellular AMP concentration and, therefore, with NAD^+ or

NAPD+ iso-citrate dehydrogenase loss of activity, promoting citric acid accumulation (Papanikolaou and Aggelis, 2009; Papanikolaou *et al.*, 2002).

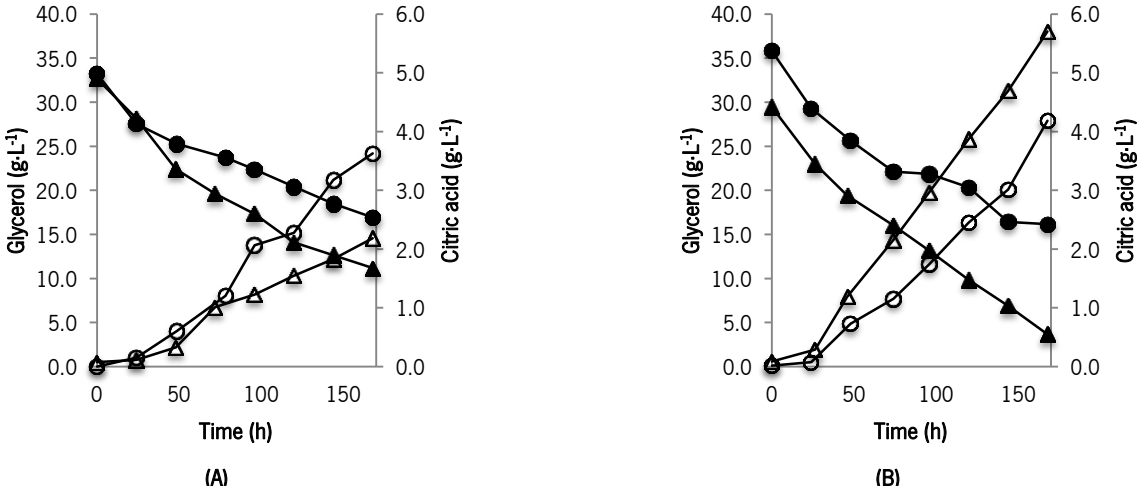


Figure 17 – Glycerol consumption (closed symbols) and citric acid concentration (open symbols) obtained in bioreactor (A) and baffled Erlenmeyer flasks (B), with yeast extract (▲, Δ) and without yeast extract (●,○) in the production medium, using cells entrapped in the spheres. Conditions: 3.3 % of sodium alginate and 0.5 M of calcium chloride, 3 mm of spheres diameter and 1.5 $g \cdot L^{-1}$ (5 $mg \cdot g^{-1}$) of cells and 40 $g \cdot L^{-1}$ crude glycerol. Standard errors with 95 % of confidence are lower than 17.8 % of the average.

Table 15 – Global results of *Y. lipolytica* batch cultures on glycerol medium, using bioreactor and baffled Erlenmeyer flask, with and without yeast extract. Q_{Glyc} - glycerol consumption rate; $Y_{CA/Glyc}$ - citric acid yield per glycerol; P_{CA} – maximum volumetric productivity of citric acid

	Bioreactor		Baffled Erlenmeyer flask	
	With yeast extract	Without yeast extract	With yeast extract	Without yeast extract
Q_{Glyc} ($g \cdot L^{-1} \cdot h^{-1}$)	0.128	0.097	0.153	0.117
$Y_{CA/Glyc}$ ($g \cdot g^{-1}$)	0.098	0.222	0.218	0.212
P_{CA} ($g \cdot L^{-1} \cdot h^{-1}$)	0.013	0.022	0.033	0.025

The new strategy adopted improved the maximum citric acid concentration in bioreactor (3.6 $g \cdot L^{-1}$) and a residual biomass concentration was detected in the medium (0.1 $g \cdot L^{-1}$ of cells). The other parameters were also improved (Figure 17 and Table 15). Nevertheless, these values kept similar when experiments in baffled Erlenmeyer flasks and bioreactor were performed. Therefore, citric acid production in bioreactor needs to be optimized in order to improve the productivity and yield of bioprocess.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In this chapter the main conclusions of this work are referred, with proposal of some perspectives to apply in future work.

The central aim of this work was to select and optimize an immobilization system for *Y. lipolytica* W29, to apply on citric acid production from glycerol. Thus, calcium alginate gel matrix and DupUM® were tested as a support for cell immobilization by entrapment and adsorption, respectively.

A strong gel matrix with 3.3 % (w/v) of sodium alginate and 0.5 M of calcium chloride contributed for a most efficient immobilization, without compromising citric acid production. Moreover, the use of a range of 3 ± 1 mm of spheres diameter did not significantly affect the behavior of the biocatalyst.

The matrix selected for cell immobilization did not interfere with cells viability, since the glucose consumption rate along 6 hours was similar for free and immobilized cells.

For experiments with free suspended cells the highest concentration of citric acid was obtained with 1.5 $g \cdot L^{-1}$ of initial cells concentration, occurring citric acid consumption when 3 $g \cdot L^{-1}$ and 4.5 $g \cdot L^{-1}$ of cell were used. However, the increase of ratio between dry cell mass per mass of wet support, thus increasing cellular concentration in the bioprocess with immobilized cells did not significantly influence citric acid production profile and but the use of no citric acid consumption was detected. Thus, the lower citric acid concentration, citric acid yield per glycerol and citric acid volumetric productivity obtained with free suspended cells show the advantages of immobilized cells by entrapment into calcium alginate gel matrix.

The increase of glycerol concentration did not repress citric acid production, being achieved the higher concentration of this metabolite with 60 $g \cdot L^{-1}$ of glycerol. Additionally, crude glycerol showed to be suitable for citric acid production with immobilized cells into calcium alginate gel matrix, since maximum citric acid concentration and maximum volumetric productivity were similar to results obtained with pure glycerol, and a higher citric acid yield was reached with crude glycerol than with pure substrate.

pH adjustment was very important for the improvement of citric acid production with immobilized cells, since it was in this condition that the maximum of citric acid concentration was found.

The stability of *Y. lipolytica* W29 entrapped into calcium alginate spheres was not affected when two consecutive fermentation cycles were performed, being obtained similar maximums volumetric productivity of citric acid in both cycles.

The adsorption of cells into DupUM® revealed to be an inefficient method for citric acid production, due to the high quantity of cells detached.

Global results of *Y. lipolytica* batch cultures on glycerol medium revealed similar values when baffled Erlenmeyer flacks and bioreactor experiments were performed. Therefore, further optimization process needs to be implemented in order to improve citric acid production, productivity and yield.

According to the results obtained in this work, the suggestions as the future prospects include:

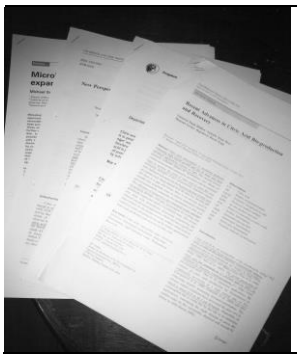
- Development of new methods to evaluate cell viability inside of calcium alginate gel matrix, including the use of trypan blue (colorimetric method) (Kong *et al.*, 2003). Trypan blue when added to the cells can pass the disrupted membrane, being maintained around the cells with intact membranes. Calcium alginate spheres with cells will be disrupted using sodium citrate solution (1 % (w/v)), leaving the cells available. The viability of cells will be quantified as the ratio of cells excluding trypan blue to the total number of cells.

- Development of new strategies to minimize the biomass leakage to outside of spheres, using additives (glutaraldehyde and polyethylenimine) for cross-linking treatment of gel spheres (Kawaguti *et al.*, 2006) .

- Implementation of citric acid production in fluidized bed reactor, like airlift and bubble columns, using entrapped cells into calcium alginate gel matrix. In fluidized bed reactors, intensive mixing of gas, liquid and solids occurs by recirculating the liquid culture medium, resulting in less abrasion of the gel matrix compared with stirred (Verbelen *et al.*, 2006).

- Operation in continuous mode for citric acid production, in order to promote the continuous feed and continuous leave of citric acid, without loss of cells and reducing the toxicity of products, improving bioprocess yields.

- The use of improved strains for citric acid production.



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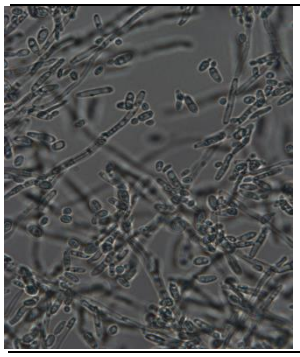

ATTACHMENTS

In this chapter covers the calibration curves constructed to support experimental work.

Biomass quantification was assessed using the calibration curve built for *Y. lipolytica* W29 (Table A 1).

Table A 1 - Expression of biomass calibration. OD_{600} - optical density at 600 nm; *m*: slope of the calibration curve; S_m - standard error associated with the slope; *CDW* - Biomass (dry mass) concentration (g·L^{-1}); *b* - ordinate at the origin; S_b - Standard error associated with the ordinate at the origin

Range of validity (g·L^{-1})	Equation $OD_{600} = (m \pm S_m) \times CDW + (b \pm S_b)$	
	$m \pm S_m$	$b \pm S_b$
0.11 – 0.81	0.84 ± 0.07	0.02 ± 0.03

Glucose quantification was assessed using the calibration curve built (Table A 2) using the DNS method (Miller, 1959).

Table A 2 - Expression of glucose calibration. OD_{540} - optical density at 540 nm; *m* - slope of the calibration curve; S_m - standard error associated with the slope; *Glu* - glucose concentration (g·L^{-1}); *b* - ordinate at the origin; S_b - Standard error associated with the ordinate at the origin

Range of validity (g·L^{-1})	Equation $OD_{540} = (m \pm S_m) \times Glu + (b \pm S_b)$	
	$m \pm S_m$	$b \pm S_b$
0.06 – 6	0.42 ± 0.02	0 ± 0.04

Glycerol and citric acid quantification was assessed using the calibration curve built (Table A 3), using HPLC.

Table A 3 - Expression of glycerol and citric acid calibration. *A* - peaks area detected by HPLC; *m*: slope of the calibration curve ; S_m: standard error associated with the slope; *[ ]* – concentration of compound (g·L^{-1}); *b*: ordinate at the origin; S_b - Standard error associated with the ordinate at the origin

Compounds	Range of validity (g·L^{-1})	Equation $A = (m \pm S_m) \times [\,] + (b \pm S_b)$	
		$m \pm S_m$	$b \pm S_b$
Glycerol	0.5 - 10	531557 ± 19629	- 36351 ± 110093
Citric acid	0.025 – 2.5	28 ± 2	1 ± 2