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REVIEW

Biotechnological production of γ-decalactone, a peach like aroma, by *Yarrowia lipolytica*

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Abstract The request for new flavourings increases every year. Consumer perception that everything natural is better is causing an increase demand for natural aroma additives. Biotechnology has become a way to get natural products. γ-Decalactone is a peach-like aroma widely used in dairy products, beverages and others food industries. In more recent years, more and more studies and industrial processes were endorsed to cost-effect this compound production. One of the best-known methods to produce γ-decalactone is from ricinoleic acid catalyzed by *Yarrowia lipolytica*, a generally regarded as safe status yeast. As yet, several factors affecting γ-decalactone production remain to be fully understood and optimized. In this review, we focus on the aromatic compound γ-decalactone and its production by *Y. lipolytica*. The metabolic pathway of lactone production and degradation are addressed. Critical analysis of novel strategies of bioprocess engineering, metabolic and genetic engineering and other strategies for the enhancement of the aroma productivity are presented.

Keywords Bioreactor · β-Oxidation · γ-Decalactone · Immobilization · *Yarrowia lipolytica* · Genetic manipulation

Introduction

The consumer demand for tasty foods has been growing, leading to an increasing need of aroma additions to replenish or add flavour to products. As a result, the production of these aromatic compounds, to be used by industrial companies such as food and beverages, cosmetics, chemical, pharmaceutical among others, has grown exponentially (Marasco and Schmidt-Dannert 2003).

The use of biocatalysts in the production of flavouring compounds similar with those present in natural sources is preferred, among others, due to regulatory reasons. In the U.S and according to European regulations (e.g. CFR 1990 and EEC 1334/2008), compounds isolated from natural resources or obtained by microbial or enzymatic processes involving precursors isolated from nature are classified as "natural". Consumer preferences reflecting the trends towards "health lifestyle" decide that the vast majority of flavour additives to be used in the food industry are compounds classified as "natural". Biotechnological production is an interesting approach for flavour production and has attracted a great deal of research interest (Longo and Sanromán 2006). Lactones are molecules comprising a carbon cycle with one oxygen atom, resulting in a hydroxy acid cyclisation. These compounds are very attractive for the food industry since they have a very characteristic "fruity" aroma and are naturally found in a wide variety of foods (fruits, milk and dairy products, meats and some fermented foods) (Marasco and Schmidt-Dannert 2003). For a long time they were obtained directly from fruits or by chemical synthesis, but over the past few years, the use of microorganisms and enzymes for the production of natural flavour compounds has been extended (Endrizzi-Joran et al. 1993). The market value of the so-called "biotechnological aroma" compounds is usually lower than those extracted from nature and commonly far above their synthetic counterparts (synthetic = US$ 150 kg^{-1}; natural = US$ 6000 kg^{-1}; "biotech" = US$ 300 kg^{-1}) (Dubal et al. 2008).

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This mini-review aims to provide a background on the new insights on the biotechnological production of γ-decalactone covering some different strategies for increasing aroma production.

The aromatic compound γ-decalactone

The most important lactone for flavour application is γ-decalactone, with a mundial market volume of several hundred tons per year and it has an oily-peach aroma, an extraordinarily tenacious odour and a very powerful, creamy-fruity, peach-like taste in concentrations below 5 mg L^{-1} (Schrader et al. 2004; Waché et al. 2003).

The interest of using yeast biotechnology for the production of lactones arose in the 60 s, after the results obtained by Okui et al. (1963) when studying the catabolism of hydroxylated fatty acids in several organisms. After that, numerous studies have been made on γ-decalactone production by yeast, often focused on the screening of yeast strains and medium optimization (Endrizzi-Joran et al. 1996). Most of the industrial processes use ricinoleic acid, the main fatty acid (about 90 %) of castor oil, or esters thereof, for its biotechnological production. This aroma can be obtained from the biotransformation of ricinoleic acid, catalysed by enzymes present in microorganisms with GRAS status, conferring this way a natural label to the compound.

γ-Decalactone ($C_{10}H_{18}O_2$) is a cyclic ester which results from the condensation of the alcohol group –OH and a carboxylic acid group –COOH of the same molecule. It is characterized by a closed ring consisting of four carbon atoms and a single endocyclic oxygen atom, coupled with an adjacent ketone (Aguedo 2002).

There are several microorganisms selected for their potentialities to produce aroma, in which the most important are *Pseudomonas*, *Sporobolomyces*, *Pichia*, *Candida* and *Rhodotorula*, being *Y. lipolytica* species the one with a higher productivity.

γ-Decalactone production through peroxisomal β-oxidation

β-oxidation pathway is the classical biochemical route involved in fatty acids degradation. It acts on an acyl-CoA molecule and consists in a four-step reaction sequence, yielding an acyl-CoA, which has two carbons less and an acetyl-CoA. This sequence is repeated several times until the complete breakdown of the compound (Fig. 1).

Lactonisation can occur at the whole C_{10} stage resulting in other decalactones of variable interest, dec-3-enolide, exhibiting very powerful fruity notes, and dec-2-enolide, characterized with mushroom notes (Gatfield et al. 1993). These lactones are probably related to a deficient 3-hydroxyacyl-CoA dehydrogenase activity. This later activity reduces NAD^+ to NADH which is regenerated through a shuttle mechanism (Hettema and Tabak 2000), that probably depends on the mitochondrial respiration. The accumulation of these lactones is observed in anoxic environments.

The enzymes involved in this pathway usually work in several β-oxidation cycles and with different chain lengths metabolites. Depending on many factors, the breakdown can be stopped before the theoretical end, liberating medium- or short-chain volatile compounds. These metabolites can exit the pathway at each two β-oxidation cycles or inside the sequence, leading to a variety of volatile compounds. The commonly accepted pathway from ricinoleic acid to γ-decalactone is presented in Fig. 1: four β-oxidation cycles occur, yielding 4-hidroxy-decanoyl-CoA, which is then cyclised to γ-decalactone. The yeast *Y. lipolytica* possesses a family of six acyl-CoA oxidases (Aox1 to 6 encoded by *POX1* to *POX6*) (Fig. 2). The first enzyme of the pathway is generally considered as the limiting step in the catalysis (Fig. 2b) (Groguenin et al. 2004) and the role comprising the other acyl-CoA oxidase were enlightened with the mutations in the *POX* genes. The disruption of *POX1* resulted in an increased β-oxidation activity but a decrease on the production of γ-decalactone (Pagot et al. 1998). Two Aox exhibited a high activity and a chain-length specificity, one being long-chain-specific (Aox2) and the other short-chain-specific (Aox3). The role of the other Aox was less evident. Aox4, Aox5 and Aox6 exhibited a weak activity in the whole spectrum of straight-chain acyl-CoA (from C_4 to C_{18}) and Aox1 did not exhibit any detectable activity. The disruption of the genes corresponding to these three Aox resulted in a two to fivefold increase in the global Aox activity, suggesting a role in the regulation of their activity. Also, in some *POX* mutants β-oxidation of C_{10} or smaller acyl-CoA is responsible for a decrease in the yield as a consequence of the γ-decalactone degradation (Waché et al. 2002; Groguenin et al. 2004).

Understanding the specific roles of each acyl-CoA oxidase has been the basis for the "construction" of strains growing at a good rate and producing γ-decalactone without degradation (Groguenin et al. 2004).

γ-Decalactone degradation pathway

In the biotransformation of γ-decalactone from ricinoleic acid, a maximum concentration can be reached after which starts to gradually decrease probably due to degradation and/or re-consumption of the compound. Therefore, the γ-decalactone concentration in the medium results from the

Fig. 1 The pathway from ricinoleic acid to γ-decalactone and enzymes involved in the yeast peroxisomal β-oxidation. Adapted from Waché et al. (1998) and Blin-Perrin et al. (2000)
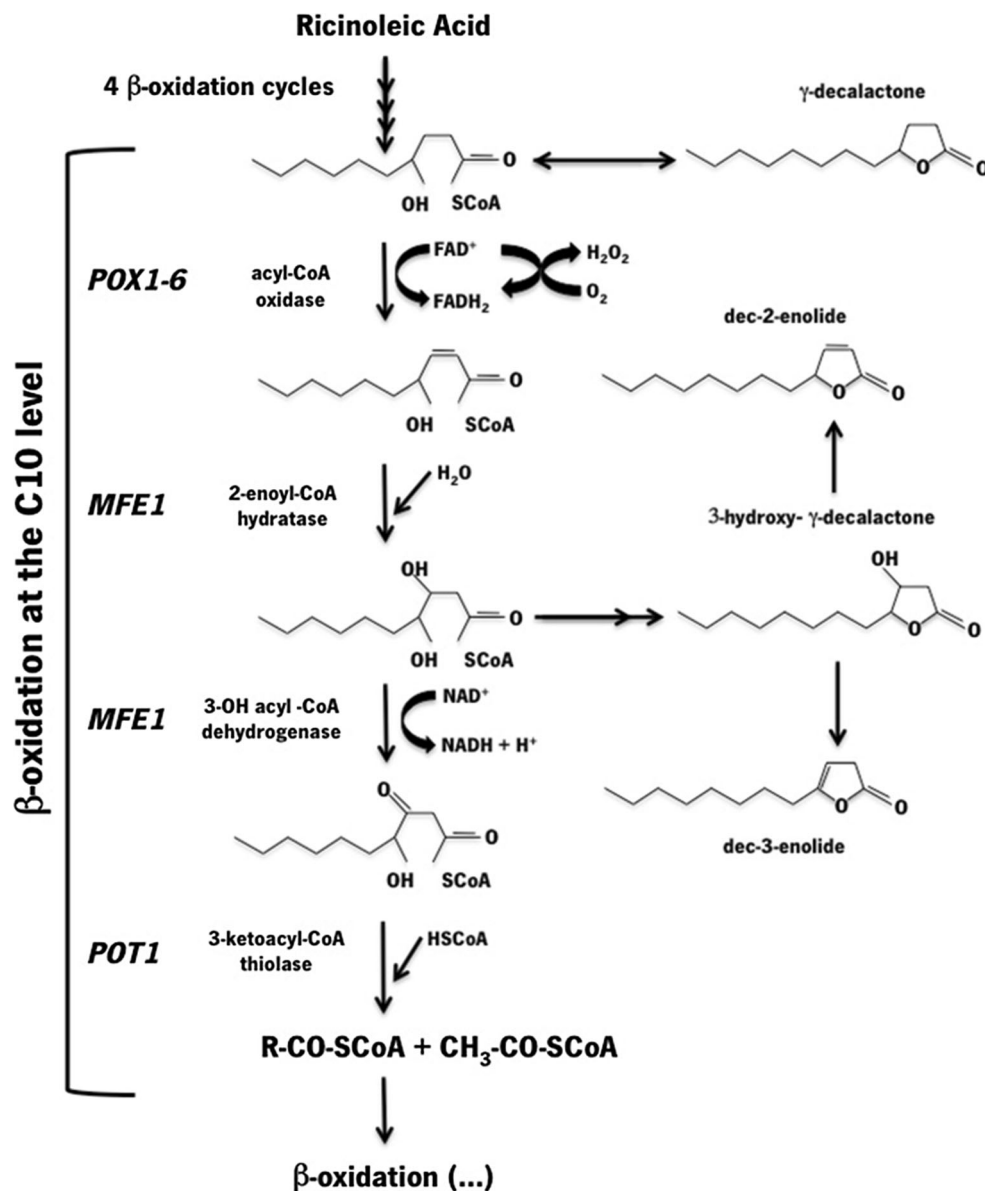
difference between what is produced and what is degraded (Endrizzi-Joran 1994). This decrease in the aroma compound concentration may be extremely prejudicial to the productivity of the process (Aguedo et al. 2005b).

Although the metabolic pathways of lactone degradation are not fully elucidated, there are some aspects related to this degradation that can be mentioned. First, the hydroxy acid form (unlactonised form) appears to be degraded faster than the lactone form (Endrizzi-Joran and Belin 1995), suggesting that the step of hydrolysis of the lactone exhibits a high control on the consumption. Then, the degradation pathway is very specific to the lactone (Fuganti et al. 1993; Latrasse et al. 1993; Fantin et al. 2001), since no degradation has been observed in the same conditions for lactones with similar structures, such

as 3-hydroxy-γ-decalactone or decen-4-olides (Waché et al. 2001).

Several pathways of degradation are possible. The most probable one includes the opening of the cyclic form through a blood γ-lactonase activity (Fishbein and Bessman 1966), followed by the activation of the CoA esters and β-oxidation. When the hydroxy group is in the α-position, a α-decarboxylation is required prior to the β-oxidation (Voet and Voet 1990). Another possible pathway involves the ω-oxidation of the lactone to yield, after delactonisation, a ω-dicarboxylic acid. The production of such diacids by cells with the inability to perform β-oxidation reactions has already been described (Picataggio et al. 1992; Fabritius et al. 1998). Nevertheless, this behaviour is not restricted to cells lacking the enzymes to
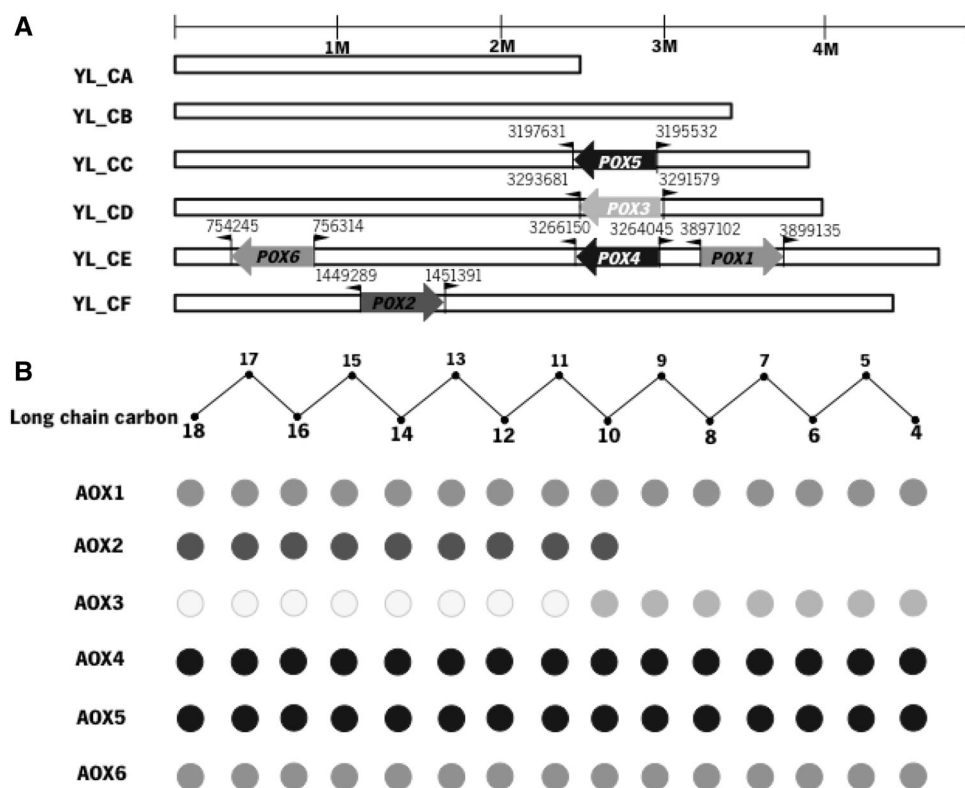
Fig. 2 Schematic representation of *POX* genes in *Y. lipolytica* genome and respective activity for the AOX enzymes. **a** Genes positioned in the different chromosomes (genes are not scaled). Code is as in **b**. The 1 M represents 1 million base pair. **b** Selective activity of AOX enzymes towards fatty acids of different chain length. The number of carbons of the substrate molecule is indicated in the *top*. The *circles below* indicate the qualitative activity for each AOX on the ricionolei acid. *Circles* are filled with a code and indicate the activity level of the AOX towards the elongation of fatty acids: ◑—short chain fatty acids (the low intensity represent low activity for long chain fatty acids); ◯—long chain fatty acids; ●—whole spectrum of straight-chain; ◕—not exhibit any detectable activity. Adapted from Groguenin et al. (2004) and Waché et al. 2001, 2002)

perform β-oxidation reactions, although in cells exhibiting an intact β-oxidation the substrate used are the dicarboxylic acids to envisage its degradation. The involvement of β-oxidation in the degradation is highly suggested by results obtained with acyl-CoA oxidase-modified mutants, in which the mutant lacking the enzyme of the β-oxidation pathway is the only one unable to degrade γ-decalactone (Waché et al. 2001).

New insights into γ-decalactone production

Metabolic engineering of β-oxidation

Developments for the lactone production processes have been made with the wild-type strain resulting in γ-decalactone concentrations of 12 g L^{-1} (Rabenhorst and Gatfield 2000). Nevertheless, rapid lactone degradation is observed due to high activity level of the acyl-CoA oxidase in *Y. lipolytica*. Also, only a portion of the ricinoleic acid is oxidized to the C_{10} level, and the C_{10} product serves as the precursor for other lactones (Farbood et al. 1989; Gatfield et al. 1993).

As an attempt to increase γ-decalactone concentration, serial knockouts for each acyl-CoA enzymes (Aox1-5) were made (Fig. 2) (Wang et al. 1999; Luo et al. 2000, 2002). Waché et al. 2001, 2002) studied the involvement of these enzymes in the biotransformation of γ-decalactone by *Y. lipolytica* and built strains that were disrupted in one or several acyl-CoA genes. They observed that the strain disrupted for *POX2*, *POX3* and *POX5* (which still possess *P0X4,* encoding a weakly active Aox) and with *POX2* gene reincorporated in a plasmid, produced more lactone, which is not consumed. Also, Groguenin et al. (2004) constructed a mutant strain (Δ*pox2–pox5,* pPOX2–*POX2*) that produced about 4× more γ-decalactone than the WT (400 vs. 100 mg L^{-1}) and was unable to degrade this aroma. Guo et al. (2011) reconstructed a mutant strain with *POX2* gene overexpressed and a knockout in the *POX3* gene, and observed that γ-decalactone production increased as a result of these two alterations and no aroma reconsumption was observed.

Waché et al. (2001) observed that the accumulation of 3-hydroxy-γ-decalactone in the wild type strain was related with the high Aox efficiency. The combined disruption of the Aox-encoding genes (like Δ*pox2pox3*) allows the reflux towards the production of γ-decalactone and the accumulation of hydroxy-lactone is no longer observed. Recently, Braga et al. (2015b) also study the effect of POX genotype on γ-decalactone production and reconsumption, and observed that the decrease of aroma was prevented and the production of hydroxylactone was minimized with MTLY40-2P strain (disrupted in the genes *POX2-5* and overexpressing *Aox2p*). Different rounds of UV irradiation and genome shuffling were also used to modified *Yarrowia* strains, which were able to produce a 6.5-fold higher γ-decalactone concentration than the wild type (Zhao et al. 2014). Efforts to decipher the complete synthetic pathway along with novel metabolic engineering approaches may improve lactone production by yeast in the next few years. *In silico* genome-scale analysis will be an important tool to understand γ-decalactone production. In recent years, three genome-scale metabolic models of *Y. lipolytica* have been developed by Loira et al. (2012), Pan and Hua (2012) and Kavscek et al. (2015), although none of them has yet been used in metabolic engineering approaches.

Bioprocess engineering developments

Besides the advances in the construction of modified strains, which allowed the production of higher γ-decalactone concentrations in the culture medium, the biotransformation of ricinoleic acid with *Y. lipolytica* has gained special attention from researchers in many different aspects.

Role of lipase

Different sources of ricinoleic acid such as methyl ricinoleate or castor oil have been used as substrates for γ-decalactone production (Alchihab et al. 2010; Braga et al. 2012, 2013b; Gomes et al. 2010; Moradia et al. 2013; Page and Eilerman 1996). When esters or castor oil are used as substrate, the rate of release of free ricinoleic acid also plays an important role in lactone formation rate. Braga et al. (2012, 2013b) pointed out the importance of extracellular lipases, namely the endogenous lipase of *Y. lipolytica* W29 and extracellular lipases (Lipozyme TL IM), for the fast release of ricinoleic acid from castor oil and consequently faster formation of γ-decalactone. Nevertheless, in an industrial point of view, this process is not the most adequate since it is cost- and time-consuming. Thus, overexpressing Lip2 enzyme would bridge the gap of this problem by improving γ-decalactone production rate with no extra costs. This was hereafter explored by Braga

et al. (2015b) that studied γ-decalactone production by the *Y. lipolytica* JMY3010 that has an additional copy of *LIP2* gene coding for the main extracellular lipase. Their results shown that the over-expression of Lip2p gene increased the γ-decalactone production rate.

Product and substrate toxicity

Beside the genetic engineering approaches the highest drawback may arise from yeast sensibility and toxicity towards elevated concentrations of lactone, which can be a limiting factor in the industrial implementation of its production.

The immobilization of microbial cells has been shown to provide some protection to the cells against physico-chemical changes, inhibitory substances, as well as enhanced substrate utilization, faster fermentation rates, prolonged cells activity and stability (Nedović et al. 2010). Fang and Zhang (2008) immobilized *Y. lipolytica* cells in a mixture of PVA and carrageenin for γ-decalactone production and increased its production in 40 %. A mixture of sodium alginate and attapulgite was also used by Zhao et al. (2012) resulting in a 2.5-fold increase in γ-decalactone production by *Y. lipolytica*. Braga and Belo (Braga and Belo 2013) compared different materials for *Y. lipolytica* immobilization by adsorption that could be used in the production of γ-decalactone from castor oil. According to their observations, the highest aroma concentration was obtained with immobilized cells in DupUM® (a thermoplastic support). More recently, Zhao et al. (2015) improved γ-decalactone production from 3.75 to 8 g L^{-1} through cell immobilization in attapulgite along with the use of ionic liquid as a co-solvent.

Oxygen role

A further factor influencing the formation of γ-decalactone in *Y. lipolytica* is the concentration of dissolved oxygen and the oxidative state of the medium, due to the oxygen role on the β-oxidation pathway (Aguedo et al. 2005a; Fickers et al. 2005; Kamzolova et al. 2003). Thus, the oxygen transfer rate (OTR) from the gas to the liquid is a key factor in the biotransformation process optimization. Aguedo et al. (2005a) studied the β-oxidation pathway on *Y. lipolytica* W29 on methyl ricinoleate medium under higher air pressure (5 bar), i.e., under increased O_2 solubility and observed that although cells grew normally, γ-decalactone production decreased in these conditions. Increased O_2 favoured the accumulation of decenolides compounds of the metabolic pathway. Thus, it was suggested that the control of the pathway by dehydrogenase seems to prevail when O_2 exceeds a threshold. In fact, the effect of oxygen on β-oxidation fluxes is quite complex and

different behaviour has been reported according to oxygen level. Escamilla-García et al. (2007) reported that under very low aeration conditions a high accumulation of γ-decalactone was observed, due to the inhibition of acyl-CoA oxidase. Under slightly higher aeration conditions (Escamilla-Garcia et al. 2009), but the aeration level still low, the impact would be on 3-hydroxyacyl-CoA dehydrogenase through the regeneration of NAD (respiration-dependent) that results in 3-hydroxy-γ-decalactone production however does not affect acyl-CoA oxidase activity. By increasing aeration, β-oxidation is optimal and few intermediates were accumulated. Finally, very high aeration disturbs the β-oxidation fluxes resulting in higher accumulation of 3-hydroxy-β-decalactone (Aguedo et al. 2005a, b; Gomes et al. 2007). Further experiments from Braga and Belo (Braga and Belo 2015) also showed a higher γ-decalactone production at low oxygenation rates, but a lower time was needed to reach the maximum aroma concentration at higher OTR, resulting in higher productivities. Escamilla-Garcia et al. (2014) reported a tenfold increase of 3-hydroxy-γ-decalactone production at high aeration rates observed in an airlift bioreactor made of plastic. The formation of adhering biofilms of Y. lipolytica stimulates the production of 3-hydroxy-γ-decalactone that is an indirect effect of aeration conditions inside bioreactors.

Process engineering

Yarrowia lipolytica cells are hydrophilic with a good attraction to hydrophobic surfaces or molecules when previously immersed in water. During the biotransformation of castor oil by Y. lipolytica direct contact occurs between the surface of the cells and the small substrate droplets and it is possible to increase the contact by choosing a surfactant having an affinity for the yeast (cationic surfactant) (Aguedo et al. 2004). The role of cells hydrophobicity in γ-decalactone production was also investigated by Gomes et al. (2010). They observed that the use of more hydrophobic cells in γ-decalactone production increased the affinity and consequently the uptake of the substrate by the cells, improving the overall process productivity. Escamilla-Garcia et al. (2014) also suggested that cells hydrophobicity could be increased under high aeration rates.

Strategies to improve aroma production also addressed the culture conditions, namely the medium pH, that is optimal for γ-decalactone production by Y. lipolytica in a value around 6 (Garcia et al. 2007; Gomes et al. 2011). The effect of medium composition on γ-decalactone production has also been studied. Braga et al. (2015b) reported that increasing castor oil concentration from 30 to 60 g L^{-1} increased the aroma production for Y. lipolytica W29 and MTLY40-2P strains.

Bioreactors and mode of operation

Different type of bioreactors have been investigated for application on the biotransformation of methyl ricinoleate or castor oil for aroma production, been the classical STR the most used. Operational conditions on these systems may have different impacts on Y. lipolytica cultures characteristics and behaviour, with influence in lactones productivities.

The production of γ-decalactone from castor oil in batch cultures of Y. lipolytica W29 was compared in stirred tank and airlift bioreactors (Braga et al. 2015b) and a twofold increase in γ-decalactone concentration (around 3 g L^{-1}) was achieved in the airlift compared to STR. Quantitative image analysis techniques were used to investigate the possible morphological changes in both systems and showed that pneumatic agitation causes less impact in the cells morphology than mechanical agitation.

To avoid γ-decalactone degradation and minimize inhibitory effects of ricinoleic acid on the cells, Gomes et al. (2012) showed that fed-batch cultivation is an interesting alternative. In fed-batch using intermittent feed, they were able to obtain an aroma concentration of 6.7 g L^{-1}, compared to 1.9 g L^{-1} in batch fermentation and the production of the side product 3-hydroxy-γ-decalactone increased simultaneously to 10 g L^{-1}. Nevertheless, in this system, the maintenance of an emulsion causes numerous constraints to ensure that the supply of fresh medium and withdraw concerns an emulsion with the same characteristics. Thus, the substrate addition by pulses (step-wise feed-batch) is a way of circumventing this problem. Using this strategy, Gomes et al. (2012) obtained a γ-decalactone productivity of 0.043 g L^{-1} h^{-1} for a step-wise fed-batch operation applied to Y. lipolytica W29, when 30 g L^{-1} methyl ricinoleate was fed twice to the bioreactor. Braga et al. (2015b) also attempted a step-wise fed-batch strategy with MTLY40-2P strain, in which 60 g L^{-1} of castor oil was added in two pulses, leading to a twofold increase in γ-decalactone concentration (around 7 g L^{-1}) compared with a batch mode.

Conclusions

γ-Decalactone production by biotransformation of castor oil using microorganisms is an attractive means to produce aroma compounds. Although, the scientific community has dedicated time and efforts around the production of "natural" aromas, the overall productivity is still very low. Different approaches were already realized to overcome hurdles of the application of microbial strains for the synthesis of flavour compounds. Higher yields can be obtained by choosing the appropriate microorganisms and

the inhibitory effect of produced lactone can be overcome by fed-batch fermentation. Next to these strategies, the influence of the oxygen concentration has to be considered as well as the availability of the substrate. In all cases, for the bioprocess engineering a detailed understanding of the regulation of different pathways is of great advantage to achieve maximum yields and product concentrations. However, until now, the yields of the products are too low to make the biotechnological process workable and further studies are necessary to overcome the limitations found to date. Additionally, fermentation technologies, downstream processes and up-scaling from lab to industrial scales require more rigorous studies not only to control and to maximize yield but also to reduce competing undesired reactions.

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