UNIVERSIDAD AUTÓNOMA METROPOLITANA

Unidad Iztapalapa

ESTUDIO DE LA BIOCONVERSIÓN DE NOOTKATON CON BIORREACTORES TRIFÁSICOS UTILIZANDO CÉLULAS COMPLETAS

STUDY OF BIOCONVERSION NOOTKATONE WITH THREE PHASE PARTITIONING BIOREACTORS USING WHOLE CELLS

TESIS PARA OBTENER EL GRADO DE DOCTORA EN BIOTECNOLOGÍA

PRESENTA

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UNIVERSIDAD AUTÓNOMA METROPOLITANA

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DE NOOTKATON CON
BIORREACTORES TRIFASICOS
UTILIZANDO CELULAS COMPLETAS.

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DOCTORA EN BIOTECNOLOGIA

DE: DULCE MARIA PALMERÍN CARREÑO

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INDEX

1. GENERAL INTRODUCTION

(+)-Nootkatone is a sesquiterpenoid that has a pleasant grapefruit like aroma and is slightly bitter with a low odor threshold of $1\ \mu g\ L^{-1}$ water. Notwithstanding this property, (+)-nootkatone is applied in pharmacology such as anti-platelet aggregation effects and anti-proliferative activity towards cancer cell lines [1,2]. (+)-Nootkatone inhibits the activity of acetylcholinesterases (AChE), AChE inhibitors are used as medicines against Alzheimer's disease [3]; and also (+)-Nootkatone inhibits the activity of human cytochrome-P450-monooxygenase. The cytochrome enzyme eliminates unwanted components from the body by oxidation [4]. (+)-Nootkatone has a stimulating effect on certain physiological conditions such as AMP-activated protein kinase (AMPK) activation in skeletal muscle and liver [5]. Furthermore, (+)-nootkatone is also used as an insecticide. It is useful because it kills insects by blocking the insects' receptors of their nerve cells for a neurotransmitter called octopamine. The insects become hyperactive and vibrate themselves to death [6,7].

Nowadays, the world production of (+)-nootkatone is carried out in 32 industries. There are three reported methods for (+)-nootkatone production: Natural (from citrus fruits and plants), chemical, and biological (using whole-cells bioconversions). Figure 1 shows how the production methods are applied by industry. The leading companies that are currently producing this sesquiterpene with whole cells are Allylix Inc., Amyris, Evolva, Isobionics B.V., ACIB and Oxford Biotrans, respectively. In the natural method, (+)-nootkatone is obtained from different plants, but only in trace amounts [8], it takes around 400,000 kg of grapefruit to produce 1.0 kg of (+)-nootkatone (http://oxfordbiotrans.com/uk-research-provides-taste-of-future-foods/). Therefore (+)-nootkatone demand is nowadays fulfilled by chemical synthesis [9], i.e., through the use of tert-butyl chromate for the Copper(I) mediated oxidation by alkyl hydroperoxides [10]. However, the main problem with chemical synthesis is that reactions include various reaction steps that require environmentally hazardous reagents, catalysts and solvents. In this sense, the use of unwanted or even toxic chemicals is not favorable and, thus, (+)-nootkatone cannot legally be marketed as a "natural" flavor [1].
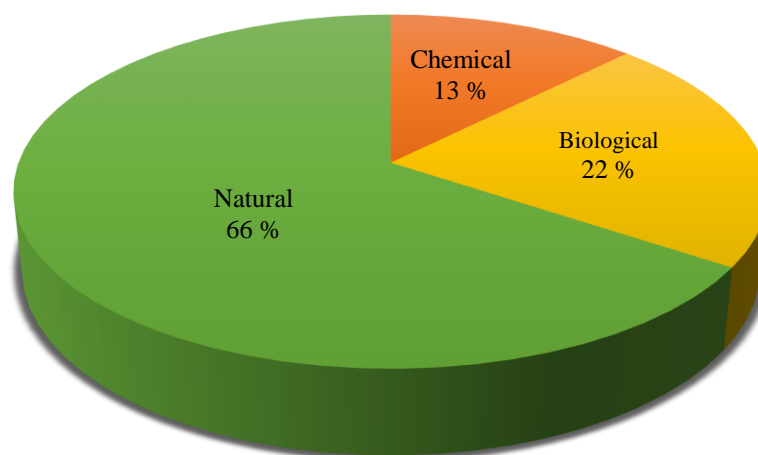
Figure 1. Distribution of industries by (+)-nootkatone production method
(http://www.radiantinsights.com/research/nootkatone-market-research-report-2015).

(+)-Valencene and (+)-Nootkatone are sesquiterpenes, natural terpenoids, that are commonly found in plant essential oils [11]. Several biotechnological processes have already been designed to achieve the oxidation of (+)-valencene to (+)-nootkatone on different microorganisms. Examples of these processes are the production of (+)-nootkatone with: green algae like *Chlorella* or *Euglena*; fungi such as *Botryodiplodia theobromae* [12] or *Chaetomium globosum* [13]; recombinant bacteria like *E. coli* [4]; a nucleic acid encoding valencene synthase is added into a host cell (*Yarrowia lipolytica*) that now will be able to encode nucleic acid sequence for sinthesizing (+)-nootkatone [13]; and, also there are some optimization methods with *Y. lipolytica* to produce or enhance the production of proteins or (+)-nootkatone metabolites [14]. Interest of industry and academia on (+)-nootkatone production during the last decade has, increased as; shown in Figure 2 through published articles and patents (SCOPUS, May 2015).

The main disadvantage of the use of recombinant microorganism is due to recombinant DNA technologies are very sophisticated and the recombinant strain conservation is stricter than the parental one. On the other hand, the use of plant cells, such as cells of *Citrus* species, for

the bioconversion present some drawbacks, since plant cells have less resistance against shear stress during mixing and temporary extremes of pH and temperature in comparison to natural microorganisms [13]. Thus, the use of a natural microorganism for bioconversion purposes therefore seems to be an interesting alternative.
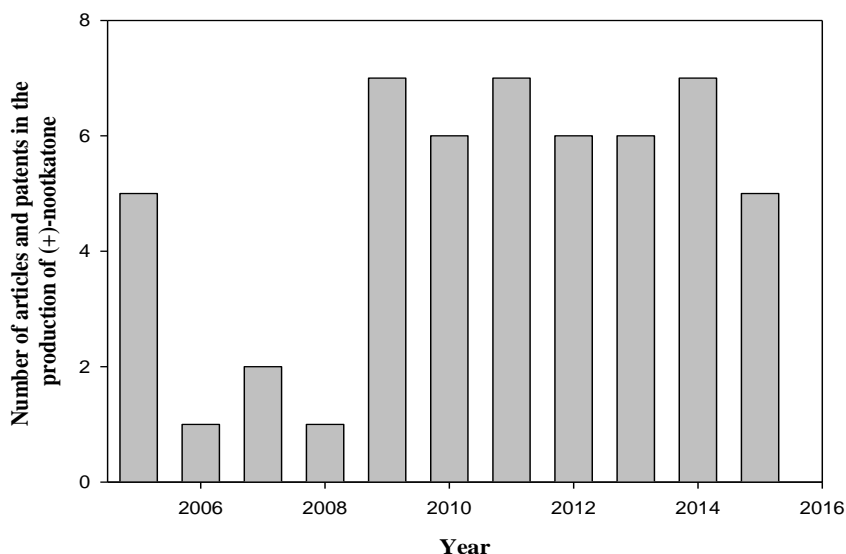
Figure 2. Number of published articles and patents for (+)-nootkatone production during the last decade (Scopus, May 2015).

The bioconversion of (+)-valencene to (+)-nootkatone is mainly due to the catalytic activity of Cytochrome P450 (CYP450), which is a superfamily of membrane-bound, haem-containing monooxygenases responsible for the oxidation and reduction of a lot of drugs, steroid hormones and fatty acids. The CYP450's are expressed in many animal tissues and are found in relatively high quantities in the liver. The main function of CYP450 is to introduce an oxygen atom into the substrate i.e. to increase the hydrophilicity of the product, thereby the enzyme eases the elimination of the product from the cell [4]. Sowden et al [15] reported a bioconversion for (+)-nootkatone production using wild type and mutants P450cam from *Pseudomonas putida* and P450BM-3 from *Bacillus megaterium.* They noted that the mutant type P450cam oxidizes (+)-valencene, while the wild type does not. They also reported that wild type P450BM-3 and mutants have higher activities than P450cam but are less selective.

Based on a literature review, two bioconversion reaction schemes from (+)-valencene to (+)-nootkatone have been described (see Figure 3): a) one of them, where the enzyme Cytochrome-P450-Monooxygenase performs this reaction [16,17], or b) another where (+)-nootkatone is produced from an intermediate, (+)-nootkatol, where this first product is formed by a second enzyme called dioxygenase [18]. In the literature, it is also mentioned that the bioconversion reaction presents inhibition by substrate, product, oxygen and intermediary [13,2,16]. Based on these findings, the mechanism proposed in this work is drawn in Figure 3c.
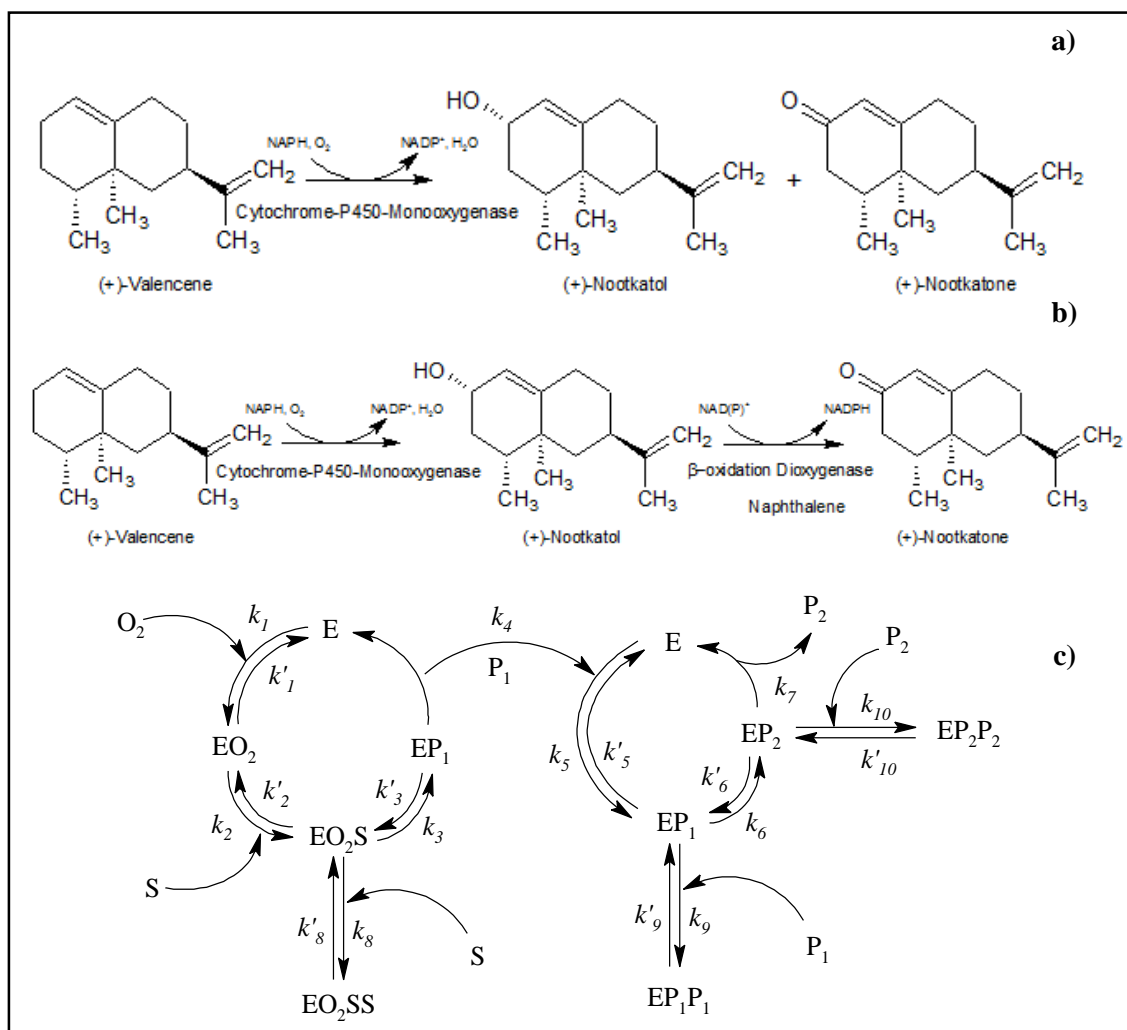
Figure 3. Possible enzyme bioconversion mechanisms performed with (a) one and (b) two enzymes. (c) Reaction mechanism proposed for (+)-nootkatone bioconversion under inhibition conditions. E enzyme; S substrate; O_2 oxygen; Products P_1 and P_2; and intermediaries EO_2, EO_2S, EO_2SS, EP_1, EP_1P_1, EP_2, EP_2P_2.

The kinetics related to the bioconversion of (+)-valencene to (+)-nootkatone using various microorganisms have already been studied by several researchers. Kaspera et al [13] studied the kinetics using the ascomycete *Chaetomium globosum*. They observed that the fungal cells maintain viability in the presence of high (+)-valencene concentrations, however, there is a fast decrement in (+)-valencene concentration at early reaction times. Besides, it was reported by Palmerín-Carreño et al. [19] that *Yarrowia lipolytica* is capable of carrying out the mentioned bioconversion. In fact strain *Y. lipolytica* 2.2ab is used through this study. Palmerín-Carreño et al [19], also investigated the effects of substrate- and product inhibition on cell growth using *Y. lipolytica* 2.2ab. Fu et al [20] described the thermal deactivation of *Y. lipolytica*. They elucidated how this microorganism loses its functionality over 40°C. Muniglia et al. [21] developed a macroscopic kinetic model for the microorganism *Y. lipolytica*. They were interested on the growth of the microorganism on glucose, obtaining a maximum specific growth rate of 0.25 h^{-1}.

Pure enzymes or whole cells are used as biocatalysts [22]. Whole cells in comparision with the pure enzymes have more stability due to their cellular matrix, which allows the re-generation of cofactors and enzyme active sites, reducing production costs [23]. Besides, in whole cells biocatalysis the enzyme system of the cell is used to carry out reactions that are not part of the metabolism. In this way, enzymes are more stable due to the presence of its natural environment within the cell and the intracellular environment provides effective method for regeneration of the cofactors, i.e. mention these cofactors [24,25].

The inhibition of the above reaction by (+)-valencene and (+)-nootkatone has been reported with various microorganisms [9,26]. A number of solutions have been recommended to decrease the substrate/product inhibition; for instance: the proposal of adsorbent resins [27]; *in situ* supply of the substrate and removal of product, maintaining both substrate and product concentrations at sub-inhibitory levels in the environment of the biocatalyst [28]; and encapsulation of biocatalyst to prevent enzyme oxidation [29]. However, one of the main limitations of these strategies is the difficulty in controlling mass transfer of substrate, product and oxygen that directly impacts on the productivity and the

cost-benefit ratio. As background, this research group proposed the addition of a second liquid phase (essential orange oil) in a partitioning bioreactor, it can be a useful tool to reduce product inhibition due to the presence of a partition coefficient favorable to the second liquid phase [30].

This thesis is aimed to evaluate, enhance and predict the potential of (+)-nootkatone production from (+)-valencene on selected microorganisms in different three-phase (aqueous-organic-air) partitioning bioreactors using orange essential oil as a cheap source of (+)-valencene.

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http://www.scopus.com/

CHAPTER I

PROBLEM STATEMENT

HYPOTHESIS

The addition of orange essential oil as second liquid phase as a substrate reservoir and *in situ* product extracting agent in a three phase partitioning bioreactor (TPPB) overcome substrate and product inhibition increasing the bioconversion performance of (+)-valencene to (+)-nootkatone. This information would be useful to develop operating windows of a TPPB which in a further study, could be used to describe and understand this technology for a commercial exploitation at industrial scales.

JUSTIFICATION

Industry and academia are interested on (+)-nootkatone bioconversion using whole cells, which involves the selection of an optimal cellular enzyme, reaction engineering, product recovery and scaling-up. Different efforts have been focused to enhance whole cell bioconversion of (+)-valencene to (+)-nootkatone among them classical genetics approach or metabolic engineering using recombinant microorganism. However, information of bioreactor design and operating condition of (+)-nootkatone bioconversion is yet scarce. The use of TPPB with whole cells as biocatalyst allows an increment of the productivity in the bioprocess. Due to thermodynamic equilibrium, the dispersed phase maintains substrates, products or toxic secondary metabolites concentrations at sub-inhibitory levels. Since these systems present several mechanisms such as mass transfer, kinetics and deactivation of the biocatalyst, it is relevant to characterize them at different conditions with the aim to understand their effect on high value metabolite production and develop strategies of these bioreactors.

GENERAL AIM

This thesis is aimed to evaluate, enhance and predict the potential of (+)-nootkatone production from (+)-valencene on selected microorganisms in different three-phase (aqueous-organic-air) partitioning bioreactors using orange essential oil as a cheap source of (+)-valencene.

SPECIFIC AIMS

1. Select a method for microorganism screening with the potential for the allylic oxidation of (+)-valencene to (+)-nootkatone using different bioconversion conditions.

2. Evaluate the bioconversion of (+)-valencene to (+)-nootkatone by *Botryodiplodia theobromae* using a Membrane Aerated Biofilm Reactor (MABR).

3. Assess physiological characterization of whole cell biocatalysis using *Yarrowia lipolytica* for the bioconversion of (+)-valencene to (+)-nootkatone in a partitioning bioreactor.

4. Enhance the bioconversion of (+)-valencene to (+)-nootkatone with *Yarrowia lipolytica* in a partitioning bioreactor using orange essential oil as the dispersed phase.

5. Determine hydrodynamic stress on the viability of the yeast *Yarrowia lipolytica* and the effect of biomass concentration on (+)-nootkatone bioconversion in a partitioning bioreactor.

6. Assess whole cell bioconversion of (+)-nootkatone in an organic phase (orange essential oil).

7. Evaluate hydrodynamic and oxygen mass transfer characteristics of a three phase gas–water–orange essential oil dispersion and determine the limiting step of the bioconversion process through a regime analysis.

GENERAL STRATEGY

Figure 1.1 depicts the general strategy followed through this work: I) The first chapter contains the main aim, hypothesis, justification, and general and specific objectives of the project. II) The second chapter deals with the evaluation and selection of microorganisms with the potential of (+)-nootkatone bioconversion using different systems. III) In the third chapter *B. theobromae,* one of the selected microorganisms is evaluated in a membrane aerated biofilm reactor (MARB). IV) In the fourth chapter a physiological characterization of whole cell biocatalysis using *Yarrowia lipolytica* is assessed in a stirred tank partitioning bioreactor. V) In the fifth chapter, the strategies to increase bioconversion are tested in a three phase partitioning bioreactor using *Y. lipolytica*. VI) In the sixth chapter, the role of hydrodynamic stress on the viability of *Y. lipolytica* and the effect of the biomass concentration on bioconversion are evaluated during the bioconversion in the three phase partitioning bioreactor. VII) In the seventh chapter whole cell bioconversion of (+)-nootkatone is assessed in an organic phase (orange essential oil). VIII) Finally, in the eighth chapter substrate, product and oxygen mass transfer mechanisms in the TPPB are studied and a regime analysis is conducted to determine the limiting step of the bioconversion at the operating condition used.
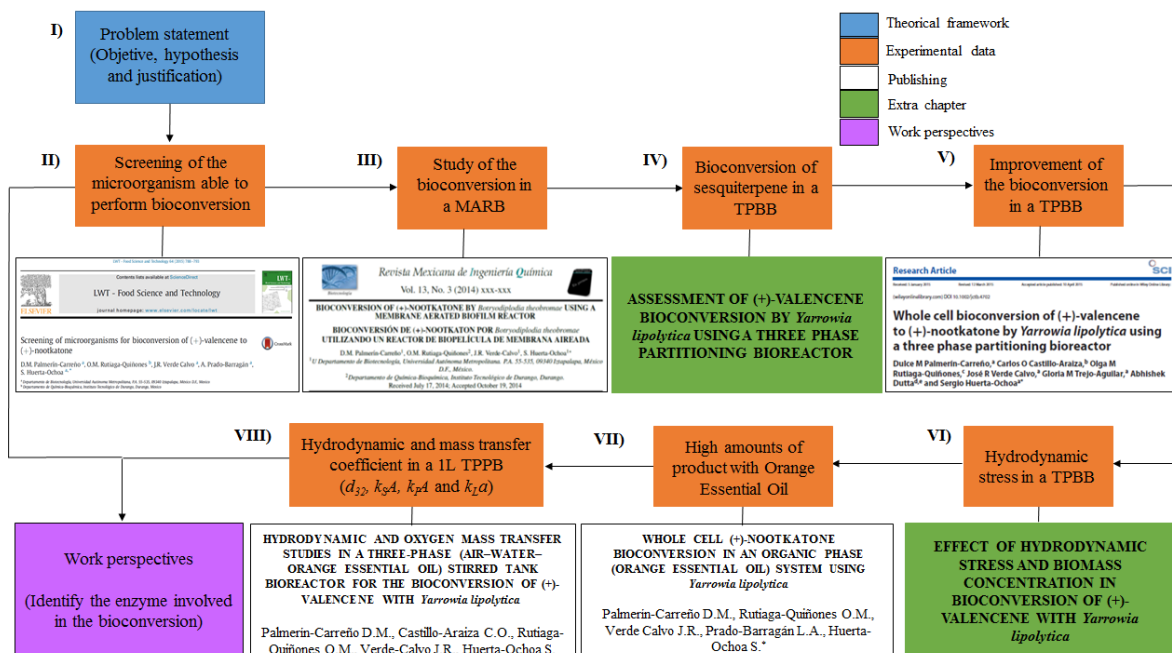
Figure 1.1. General strategy followed in this Thesis for the study of (+)-nootkatone bioconversion using whole cells in three phase partitioning bioreactors.

NOTE: Each chapter has its self-nomenclature, figures, tables and references.

CHAPTER II

SCREENING OF MICROORGANISMS FOR BIOCONVERSION OF (+)-VALENCENE TO (+)-NOOTKATONE

Palmerín-Carreño D.M., Rutiaga-Quiñones O.M., Verde Calvo J.R., Prado-Barragán A.,
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SCREENING OF MICROORGANISMS FOR BIOCONVERSION OF (+)-VALENCENE TO (+)-NOOTKATONE

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ABSTRACT

The production of (+)-nootkatone, highly appreciated by fragrance and flavour industries, can be performed by whole-cell bioconversion from the sesquiterpene (+)-valencene, a compound readily available in orange essential oil. The aim of this work was to screen for microorganisms that convert (+)-valencene to (+)-nootkatone using different bioconversion systems. The screening was conducted using six different microorganisms, and bioconversion experiments were set up on surface culture using serological flasks containing PDA at 30 °C. It was observed that *Botryodiplodia theobromae* 1368, *Yarrowia lipolytica* 2.2ab, and *Phanerochaete chrysosporium* oxidised (+)-valencene to (+)-nootkatone, reaching (+)-nootkatone concentrations of 231.7 ± 2.1, 216.9 ± 5.8 and 100.8 ± 2.6 mg L^{-1}, respectively. Different bioconversion conditions were also tested—aqueous, organic, and biphasic—all resulting in similar (+)-nootkatone production. Both *B. theobromae* 1368 and *Y. lipolytica* 2.2ab showed substrate inhibition above 4.2×10^{-2} and 0.13 g of (+)-valencene (g of biomass)$^{-1}$, respectively, in aqueous phase experiments. Furthermore, *B. theobromae* 1368 and *Y. lipolytica* 2.2ab showed product inhibition when concentrations reached above 17.02 and 34.78 mg of (+)-nootkatone (g of biomass)$^{-1}$, respectively. The experimental method presented here allowed for the identification of microorganisms with the potential for the bioconversion of (+)-valencene to (+)-nootkatone. This study will be useful for ongoing studies on the selection and operation of the proper bioreactor at different bioconversion conditions.

Keywords: Screening, surface culture, orange essential oil, bioconversion, (+)-nootkatone

Chemical compounds studied in this article

(+)-Valencene (PubChem CID: 9855795); (+)-Nootkatone (PubChem CID: 1268142); Ethyl acetate (PubChem CID: 8857); Glucose (PubChem CID: 53782692); Sucrose (PubChem CID: 5988); Tween 80 (PubChem CID: 5281955); Sodium chloride (PubChem CID: 5234); Potassium dihydrogen phosphate (PubChem CID: 516951); Magnesium sulphate (PubChem CID: 24083); Copper sulfate (PubChem CID: 24462)

2.1. Introduction

Flavours and aromas are highly valued in food, cosmetic, chemical, and pharmaceutical industries. Most of the aromatic compounds available are obtained by chemical synthesis or solvent extraction. However, consumers' growing aversion towards synthetic chemicals added to foods, cosmetics, and other household products has encouraged research efforts towards the production of aromatic and flavouring compounds using biological organisms (Maróstica & Pastore, 2007). The sesquiterpene molecules and their oxygenated derivatives are widely used in the aroma industry. Of particular interest is (+)-valencene ((2R)-8,8,8a-trimethyl-2-prop-1-en-2-yl-1,2,3,4,6,7-hexahydronaphthalene), the main bicyclic sesquiterpene found in orange peel oil (Elston et al., 2005). Sesquiterpenes are low-priced compounds commonly used as substrates for the bioconversion of various aromas (Misawa et al., 2011). The production of (+)-nootkatone has been conducted by chemical synthesis mainly from the sesquiterpene (+)-valencene, but also through the use of oxidising agents known to be unsafe to the environment, such as tert-butyl peracetate (Wilson & Shaw, 1978) or tert-butyl hydroperoxide in combination with metal catalysts supported on silica (Salvador & Clark, 2002). As such, the (+)-nootkatone molecules obtained through the mentioned methods may not be considered natural products, and therefore, do not satisfy the increasing demand for natural aromatic compounds. In order to meet this demand, many efforts are underway to find a biotechnological process using bacteria, fungi, or plants (Fraatz et al., 2009).

Microorganisms, plants, or animal cells can make these new biotechnological products in natural forms at a relatively low cost. Microorganisms are of particular interest because of the multitude of naturally-occurring strains and their great metabolic diversity for modifying and upgrading a variety of complex organic molecules; consequently, it is expected that one of the many native strains may be responsible for catalysing any specific reaction (Tan & Day, 1998). The use of diverse microorganisms as catalytic agents for the bioconversion of (+)-valencene to (+)-nootkatone (Fig. 2.1) has previously been studied (Kaspera et al., 2005). Fraatz et al. (2009) used *Pleurotus sapidus* to obtain 600 mg of (+)-nootkatone L^{-1} in a fed-batch system. Sowden et al. (2005) used *Pseudomonas putida* and *Bacillus megaterium* for the same bioconversion process. Girhard et al. (2009) worked with enzymes from *Bacillus*

subtilis inserted into *Escherichia coli* to convert 94.2 mg L^{-1} of the intermediate nootkatol and the product (+)-nootkatone. The yeast *Saccharomyces cerevisiae* (OGM) was also used as a catalyst for this bioconversion, producing a low concentration (10 mg L^{-1}) of (+)-nootkatone (Gavira et al., 2013). The aim of this work was to screen for microorganisms with the potential for the allylic oxidation of (+)-valencene using different bioconversion conditions.
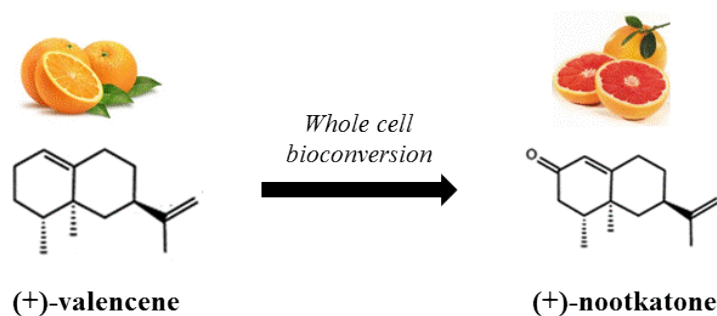
Fig. 2.1. Bioconversion of (+)-valencene to (+)-nootkatone using microbial whole cells

2.2. Materials and methods

2.2.1. Chemicals

Sesquiterpenes (+)-valencene (CAS 75-05-6) and (+)-nootkatone (CAS 93-78-5) with a purity of > 70% and > 85%, respectively, were purchased from Fluka (Switzerland). Ethyl acetate (99.5%) was purchased from Quimex (México). Orange essential oil (*Citrus aurantium*, var. Amara) was purchased from Cosmopolita Drugstore (Mexico DF) and used as the organic phase.

2.2.2. Microorganisms

The strains *Botryodiplodia theobromae* 1368, *Aspergillus tamari* V12307, *Phanerochaete chrysosporium*, *Yarrowia lipolytica* 2.2ab, *Kluyveromyces marxianus* NCYC 1429, and *Rhyzomucor* sp. that were used came from the culture collection of the Solid State Fermentation Plant at the Autonomous Metropolitan University Iztapalapa. *Y. lipolytica* 2.2ab strain was previously sequenced in the Laboratory of Industrial Biotechnology of the National Polytechnic Institute (CBG-IPN), Mexico. The access in the National Centre for Biotechnology Information (NCBI) is EF643594.1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed

spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence, identities 94 % with *Y. lipolytica* strain LN-13. The strains were propagated in Potato Dextrose Agar plates (PDA, Bioxon-Dickinson, México) incubated at 30 °C for 3−7 days.

For the determination of substrate and product inhibition, microorganisms were grown in 250 mL baffled Erlenmeyer flasks containing 100 mL of culture medium. The culture medium for *Y. lipolytica* 2.2ab was composed of (in g L^{-1}): glucose, 30; Na_2MoO_4, 0.2; $MnSO_4$, 0.4; $FeCl_3$, 0.2; KI, 0.1; $CuSO_4$, 0.04; H_3BO_3, 0.5; $CaCl_2$, 0.1; NaCl, 0.1; $MgSO_4*7H_2O$, 0.5; KH_2PO_4, 1.0; $ZnSO_4$, 0.4; $(NH_4)_2SO_4$, 5; yeast extract, 4.004. Prior to sterilisation, the pH of the culture medium was adjusted to 5.5 with 2 M HCl (Tsigie et al., 2012). The culture medium for *B. theobromae* 1368 was composed of (in g L^{-1}): sucrose, 50; $NaNO_3$, 7.5; KH_2PO_4, 2.0; KCl, 0.3; $MgSO_4*7H_2O$, 0.6; $FeSO_4*7H_2O$, 0.6; $ZnSO_4*7H_2O$, 0.03; $MnSO_4*7H_2O$, 0.003; $CuSO_4*7H_2O$, 0.003; $Na_2MoO_4*2H_2O$, 0.003; yeast extract, 1.0. Prior to sterilisation, the pH of the culture medium was adjusted to 5.5 with 2 M HCl (Eng et al., 1998). Flasks were then inoculated with 1 x 10^6 cells mL^{-1} and incubated for 3 days (*Y. lipolytica* 2.2ab) or 7 days (*B. theobromae* 1368) at 200 rpm and 30 °C. After incubation, a concentrated solution of (+)-valencene was added to provide different initial concentrations of (+)-valencene: 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 3.2, 4.5 and 6.4 g L^{-1}. To determine product inhibition on the bioconversion process, a concentrated solution of (+)-nootkatone was added in other experimental units to ensure different initial concentrations of (+)-nootkatone: 50, 100, 200, 300, 400, 600 and 1000 mg L^{-1}. The flasks were incubated for 4 days to carry out the bioconversion process, and samples (2.0 mL) were taken every 24 h. The concentrations of (+)-valencene and (+)-nootkatone were determined. Control experiments containing sterile broth without inoculum were also conducted. All the experiments were conducted in triplicate. Every day, three Erlenmeyer flasks were taken and centrifuged at 5000 rpm for 10 min. Biomass determination was carried out by measuring the dry weight of cells. Sesquiterpenes were extracted from the supernatant by adding 500 µL of ethyl acetate to 500 µL of sample. The mixture was vortexed twice for 20 s and allowed to settle until phase separation occurred, after which 1 µL of the ethyl phase was injected into a GC (Sowden et al., 2005; Girhard et al., 2009).

2.2.3. Partition coefficients determination

Partition coefficients of (+)-valencene and (+)-nootkatone between a buffer solution (aqueous phase) and orange essential oil (organic phase) were determined taking into account the phase ratio (1:1 v/v) and the initial (+)-valencene concentration conditions used in the serological flasks. During the bioconversion process, (+)-valencene is transferred from the organic phase to the aqueous phase, and (+)-nootkatone is transferred from the aqueous phase to the organic phase. Therefore, the aqueous phase was prepared as follows: 3.2 g L^{-1} of nootkatone was dissolved in a 0.1 M phosphate buffer adjusted to pH 7.5. Also, the organic phase was orange essential oil containing 3.2 g L^{-1} of (+)-valencene. Then, equal amounts of both solutions were mixed, yielding a final emulsion with a total volume of 6 mL. The emulsion was subjected to mechanical agitation for 6 h at 30 °C in order to reach thermodynamic equilibrium. The phases were then separated by centrifugation. (+)-valencene and (+)-nootkatone were extracted from the aqueous and organic phases, respectively, using 500 µL of ethyl acetate. Both phases were analysed quantitatively by GC and the partition coefficient (K_p) was subsequently determined. The partition coefficient was calculated as the ratio of the solute concentration in the organic phase divided by the solute concentration in the aqueous phase at equilibrium.

2.2.4. Sesquiterpene analysis

Concentrations of (+)-valencene and (+)-nootkatone were determined by gas chromatography (GC-FID) using a Perkin Elmer Auto System XL gas chromatographer equipped with a flame ionisation detector (Cyclosil-B capillary column; 30 m x 0.32 mm x 0.25 µm; J & W Scientific). The temperature of the injector and detector was constant at 250 °C to 270 °C, respectively. One µL of sample was automatically injected with a split ratio of 1:4, using helium gas as a carrier. The column temperature was initially set to 120 °C for 4 min then ramped up to 250 °C at a rate of 10 °C per min and maintained at 250 °C for 5 min, using the technique reported by Girhard et al. (2009). Analyses were performed in triplicate. GC/MS spectra were obtained using a GC/MS-HP6890 (Agilent) gas chromatograph equipped with a HP-5 column (30 m x 250 µm x 0.25 µm, Agilent). The same conditions of separation as the GC-FID analysis were used. Compounds in the samples were identified by comparison of mass spectra to that of pure compounds.

2.2.5. Microorganism pre-selection experiments

Inoculum from each strain was obtained in 250 mL Erlenmeyer flasks containing 50 mL of PDA incubated at 30° C for 3–7 days. Cells (yeast) or spores (fungi) were harvested with 5.0 mL of saline solution (0.85 g 100 mL^{-1}) or Tween 80 solution (0.1% v/v), respectively. Cells or spores were counted (Neubauer chamber). Bioconversion studies were conducted in serological flasks following the method reported by Castellanos (2007) with some modifications. Serological flasks containing 5 mL of PDA medium were inoculated with 1 x 10^6 cells or spores mL^{-1}. After incubation periods mentioned previously at 30 °C, a solution of 0.1 M phosphate buffer adjusted to pH 7.5 was dissolved with a (+)-valencene concentration of 3.2 g L^{-1} and then added.

2.2.6. Microorganism selection experiments

Serological flasks containing 5 mL of PDA medium were inoculated with 1 x 10^6 cells or spores mL^{-1} of *Botryodiplodia theobromae* 1368, *Yarrowia lipolytica* 2.2ab*,* or *Phanerochaete chrysosporium*. After strain growth, either a (+)-valencene solution or orange essential oil was added, covering the surface of the culture. Three different conditions were prepared as follows: (a) 3.0 mL of 0.1 M phosphate buffer solution with a (+)-valencene concentration of 3.2 g L^{-1}; (b) 3.0 mL of a mixture in equal volumes of orange essential oil and a phosphate buffer solution, forming a biphasic system; and finally (c) 3.0 mL of orange essential oil (3.2 g L^{-1} of valencene). The bases of all serological flasks were filled with water and placed in a desiccator, to avoid volatilisation of the aromatic compounds. The flasks were closed with a plug and incubated at 30 °C. All experiments were conducted in triplicate, and controls without microorganisms at the same reaction conditions were also performed. Aliquots (500 µL) were taken every 24 h for 12 days.

2.3. Results and discussion

2.3.1. Sesquiterpenes present in orange essential oil (Citrus aurantium, var. *amara)*

Orange essential oil contained (+)-valencene and (+)-nootkatone concentrations of 3.2 g L^{-1} and 0.94 mg L^{-1}, respectively. A similar (+)-valencene concentration (2.99 g L^{-1} of (+)-valencene) with the same kind of orange oil was reported by Del Rio et al. (1991). Sesquiterpene concentration varies for different species of the *Citrus* genus depending on the

time of harvest of the fruit and its variety (Elston et al., 2005; Espina et al., 2010). The highest (+)-valencene concentration (6 g L^{-1}) has been reported for *Citrus sinensis* (Sharon-Asa et al., 2003). Essential oil contains a large number of sesquiterpene compounds and can be potentially used as a broad spectrum green pesticide (Koul et al., 2008). The largest compound present in orange essential oil is limonene, a terpene with high antimicrobial activity (Mustafa et al., 2012).

2.3.2. *Partition coefficient determination*

Partition coefficients of valencene and nootkatone between orange essential oil (*Citrus aurantium* var. *amara*) and a phosphate buffer solution were determined (Table 2.1). The partition coefficient (K_P) of (+)-valencene was 6.37, which is 3.5 times lower than that obtained for (+)-nootkatone (22.5). This high value of K_P for nootkatone indicates a high affinity of the product for orange essential oil (organic phase), which would be useful as a potential recovery *in situ* technique.

Table 2.1. Partition coefficient values of (+)-valencene and (+)-nootkatone between orange essential oil and culture medium at 30 °C.

Sesquiterpenes	Value
(+)-valencene	6.37
(+)-nootkatone	22.50

2.3.3. *Selection of the microorganisms in a monophasic system*

Selection experiments of six strains (*Botryodiplodia theobromae* 1368, *Phanerochaete chrysosporium*, *Yarrowia lipolytica* 2.2ab, *Aspergillus tamari* V12307, *Rhyzomucor* sp. and *Kluyveromyces marxianus* NCYC1429) were conducted to observe their ability to oxidise (+)-valencene on surface cultures in serological flasks at 30 °C. In order toper form the screening of microorganisms, we proceeded to use a simple technique with solid culture medium. The results of this selection are shown in Table 2.2. The highest (+)-nootkatone production was obtained by *Botryodiplodia theobromae* 1368, *Phanerochaete chrysosporium,* and *Yarrowia lipolytica* 2.2ab with a bioconversion percentage of 2.21, 1.71, and 1.6%, respectively. The low bioconversion percentage obtained was probably due to (+)-

valencene volatility, as it was also observed in the control. Furusawa et al. (2005) also reported the ability of *Botryodiplodia theobromae* to convert (+)-valencene to (+)-nootkatone in an enriched Czapeck-peptone culture medium. However, to the best of our knowledge, our research group is the first one reporting of the use of *Y. lipolytica* for this bioconversion process.

Table 2.2. Screening results for the biotransformation of (+)-valencene to (+)-nootkatone.

Microorganism	Maximum Nootkatone (mg L^{-1})[ii]	Bioconversion (%)[i]
Kluyveromyces marxianus NCYC1429	14.51 ± 0.83 (d)	1.76 ± 1.09 (a)
Aspergillus tamarii V12307	4.70 ± 0.56 (e)	0.46 ± 0.32 (b)
Botryodiplodia theobromae 1368	29.03 ± 1.33 (c)	2.21 ± 1.13 (a)
Yarrowia lipolytica 2.2ab	42.35 ± 0.91 (a)	1.60 ± 0.23 (a)
Phanerochaete chrysosporium	32.78 ± 1.23 (b)	1.71 ± 0.32 (a)
Rhyzomucor sp.	0.315 ± 0.23 (f)	0.91 ± 0.56 (b)

[i] Percentage of (+)-nootkatone produced relative to (+)-valencene consumed.

[ii] mg of maximum nootkatone L^{-1} produced after 12 days.

Values represent mean ± standard deviation.

Same letters represent no significant differences ($p \leq 0.05$; Tukey, SAS System Version 9).

2.3.4. *Microorganism screening for the bioconversion of (+)-valencene to (+)-nootkatone in a single aqueous phase system.*

The bioconversion kinetics of (+)-valencene to (+)-nootkatone for 12 days for the three selected strains (*B. theobromae* 1368, *P. chrysosporium* and *Y. lipolytica* 2.2ab) are shown in Fig. 2.2. It was observed that (+)-nootkatone production began between the second and fourth day and finished after the ninth day. Among them, *B. theobromae* 1368 produced the highest (+)-nootkatone concentration of 223.7 mg L^{-1}. Comparing these results with previous experiments (section 2.3.3), it was observed that (+)-nootkatone production increased 7.7 and 4.1 times for *B. theobromae* 1368 and *Y. lipolytica* 2.2ab, respectively (see Tables 2.1 and 2.2), most likely due to the lack of evaporation of substrate and product.
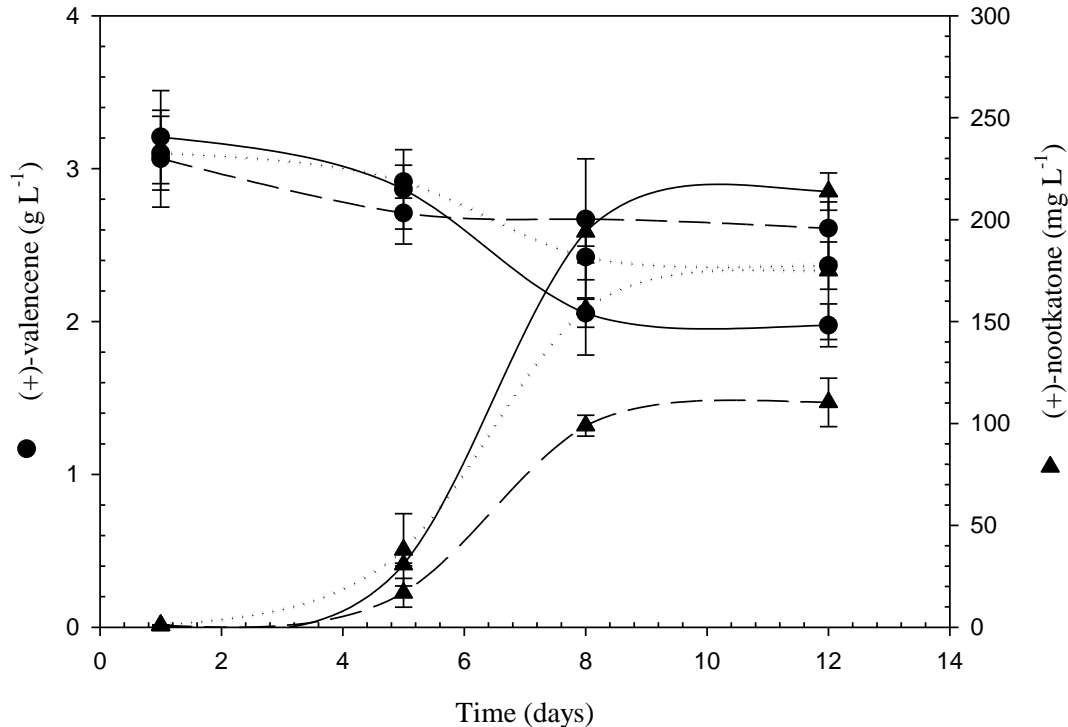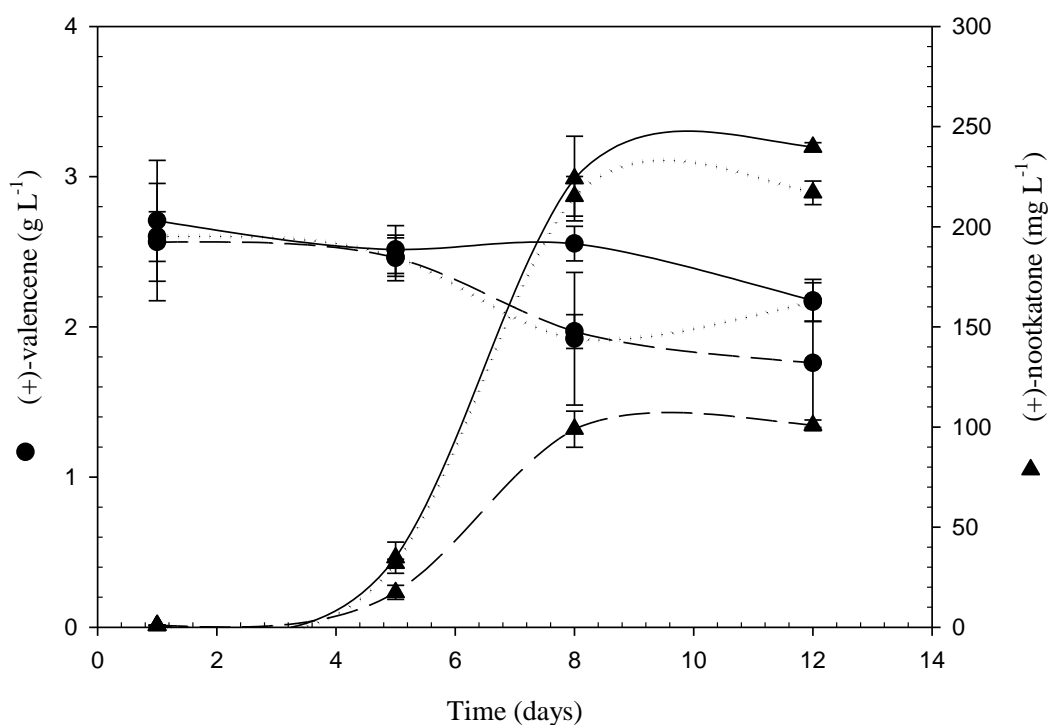
Fig. 2.2. Bioconversion kinetics of (+)-valencene to (+)-nootkatone in a monophasic system: (——) *B. theobromae* 1368, (·····) *Y. lipolytica* 2.2ab, (———) *P. chrysosporium.* Experiments were carried out in triplicate.

2.3.5. *Microorganism screening for the bioconversion of (+)-valencene to (+)-nootkatone in a biphasic system.*

The bioconversion kinetics of the selected strains (*B. theobromae* 1368, *P. chrysosporium* and *Y. lipolytica* 2.2ab) using the biphasic system is shown in Fig. 2.3. The (+)-valencene and (+)-nootkatone concentrations were determined from the organic phase (orange essential oil). It was also observed that *B. theobromae* 1368 and *Y. lipolytica* 2.2ab had the highest (+)-nootkatone production, 239.7 and 216.9 mg L^{-1}, respectively. For both strains, no significant difference in maximum nootkatone concentration was observed when comparing these results to those obtained with the single aqueous phase. A two-phase bioconversion process was studied by Girhard at al. (2009) using recombinant *E. coli* with a 10% dodecane organic phase in a stirred tank bioreactor. These authors reported a (+)-nootkatone concentration of 94.2 mg L^{-1} after growth for 8 h. Recently, Wriessnegger et al. (2014)

reported the production of (+)-nootkatone by metabolic engineering of *Pichia pastoris* obtaining 208 mg L^{-1} in 48 h using a 20% n-dodecane organic phase. Our results show the potential for a (+)-nootkatone production *in situ* extraction system using an orange essential oil organic phase. A biphasic bioprocess for the production of high concentrations of R-(+)-α-terpineol from R-(+)-limonene was described by Bicas et al. (2010). The system used consisted of a biphasic medium in which the aqueous phase contained a high concentration of *Sphingobium* sp. resting cells and a sunflower oil organic phase.
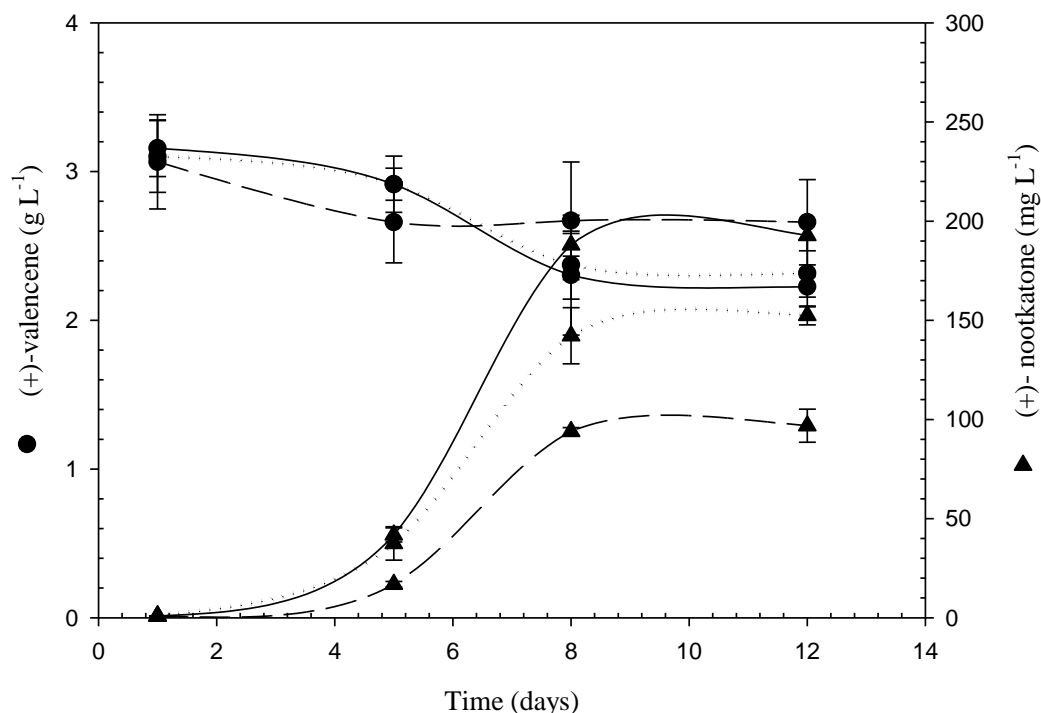

Fig. 2.3. Bioconversion kinetics of (+)-valencene to (+)-nootkatone in a biphasic system: (——) *B. theobromae* 1368, (·····) *Y. lipolytica* 2.2ab, (———) *P. chrysosporium.* Experiments were carried out in triplicate.

2.3.6. *Microorganism screening for the bioconversion of (+)-valencene to (+)-nootkatone in a single organic phase system.*

The bioconversion kinetics of (+)-valencene to (+)-nootkatone in a single organic phase using orange essential oil is shown in Fig. 2.4. Interestingly, similar (+)-nootkatone production was observed. Recently, Brault et al. (2014) reported the synthesis of a short-chain ester flavour in organic media using an *E. coli* whole-cell biocatalyst expressing a newly characterised

heterologous lipase. The whole-cell biocatalyst showed excellent tolerance to alcohol and short-chain fatty acid denaturation in a micro-aqueous media. Therefore, further studies should focus on the regioselectivity of the bioconversion.

Fig. 2.4. Bioconversion kinetics of (+)-valencene to (+)-nootkatone in a single organic phase: (——) *B. theobromae* 1368, (·····) *Y. lipolytica* 2.2ab, (– – –) *P. chrysosporium.* Experiments were carried out in triplicate.

For each strain, a similar percentage of bioconversion and maximum (+)-nootkatone concentration using three different bioconversion conditions—aqueous, organic, and biphasic phase—were obtained, probably due to the following: a) in the aqueous phase, substrate and product concentration were under non-inhibition conditions; b) in the biphasic phase, unknown components in orange essential oil might not be as toxic as expected; and c) in the organic phase, the hydrophobic characteristic of the strain (Table 2.3). Differences in percentage of bioconversion and maximum (+)-nootkatone concentration between strains might be due to cell catalytic activity, differences in g of biomass per cm^2 on the agar surface, different tolerance to orange essential oil components, or hydrophobic characteristic of the strain. Girhard et al. (2009) studied this bioconversion process using recombinant *E. coli* in

a stirred tank bioreactor. A similar bioconversion of 24.7% for the monophasic system and 23% for the biphasic system, under optimised conditions with 10% dodecane as an organic phase, was observed in this study.

Table 2.3. Maximum (+)-nootkatone production and percentage of bioconversion of (+)-valencene for the three systems studied.

Microorganism	Maximum Nootkatone (mg L^{-1})[ii]	Bioconversion (%)[i]	System
B. theobromae 1368	223.7 ± 9.1 (a,b)	18.17 ± 3.0 (a)	Single aqueous phase
	239.7 ± 2.1 (b)	23.25 ± 7.5 (a)	Biphasic
	237.7 ± 7.6 (a)	25.53 ± 5.1 (a)	Single organic phase
Y. lipolytica 2.2ab	174.9 ± 33.7 (a)	27.19 ± 3.3 (a)	Single aqueous phase
	216.9 ± 5.8 (a)	29.50 ± 13.9 (a)	Biphasic
	217.4 ± 4.7 (a)	27.68 ± 14.7 (a)	Single organic phase
P. chrysosporium	110.3 ± 11.8 (a)	24.21 ± 11.2 (a)	Single aqueous phase
	100.8 ± 2.6 (a)	22.13 ± 0.03 (a)	Biphasic
	96.8 ± 8.3 (a)	23.87 ± 1.3 (a)	Single organic phase

[i] Percentage of (+)-nootkatone produced relative to (+)-valencene consumed.

[ii] mg of maximum nootkatone L^{-1} produced after 12 days.

Values represent mean ± standard deviation.

Same letters represent no significant differences ($p \leq 0.05$; Tukey, SAS System Version 9).

The strains *B. theobromae* 1368 and *Y. lipolytica* 2.2ab were selected for further study, as they provided the highest (+)-nootkatone production and percentage of bioconversion. The screening of microorganisms was conducted using a technique in serological flasks with spores or cells on a surface culture. There are few references on bioconversion using surface cultures. For instance, Demyttenaere et al. (2000) studied the biotransformation of cetrol, geraniol, and citral oils by *Aspergillus niger* and *Penicillium* sp. on surface cultures. The biotransformation was monitored by dynamic headspace, and the main product obtained linalool yield values up to 8.57 µL from 200 µL of nerol.

In all the kinetic profiles, a stationary phase of (+)-nootkatone production was observed after 8 days when inhibition by substrate, product, or oxygen limitation could be involved. The results of the control without addition of inoculum showed no bioconversion. Therefore, in order to elucidate the cause of the stationary phase, a new set of bioconversion experiments was performed.

2.3.7. Substrate and product inhibition

Biomass was previously produced in liquid media in baffled Erlenmeyer flasks. Growth kinetics for the two strains was obtained and biomass concentrations chosen were 23.5 ± 0.71 g L^{-1} at 7 days for *B. theobromae* 1368 and 11.5 ± 1.41 g L^{-1} at 3 days of culture for *Y. lipolytica* 2.2ab. Subsequently, different amounts of a concentrated solution of (+)-valencene were added to ensure different initial concentrations, and experimental units were incubated at 30 °C. Fig. 2.5A shows that the maximum concentration of (+)-nootkatone for *Y. lipolytica* 2.2ab from an initial concentration of 1.5 g L^{-1} of (+)-valencene (0.13 g of valencene per g of biomass) reached a bioconversion maximum of 34.7%. On the other hand, *B. theobromae* 1368 presented a lower tolerance at 1 g L^{-1} of (+)-valencene (0.042 g of valencene per g of biomass), but a greater bioconversion of 45.8%. An inhibitory effect above these concentrations was evident. It has been reported that sesquiterpenes present antifungal activities, and their biosynthesis is often induced after fungal infection (Manter et al., 2007; Huffaker et al., 2011). Recently, Liu et al. (2012) demonstrated that (+)-valencene contained in sweet orange essential oil resulted in inhibition of fungal growth in five different microorganisms.
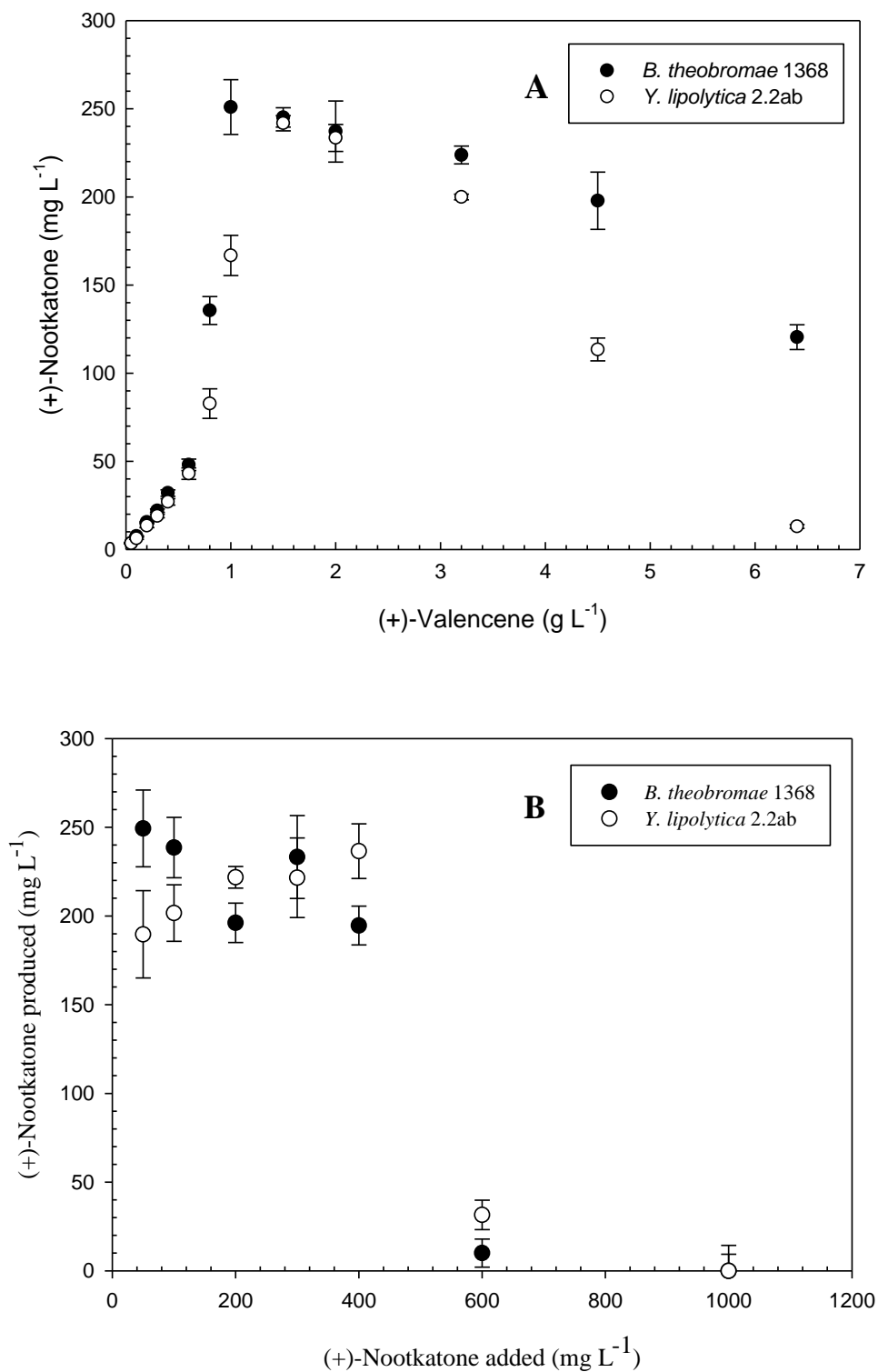
Fig. 2.5. Effect of (**A**) Substrate and (**B**) Product inhibition on (+)-Nootkatone bioconversion for (●) *B. theobromae* 1368 and (○) *Y. lipolytica* 2.2ab in an aqueous phase system using baffled Erlenmeyer flasks.

In order to determine product inhibition, further experiments were carried out by adding different amounts of a concentrated solution of (+)-nootkatone to culture medium, resulting in different initial concentrations. Fig. 2.5B shows a decrease in (+)-nootkatone produced when the initial (+)-nootkatone added was above 600 mg L^{-1}, indicating that *B. theobromae* 1368 and *Y. lipolytica* 2.2ab present product inhibition above 17.0 and 52.17 mg of (+)-nootkatone (g of biomass)$^{-1}$. Studies performed by Gavira et al. (2013) with *S. cerevisiae* showed that a concentration of 1.0 g L^{-1} of (+)-nootkatone results in product inhibition and a loss of cell viability.

Therefore, the results obtained using this screening methodology are useful for the bioconversion process design. Consequently, it is possible to visualise studies focused on multiphasic systems in order to avoid substrate and product inhibition during the bioconversion process.

2.4. Conclusions

The screening method that was used evaluated the potential ability of the strains studied to biotransform (+)-valencene to (+)-nootkatone. This regioselective synthesis was performed naturally via surface culture, which provided greater versatility using different bioconversion systems. Remarkably, similar (+)-nootkatone concentrations in the single organic phase were observed compared to both the aqueous and biphasic systems. The addition of a second liquid phase (orange essential oil) presents a potential application for the production and *in situ* extraction of the sesquiterpene (+)-nootkatone. Due to the different physiological and morphological characteristics of the selected strains, further work must focus on the use of a membrane-aerated biofilm reactor for the fungus *B. theobromae* 1368 and a stirred tank bioreactor for the yeast *Y. lipolytica* 2.2ab, using a single aqueous phase or partitioning bioreactors with whole cells.

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CHAPTER III

BIOCONVERSION OF (+)-VALENCENE BY *Botryodiplodia theobromae* USING A MEMBRANE AERATED BIOFILM REACTOR

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BIOCONVERSION OF (+)-VALENCENE BY *Botryodiplodia theobromae* USING A MEMBRANE AERATED BIOFILM REACTOR

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Abstract

The aim of this work was to evaluate the bioconversion of (+)-valencene to (+)-nootkatone by *B. theobromae* using a membrane aerated biofilm reactor (MABR) in a two liquid phase system with orange essential oil as organic phase. In an aqueous phase system, a (+)-nootkatone production rate up to 3.98 mg L^{-1} h^{-1} was achieved obtaining a final product concentration of 398.08 mg L^{-1} with a conversion rate: 62%. Also, a two-phase liquid system, using orange essential oil as dispersed phase was studied and a final (+)-nootkatone concentration of 310.37 mg L^{-1} in the organic phase, with 30.5% of bioconversion and production rate of 2.46 mg L^{-1} day^{-1} was achieved. The present work is the first report of a MABR for the bioconversion of (+)-valencene (+)-nootkatone. Further studies of bioconversion products and optimization of biofilm reactor operations are needed to enhance bioconversion.

Keywords: Bioconversion, (+)-nootkatone, *Botryodiplodia theobromae*, Membrane aerated biofilm reactor

3.1. Introduction

Bioconversions play an increasingly important role in organic synthesis for the production of fine chemicals, such as the synthesis of chiral precursors (Faber, 2004). However, an important number of substrates and products of bioconversions are poorly soluble in water, the medium in which most biocatalysts have optimal biological performance. Additionally, substrate and product above critical concentrations are known to inhibit biocatalytic activity (D´Arrigo *et al.,* 2000; Held *et al.,* 2000). Therefore, a significant obstacle to the application of bioconversions in industry is the limiting loading of substrate and product allowed in the reactor. Partitioning bioreactors overcome this limitation since favorable partitioning coefficients of substrates and products toward the organic phase allow the use of high concentrations in this phase, while maintaining low concentrations in the aqueous phase. However, there might be some drawbacks such as the intensive mixing of medium and organic phases can cause strong emulsions that make downstream separation a practical

constraint. And the presence of the organic solvent might inhibit or even stop the biocatalyst activity (D´Arrigo *et al.,* 2000; Held *et al.,* 2000).

MABR's have been applied for the degradation of volatile contaminants in waste water (Qureshi *et al.,* 2005; Judd, 2008) but has not been used yet in oxidative bioconversion. MABR showed to be a suitable method to decrease the loss of volatile substances during a transformation process (Casey *et al.,* 1999; Lilly and Woodley, 1996). Onken and Berger (1999) studied the use of a MABR for the biotransformation of the citronellol by basidiomycete *Cystoderma carcharias*, production rates up to 150 mg of citronellol L^{-1} day^{-1} were reached and led to a product concentration of 866 mg L^{-1} with a conversion rate of 52%. The total loss of the added volatile substrate via the exhaust air was 4.5% when this aeration method was used. In addition, González-Brambila and López-Isunza (2007) reduced oxygen transfer limitations across the biofilm supplying oxygen from inside the membrane and simultaneous sparing of air to the residual water improving the MABR performance.

The aim of this work was to evaluate the bioconversion of (+)-valencene to (+)-nootkatone by *B. theobromae* using a MABR. The bioconversion was tested in both a single liquid phase system and a two liquid phase system using orange essential oil as organic phase.

3.2. Materials and methods

3.2.1. Chemicals

(+)-valencene (CAS 75-05-6) and (+)-nootkatone (CAS 93-78-5) with a purity >70% and >85%, respectively, were purchased from Fluka (Switzerland) and used as standards. Ethyl acetate (99.5%) was purchased from Quimex (México). Analytical grade orange essential oil (*Citrus aurantium*, var. Amara) was purchased from Cosmopolita Drugstore (Mexico, DF) and used as organic dispersed phase to carry out bioconversion studies.

3.2.2. Microorganism

Botryodiplodia theobromae 1368 from the lnstituto Nacional de Investigaciones Fundamentales de la Agricultura Tropical (La Habana, Cuba) isolated from Cuban *Citrus cinensis* Osbeck cv Valencia, was used. The strain was stored on dextrose potato agar slants

at 4 °C and sub-cultured every month at 30 °C for 7 days. Spores were harvested with 5.0 mL of Tween-80 (0.01% v/v) and were used as inoculum ($1x10^6$ spores mL^{-1}).

3.2.3. Culture media

Culture media with the following basal salt composition were used (in g L^{-1}): sucrose, 50; $NaNO_3$, 7.5; KH_2PO_4, 2.0; KCl, 0.3; $MgSO_4.7H_2O$, 0.6; $FeSO_4.7H_2O$, 0.6; $ZnSO_4.7H_2O$, 0.03; $MnSO_4.7H_2O$, 0.003; $CuSO_4.7H_2O$, 0.003; $Na_2MoO_4.2H_2O$, 0.003; yeast extract, 1.0 (Eng *et al.,* 1998). After autoclaving initial pH was adjusted to 5.5-5.6 with HCl 2 M.

3.2.4. Suspended biomass determination

Samples of known volume were dried until a constant weight in metal plates which had been previously weighed after being left in an oven at 90 °C overnight. All analyses were performed in triplicate.

3.2.5. Sucrose consumption

Sucrose consumption of *B. theobromae* was estimated by analyzing the sucrose concentration in the medium using the technique described by Miller *et al.* (1960). Analyses were performed in triplicate.

3.2.6. Cell viability

Cell viability during growth and the bioconversion process was determined by measuring the concentration of living cells using the methylene blue technique, as described by Bonora and Mares (1982). Analyses were performed in triplicate.

3.2.7. Membrane Aerated Biofilm reactor (MABR)

The MABR used was previously described by González-Brambila and López-Isunza (2007). The Pyrex tube-bioreactor had the following dimensions: 200 mL volume, 300 mm height, 30 mm outside diameter, and 2 mm thickness. An oxygen permeable membrane of silicon rubber (290 mm length, 1.5 mm inner radius, and 0.3 mm thick) was used. The membrane tube was placed inside the glass bioreactor. One side of the tube was connected to the air supply valve, and the other side was partially blocked to force oxygen to flow through the

wall membrane. The system operated continuously at 30 °C and pH 5.5. Atmospheric air was filtered through a sterile filter of 0.22 μm and was pumped through the membrane at a flow rate of 135 mL min^{-1}. The culture medium was fed up ward with hydraulic retention time (HRT) of 0.756 h^{-1}.
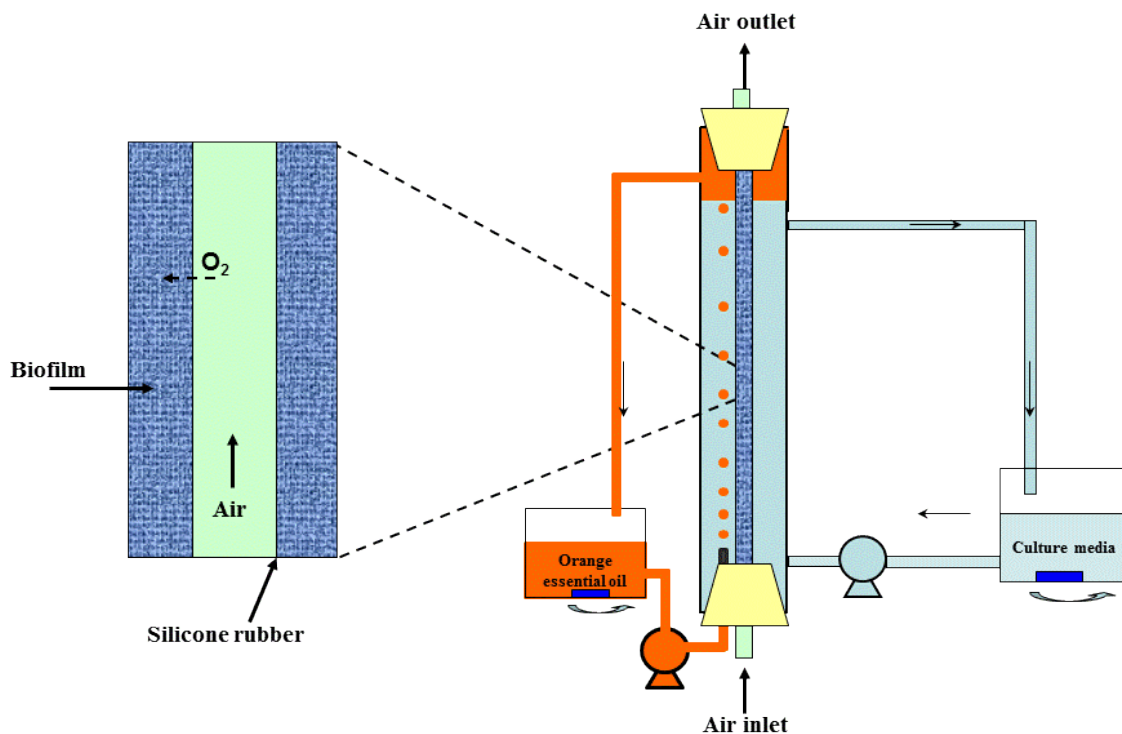
3.2.8. Bioconversion processes

The MABR was inoculated using a concentration of 1x10^6 spores of *B. theobromae* mL^{-1}. After 7 days of growth a biofilm was formed outside of the membrane. Then, bioconversion studies were conducted by: (a) adding a concentrated solution of (+)-valencene to reach a final concentration of 1.0 g L^{-1} or (b) dropping a total volume of 135 mL of orange essential oil through the MABR during of bioconversion process (Figure 3.1). In order to determine the effect of adding valencene and other components of orange essential oil to the culture medium, a control without the addition of (+)-valencene was included using 50 g of sucrose L^{-1}, which was performed in duplicate. Every day, 2 mL of sample was taken and centrifuged at 5000 rpm for 10 min. Sesquiterpenes extraction was carried out using 500 μL of sample and 500 μL of ethyl acetate. The mixture was vortexed twice for 20 s in a vortex, and settled until phase separation occurred, after that 1 μL of the ethyl acetate phase was injected to GC (Girhard *et al.,* 2009; Kaspera *et al.,* 2005).

3.2.9. Sesquiterpenes analysis

The concentrations of (+)-valencene and (+)-nootkatone were determined by gas chromatography (GC-FID) using a Perkin Elmer Auto System XL Gas Chromatograph equipped with a flame ionization detector (Cyclosil-B capillary column; 30 m x 0.32 mm x 0.25 μm; J & W Scientific). The temperatures of the injector and detector were constant at 250 °C to 270 °C, respectively. 1 μL of sample was automatically injected with a split ratio of 1:4, using helium gas as a carrier. The column temperature was initially set to 120 °C for 4 min then ramped up to 250 °C at a rate of 10 °C per min and maintained at 250 °C for 5 min, using the technique reported by Girhard *et al.* (2009). Analyses were performed in triplicate. GC/MS spectra were obtained using a GC/MS-HP6890 (Agilent) gas chromatograph equipped with a HP-5 column (30 m x 250 μm x 0.25 μm, Agilent). The same

conditions of separation as the GC FID analysis were used. Compounds in the samples were identified by comparison of mass spectra to that of pure compounds.

Figure 3.1. Diagram of the membrane aerated biofilm reactor for the bioconversion of (+)-valencene to (+)-nootkatone by *B. theobromae*.

3.3. Results and discussion

3.3.1. *Sesquiterpenes in orange essential oil (Citrus aurantium,* var. *amara)*

Orange essential oil used in this work was analysed by GC coupled to mass spectrometry. Concentrations of (+)-valencene and (+)-nootkatone were determined to be 3.2 g L^{-1} and 0.94 mg L^{-1}, respectively. The (+)-valencene concentration obtained was similar to that reported by Del Rio *et al.* (1991) at 2.99 g L^{-1} for the same variety of orange. Depending on the harvest of the fruit and its specific variety, the concentration of sesquiterpenes varies for different species of the *Citrus* genus (Espina *et al.,* 2010; Elston and Rouseff 2005). The highest concentration reported for (+)-valencene was 6.0 g L^{-1} for the species *sinensis* (Sharon-Asa *et al.,* 2003). In orange essential oil, there are a large number of compounds used in the food and beverage industry as antimicrobial agents. Limonene, a terpene with the highest antimicrobial activity, is largest compound in abundance (Mustafa *et al.,* 2012).
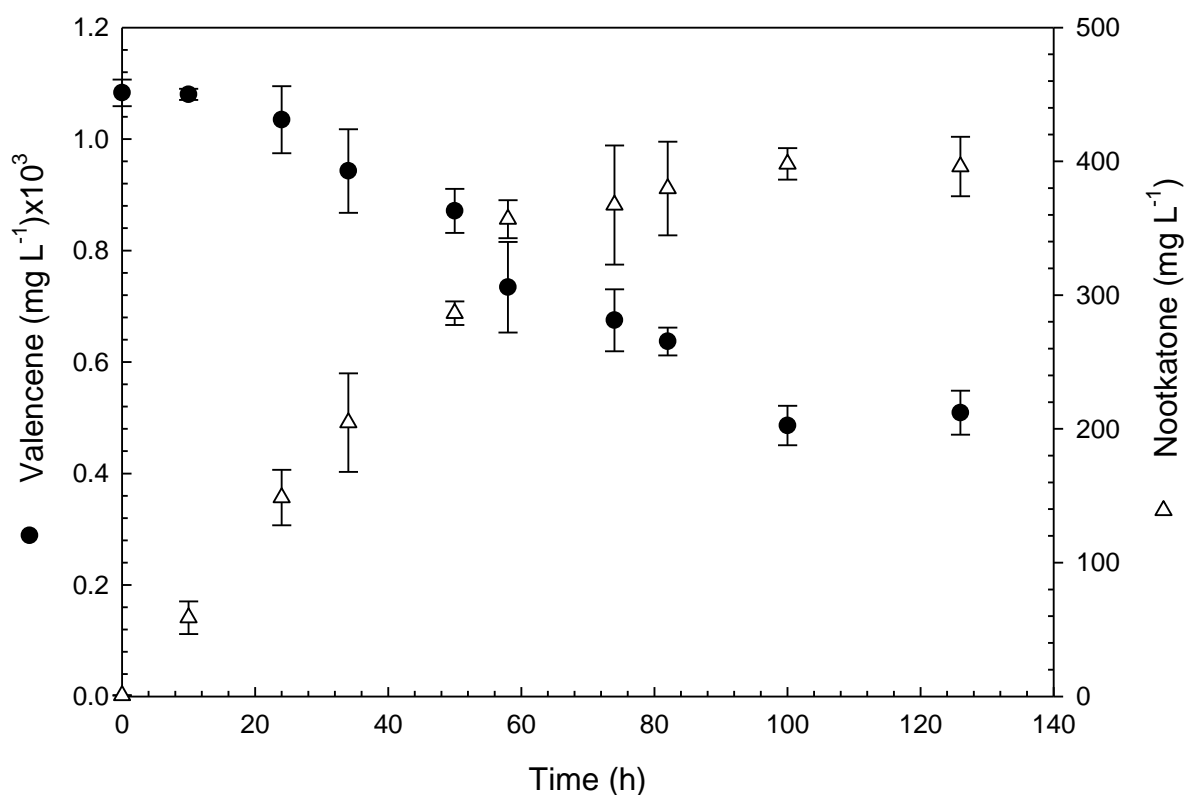
3.3.2. Membrane aerated biofilm reactor during the bioconversion in a monophasic system

To investigate the effect of (+)-valencene on the whole bioconversion process in the MABR, an aqueous phase system was studied. The MABR was inoculated with 1×10^6 spores suspended in 250 mL of mineral medium with 50 g of sucrose L^{-1} as a carbon source. The biofilm was allowed to develop for 7 days at 30 ºC. However, suspended biomass was observed reaching a maximum biomass concentration of 23.5 g L^{-1} after approximately 90% sucrose consumption. At this point, a stock solution of valencene was added to reach an initial (+)-valencene concentration of 1.0 g L^{-1} in the MABR to initiate the bioconversion process. The reactor was operated with a recycle rate of 12.6 mL min^{-1} and an overall HRT of 0.185 h, and the ascending liquid velocity parallel to the membrane was 0.5 m h^{-1}. The bioconversion of (+)-valencene was measured by GC. Kinetics of cell growth, sucrose consumption, and cell viability during the bioconversion process are plotted in Figure 3.2. It was observed that cell viability decreased linearly to roughly 30% between 8 and 12 days. Final cell viability might indicate that (+)-valencene or an intermediary may induce the inhibition of (+)-nootkatone bioconversion.
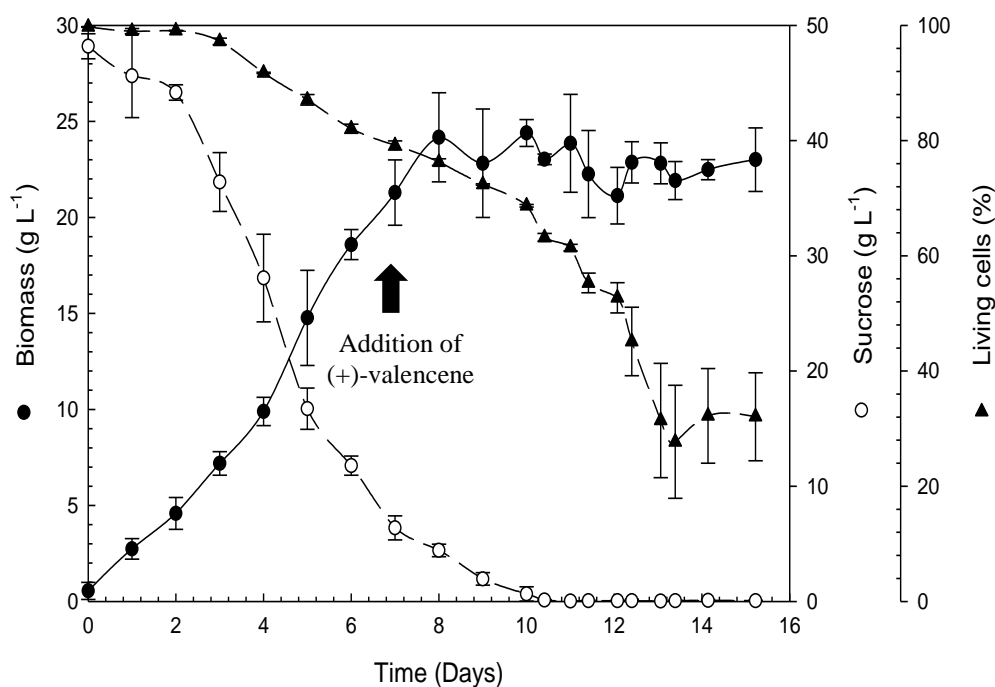
Figure 3.2. Kinetics of suspended biomass, sucrose consumption, and cell viability in the aqueous phase system. 1.0 g L^{-1} of (+)-valencene was added to initiate the bioconversion process.
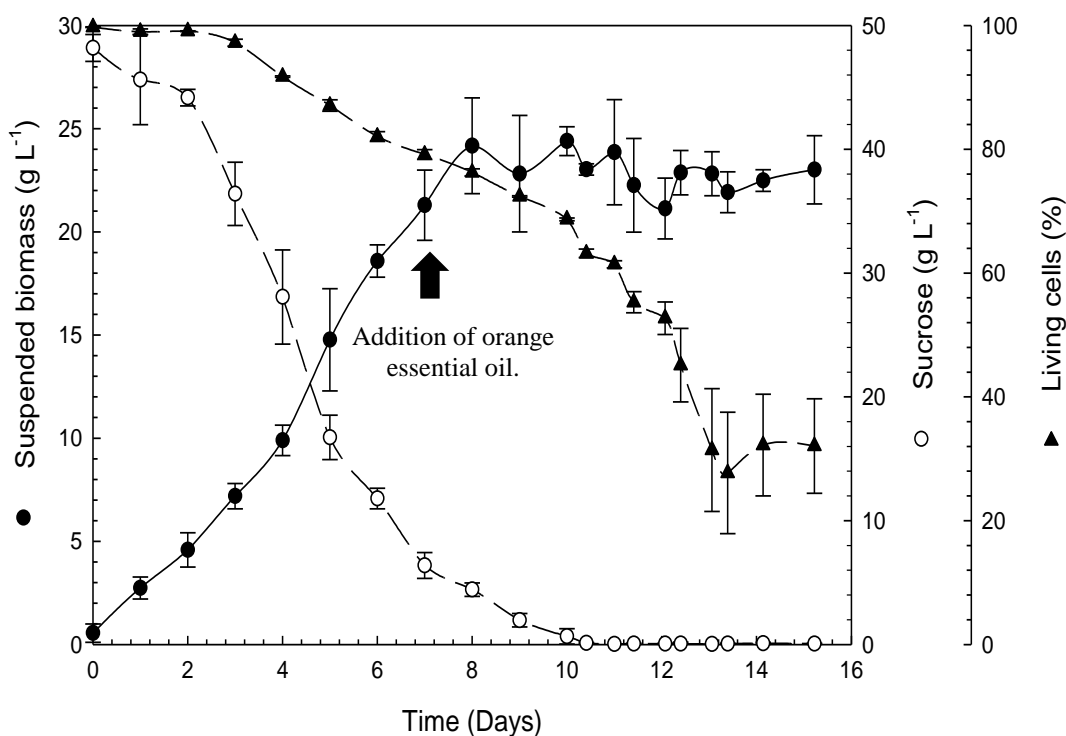
Kinetics of (+)-valencene consumption and (+)-nootkatone production during the bioconversion process are plotted in Figure 3.3. After 96 h, a concentration of 398.08 mg L^{-1} of (+)-nootkatone was reached, converting 0.526 g L^{-1} of valencene (i.e. bioconversion of 62 %). It was observed that the initial (+)-valencene concentration (1.0 g L^{-1}) decreased in about 50% after 96 h of bioconversion at 30 °C. After this period, no further changes in (+)-valencene concentration were observed. However, after 56 h no statistically significant difference from the (+)-nootkatone produced by *B. theobromae* is observed. The production of (+)-nootkatone from (+)-valencene has been reported by Furusawa *et al.* (2005), using to *Botryosphaeria dothidea* and *Botryodiplodia theobromae*, fungi cells. The authors reported that the biotransformation yielded 93 and 72% respectively of enantiomerically pure (+)-nootkatone. In this system, NADP(H), the reduced form of the cofactor, is used to provide the reaction reduction potential and is regenerated by the cells.

Figure 3.3. Bioconversion of (+)-valencene to (+)-nootkatone in an aqueous phase system using *B. theobromae*. Zero time corresponds to 7 days of growth.

3.3.3. Membrane aerated biofilm reactor during the bioconversion in a biphasic system

Bioconversion experiments were conducted in a two-phase system using orange essential oil as a source of (+)-valencene for bioconversion to (+)-nootkatone. First, the biofilm was allowed to develop for 7 days at 30 ℃ using 50 g of sucrose L^{-1} as a carbon source. Then, orange essential oil was added. Figure 3.4 shows the results of the growth kinetics, sucrose concentration and cell viability during the bioconversion process. At 10 days, approximately 90% sucrose consumption and a suspended biomass concentration of 23.25 g L^{-1} was observed. After the addition of orange essential oil, cell viability decreased linearly between 8 and 12 days.


Figure 3.4. Kinetics of suspended biomass, sucrose consumption, and cell viability in the biphasic system. Orange essential oil was added to initiate the bioconversion process.

(+)-Valencene and (+)-nootkatone concentrations in the organic phase during the bioconversion process are plotted in Figure 3.5. At 80 h, a concentration of 310.37±13.4 mg L^{-1} of (+)-nootkatone and an approximate consumption of 0.716 g L^{-1} of (+)-valencene was observed (i.e. bioconversion of 30.5 %). During the bioconversion process in the partitioning

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bioreactor, the (+)-valencene present in orange essential oil was transferred by partition (K_p = 6.37) to the aqueous phase based on the equilibrium and the metabolic demand of the cells. Also, the (+)-nootkatone produced in the aqueous phase was mainly transferred by partition (K_p = 22.5) into the organic phase for an *in situ* extraction. The relatively low nootkatone concentrations in the organic phase, observed during the bioconversion in the MABR might be due to lipophilic compounds, such as terpenoids, are preferentially dissolved in the lipophilic membrane systems of fungal cells (Abraham et al. 1997). The terpenoids, but also other lipophilic compounds, such as polycyclic aromatic hydrocarbons, alkanes and phenols, induce changes of the membrane properties and, thus, cause toxic effects. The fungi counteract these effects by co-metabolizing the poorly water-soluble compounds to water-soluble ones or to carbon dioxide and water, using easily metabolizable substrates (e.g. sucrose) as the main source of energy. The enzyme systems involved in these detoxification processes are comparable to those of other eucaryotic cells, e.g. mammals (Wackett and Gibson, 1982). In the first step, catalyzed by cytochrome P-450 mono-oxygenases, the molecule is oxyfunctionalised and, in the second step, hydrolysis (e.g. by epoxide hydrolases) or conjugation (e.g. by glutathione-S-transferases or UDP-glucuronosyltransferases) leads to water-soluble products, which are excreted into the medium.

The bioreactor experiments were performed in dead-end mode to provide a trans-membrane flow to diminish blocking of membrane pores by growth of the fungus or by extra-cellular products which provoke a partial blocking of the membrane pores causing a decreased oxygen transfer. The aeration of fungal cultures in bioreactors using hydrophobic micro-porous membranes offers attractive perspectives for bioconversions of volatile sesquiterpenes substrates. Further work has to focus on the long-term supply of sufficient amounts of oxygen through the aeration membrane to make this approach more applicable to the bioconversion of lipophilic compounds by submerged fungal cultures. This method of regioselective synthesis was performed naturally by microbial pathway converting the product of commercial interest, presenting a better versatility, so this method is to find alternatives in the industrial production of nootkatone.
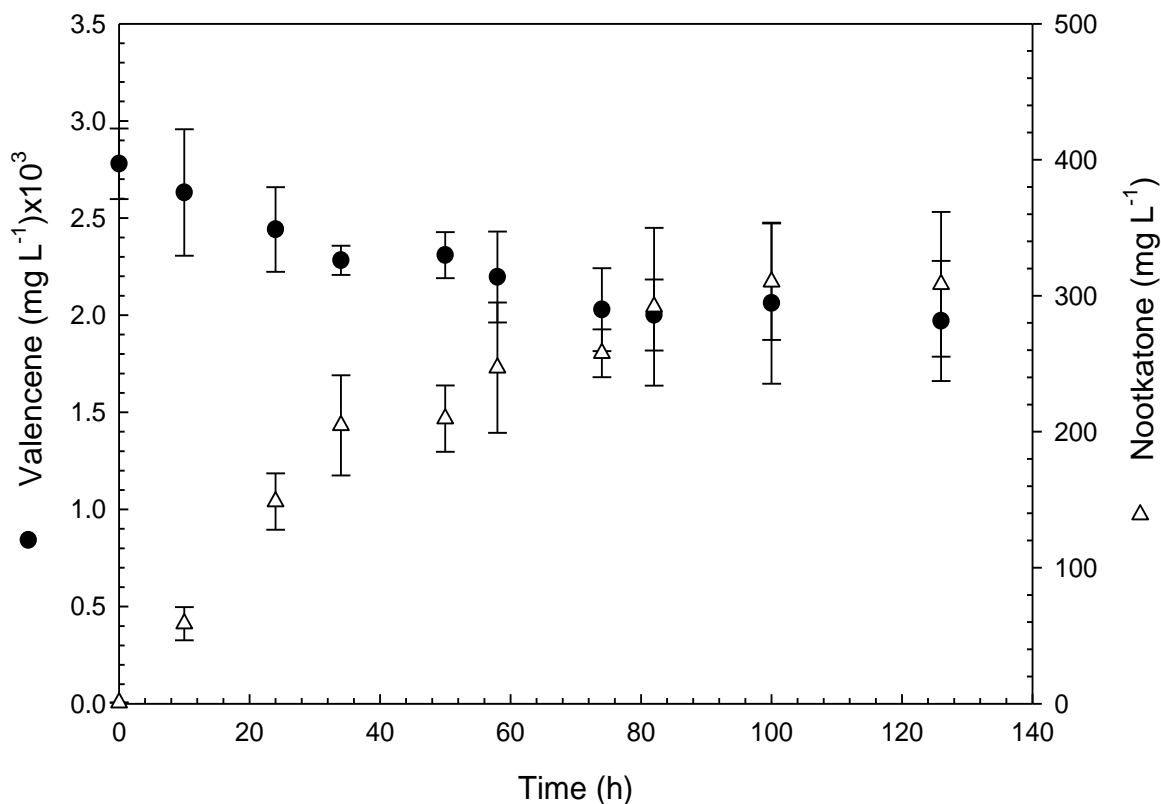
Figure 3.5. Bioconversion of (+)-valencene to (+)-nootkatone in a biphasic system using *B. theobromae*. Zero time corresponds to 7 days of growth.

3.4. Conclusions

In a biphasic system using *B. theobromae*, a (+)-nootkatone concentration of 310.37 mg L^{-1} was recovered in the organic phase. The addition of a second liquid phase (orange essential oil) presents a potential application for the production and *in-situ* extraction of the sesquiterpene (+)-nootkatone. However, further bioconversion studies and optimization of the MABR operation are needed to enhance this process.

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CHAPTER IV

ASSESSMENT OF (+)-VALENCENE BIOCONVERSION BY *Yarrowia lipolytica* USING A THREE PHASE PARTITIONING BIOREACTOR

ASSESSMENT OF (+)-VALENCENE BIOCONVERSION BY *Yarrowia lipolytica* USING A PARTITIONING BIOREACTOR

ABSTRACT

The aim of this work was to perform the physiological characterization of *Yarrowia lipolytica* 2.2ab during the bioconversion of (+)-valencene to (+)-nootkatone in a partitioning bioreactor using orange essential oil as the dispersed phase. When the exponential growth phase finished after 33 h of cell cultivation, orange essential oil (50% v/v) was added. A (+)-nootkatone concentration of 326.34 mg L^{-1} in the organic phase was obtained after 4 days, reaching a volumetric productivity of 3.40 mg L^{-1} h^{-1} and a bioconversion of 38.82%. At the end of the process, a loss of 70% of viable cells was observed, likely due to the accumulation of an intermediary. To the best of our knowledge, our research group is the first one reporting the wild type *Y. lipolytica* strain as a microorganism with the enzymatic machinery to carry out the oxidation of (+)-valencene. The bioconversion system based on the addition of an organic phase presents an attractive way to produce and recover (+)-nootkatone *in situ*.

4.1. Introduction

The compound (+)-nootkatone is a sesquiterpenoid that possesses an intense grapefruit-like taste and other valuable properties that are exploited by the fragrance and flavour industries [1]. The production of (+)-nootkatone is performed via chemical synthesis, mainly from the sesquiterpene (+)-valencene, which is readily available from the orange industry and also through the use of environmentally unfriendly oxidizing agents, such as tert-butyl peracetate [2] and tert-butyl hydroperoxide in combination with catalytic metal supported on silica [3]. However, the resulting (+)-nootkatone produced via chemical synthesis cannot be marketed as a "natural" product and does not satisfy increasing market demands for natural aromatic compounds. In order to meet this demand, many efforts have focussed on the use of biotechnological processes with bacteria, fungi or plants [4]. Some researchers have investigated genetically modified microorganisms, and several published reviews [5,6] have provided an overview of recently acquired knowledge about whole cell bioconversions and future industrial applications. A biocatalysis process in whole cells involves the selection of an optimal cellular enzyme, reaction engineering, product recovery and scaling-up. However,

to date, there is a lack of literature related to the appropriate selection of a bioreactor for biosynthesis of (+)-nootkatone.

Gavira et al. [7] reported a bioconversion process for (+)-nootkatone production using plant enzymes expressed in *S. cerevisiae*, the process was inhibited by substrate (> 4 g L^{-1}) and product (1.0 g L^{-1}). Alternatives are needed to overcome this inhibition by the substrate and product, as well as the over-oxidation of (+)-valencene. For instance, essential oils can be used as both a substrate reservoir and an *in situ* extraction agent in a partitioning bioreactor, thereby increasing the efficiency of the process [8]. Oppermann et al. [9] previously reviewed the use of biocompatible organic solvents in biphasic systems and their application in purification, extraction and bioconversion. However, bioconversion productivity of sesquiterpenes in a multi-phase system can be limited by low mass transfer rates at interfaces (gas-liquid or liquid-liquid). Partitioning bioreactors have shown significant potential for improving the productivity of many biological processes by overcoming problems related to low solubility or the toxicity of substrates and products [10].

The aim of this work was to perform the physiological characterisation of *Yarrowia lipolytica* 2.2ab during the bioconversion of (+)-valencene to (+)-nootkatone in a partitioning bioreactor.

4.2. Materials and methods

4.2.1. Chemicals

(+)-valencene (CAS 75-05-6) and (+)-nootkatone (CAS 93-78-5) with a purity >70% and >85%, respectively, were purchased from Fluka (Switzerland) and used as standards. Ethyl acetate (99.5%) was purchased from, Quimex (México). Analytical grade orange essential oil (*Citrus aurantium*, var. Amara, Cosmopolita Drugstore, Mexico DF) was used as organic dispersed phase. This orange essential oil was used to carry out thermodynamic, hydrodynamic, mass transfer, and bioconversion studies.

4.2.2. Microorganism, culture media and inoculum

Yarrowia lipolytica 2.2ab was previously selected for its capability to convert (+)-valencene to (+)-nootkatone from a screening between 6 selected strains (fungi and yeasts). *Yarrowia*

lipolytica 2.2ab was isolated from copra meal that was provided by the Laboratory of Bio-Process of the University of Guadalajara (U de G), Mexico. The strain was maintained in potato dextrose agar (PDA) at 4 °C, and cultures were propagated in 125-mL Erlenmeyer conical flasks containing 30 mL of PDA, before being incubated at 30 °C for 4 days. Cells were harvested with 25 mL 0.01% Tween-80 and used as inoculum (stock solution). Culture medium for *Y. lipolytica* was composed of (g L^{-1}): Glucose, 20; Na_2MoO_4, 0.2; $MnSO_4$, 0.4; $FeCl_3$, 0.2; KI, 0.1; $CuSO_4$, 0.04; H_2BO_3, 0.5; $CaCl_2$, 0.1; NaCl, 0.1; $MgSO_4*7H_2O$, 0.5; KH_2PO_4, 1; $ZnSO_4$, 0.4; $(NH_4)_2SO_4$, 5; and Yeast extract, 4.004 [11]. Prior to sterilization, the pH of culture medium was adjusted to 5.5 using 2 M HCl. For experiments in baffled conical flasks, 100 µL of *Y. lipolytica* 2.2ab cell suspension ($1x10^6$ cells mL^{-1}) was added as inoculum to 100 mL of culture medium. Flasks were incubated at 30°C for 3 days on a rotary shaker at 200 rpm. For bioreactor experiments, 700 µL of inoculum was added to 350 mL of sterile medium.

4.2.3. Growth kinetics and biomass determination

To increase the biomass of *Y. lipolytica* 2.2ab, and thus, to obtain a greater yield of (+)-nootkatone, it was carried out an analysis of growth kinetics with different concentrations of glucose (20–60 g L^{-1}) in the culture medium. Baffled Erlenmeyer flasks of 250 mL containing 100 mL of culture media were used. Each baffled Erlenmeyer flask was inoculated with $1x10^6$ cells mL^{-1} and incubated at 30 °C for 3 days on a rotary shaker at 200 rpm. Biomass determination was carried out by measuring the dry weight of cells. Samples of known volume were dried to a constant weight in metal plates which had been previously weighed after being left in an oven at 90 °C overnight. All analyses were performed in triplicate.

4.2.4. Glucose consumption

Glucose consumption of *Y. lipolytica* 2.2ab was estimated by analysing the glucose concentration in the medium using the technique described by Miller [12]. Analyses were performed in triplicate.

4.2.5. Cell viability

Cell viability during growth and the bioconversion process was determined by measuring the concentration of living cells using the methylene blue technique, described by Bonora and Mares [13]. Analyses were performed in triplicate.

4.2.6. Partitioning bioreactor

A 1.0 L glass stirred tank bioreactor (model ADI 1025, Applikon) was used for hydrodynamic characterisation, mass transfer and bioconversion processes. The bioreactor had an inside diameter of 9.5 cm and an operation volume of 0.7 L ($H_L/D_T = 0.96$). The bioreactor was equipped with a single Rushton turbine with 6 paddles, $D_i = 4.53$ cm (Di / DT $= 0.46$), located 4.53 cm from the base of the container. The bioreactor had 2 equidistant deflectors of 1.0 cm in width to improve liquid-liquid mixing. Temperature and agitation were maintained at 30°C and 300 rpm, respectively. The pH was monitored using an AppLiSens Z001023511 pH electrode (Applikon) and controlled at 5.5 using 2M NaOH. Atmospheric air entering the system was filtered through a sterile 0.22 µm filter. The air flow rate in the bioreactor was 1 vvm through a perforated "L" tube with seven holes of 1.0 mm in diameter.

4.2.7. Bioconversion processes

Bioconversion studies were conducted after the growth phase of *Y. lipolytica* 2.2ab (33 h), by adding (a) a concentrated solution of (+)-valencene to reach a final concentration of 1.5 g L^{-1} or (b) adding 350 mL of orange essential oil (phase ratio of 1:1 v/v) to the culture medium to reach a final concentration of 2.7 g of (+)-valencene L^{-1} in the organic phase and 0.4 g of (+)-valencene L^{-1} in the aqueous phase. Every day, 5 mL of sample from the bioconversion media was taken and centrifuged at 5000 rpm for 10 min. Then, sesquiterpene extraction was carried out using 500 µL of the aqueous or organic phase and 500 µL of ethyl acetate. The mixture was vortexed twice for 20 s in a vortex, and settled until phase separation occurred, after that 1 µL of the ethyl phase was injected to GC [14,15].

4.2.8. Sesquiterpenes analysis

The concentrations of (+)-valencene and (+)-nootkatone were determined by gas chromatography (GC-FID) using a Perkin Elmer Auto System XL Gas Chromatograph equipped with a flame ionization detector (Cyclosil-B capillary column; 30 m x 0.32 mm x 0.25 µm; J & W Scientific). The temperatures of the injector and detector were constant at 250 °C to 270 °C, respectively. 1 µL of sample was automatically injected with a split ratio of 1:4, using helium gas as a carrier. The column temperature was initially set to 120 °C for 4 min then ramped up to 250 °C at a rate of 10 °C per min and maintained at 250 °C for 5 min, using the technique reported by Girhard et al. [14]. Analyses were performed in triplicate, and concentrations were determined using external calibration curves of (+)-valencene and (+)-nootkatone. GC/MS spectra were obtained using a GC/MS-HP6890 (Agilent) gas chromatograph equipped with a HP-5 column (30 m x 250 µm x 0.25 µm, Agilent). The same conditions of separation as for the GC-FID analysis were used. Compounds in the samples were identified by comparison of mass spectra to that of pure compounds.

4.3. RESULTS AND DISCUSIONS

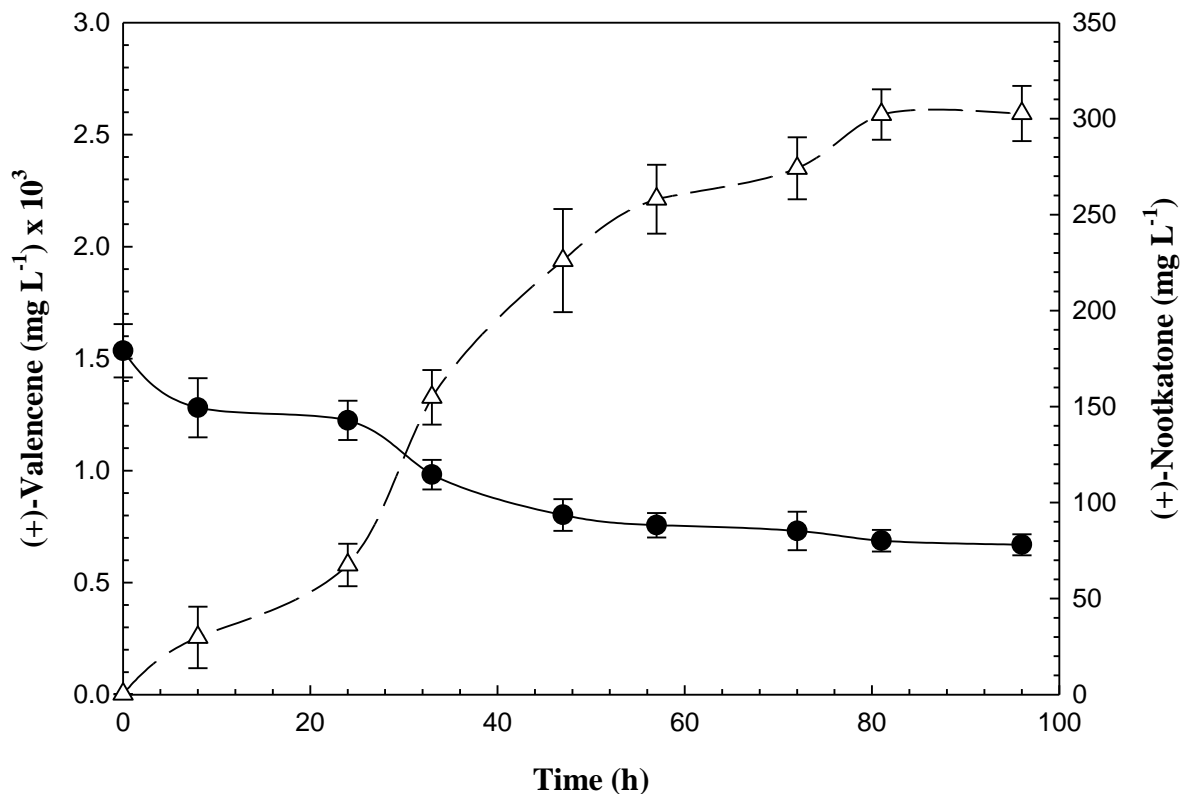*4.3.1. Bioconversion of (+)-valencene in a biphasic system with Y. lipolytica*

In order to investigate the effect of (+)-valencene on the whole bioconversion process in the bioreactor, a biphasic bioreactor was studied. Kinetics of cell growth, glucose consumption, and cell viability during the bioconversion process are plotted in Figure 4.1. After 33 h of growth, a maximum biomass concentration of 9.36 g L^{-1} was reached after approximately 90% glucose consumption. At this point, a stock solution of valencene was added to a final concentration of 1.5 g of (+)-valencene L^{-1} in the bioreactor to initiate the bioconversion process. Cell viability decreased linearly to roughly 30% between 30 and 80 h. Final cell viability might indicate that (+)-valencene or an intermediary may induce the inhibition of (+)-nootkatone bioconversion.
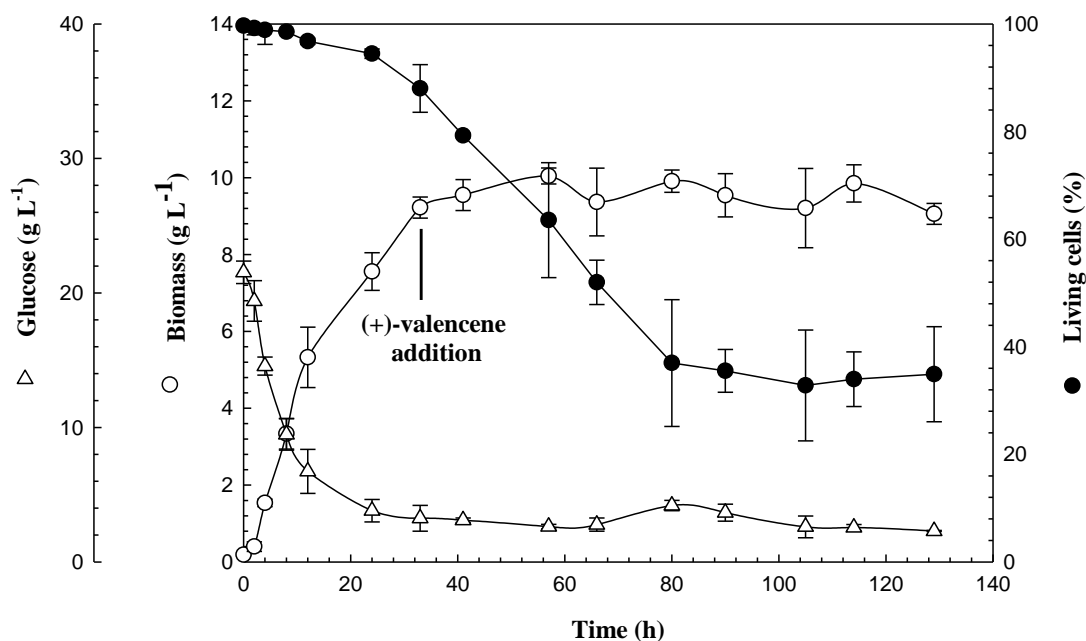
Figure 4.1. Kinetics of cell growth, glucose consumption, and cell viability in the biphasic system. 1.5 g of (+)-valencene L^{-1} was added to initiate the bioconversion process.
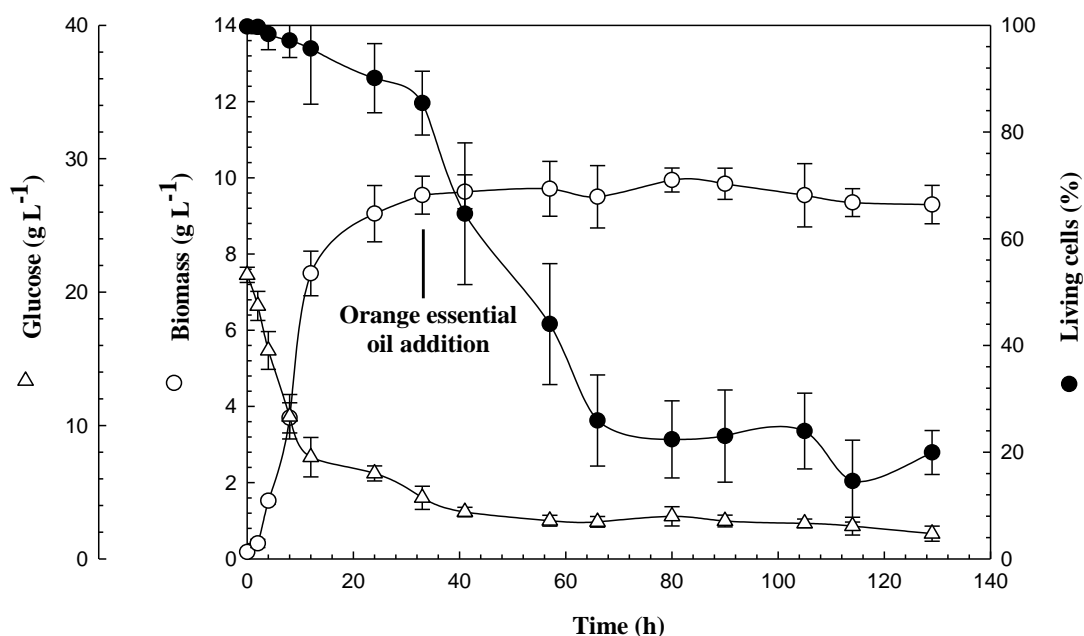
Besides, kinetics of (+)-valencene consumption and (+)-nootkatone production during the bioconversion process are plotted in Figure 4.2. After 96 h, a concentration of 302.65 mg of (+)-nootkatone L^{-1} was reached, converting 0.866 g of valencene L^{-1} (i.e. bioconversion of 32.7 %). However, a stationary phase was observed after 70 h, likely due to: loss of cell viability; alteration in the transport mechanism through the cell membrane; accumulation of intermediaries of the bioconversion reaction in the endo-membranes; or excessive product formation to potentially toxic levels.

Figure 4.2. Bioconversion of (+)-valencene to (+)-nootkatone in a biphasic system using *Y. lipolytica*. Zero time corresponds to 33 h of growth.

4.3.2. Bioconversion of (+)-valencene in a three-phase system with Y. lipolytica

Bioconversion experiments were conducted in a three-phase system using orange essential oil as a source of (+)-valencene for bioconversion to (+)-nootkatone. First, *Y. lipolytica* was grown using 20 g of glucose L^{-1} as a carbon source. Then, after 33 h, orange essential oil (50% v/v) was added as a source of (+)-valencene to produce (+)-nootkatone. Figure 4.3 shows the results of the growth kinetics, glucose concentration and cell viability during the bioconversion process. At 33 h, approximately 90% glucose consumption and a biomass of 9.28 g L^{-1} were observed. After this time, when orange essential oil was added, cell viability decreased linearly between 30 and 80 h.

Figure 4.3. Kinetics of cell growth, glucose consumption, and cell viability in the three-phasic system. Up to 50% v/v orange essential oil (3.2 g of (+)-valencene L^{-1}) were added to initiate the bioconversion process.

Cell viability value is half to the value observed from the control experiment (*see Section 5.3.4*) and 10% lower than the value observed in the biphasic system (Figure 4.1), likely due to unknown components in the orange essential oil.

During the bioconversion process in the partitioning bioreactor, the (+)-valencene present in orange essential oil was transferred by partition ($K_p = 6.37$) to the aqueous phase based on the equilibrium and the metabolic demand of the cells. Also, the (+)-nootkatone produced in the aqueous phase was mainly transferred by partition ($K_p = 22.5$) into the organic phase for an *in situ* extraction. Substrate ((+)-valencene) and product ((+)-nootkatone) concentrations in the organic phase during the bioconversion process are plotted in Figure 4.4. At 96 h, a concentration of 280.27 mg of (+)-nootkatone L^{-1} and an approximate consumption of 1.016 g of (+)-valencene L^{-1} were observed (i.e. bioconversion of 31.93 %). However, after 70 h, the bioconversion process reached a stationary phase, indicating that there was a limiting factor in the production of (+)-nootkatone.
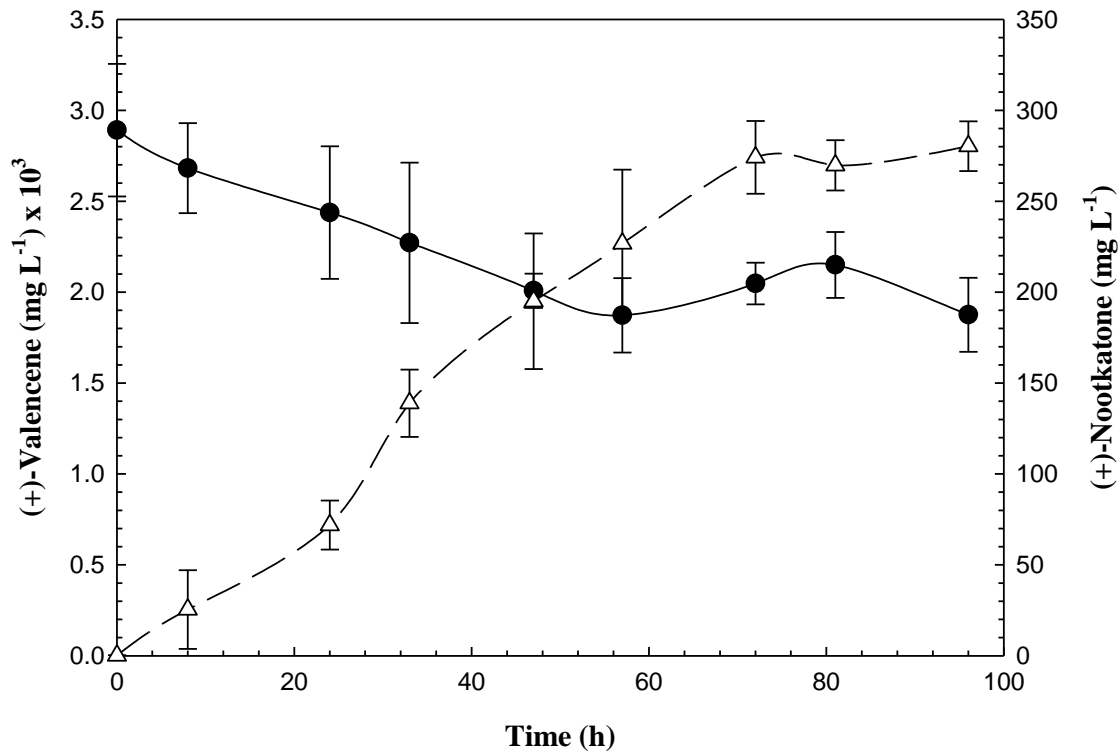
Figure 4.4. Bioconversion of (+)-valencene to (+)-nootkatone in a three-phasic system using *Y. lipolytica*. Zero time corresponds to 33 h of growth.

Based on these results, the first strategy to increase the yield of (+)-nootkatone involved increasing the amount of biomass during the bioconversion process. To this end, growth kinetics in shaken, baffled Erlenmeyer flasks were measured using various initial glucose concentrations (20 to 60 g L^{-1}) in order to determine the concentration with the greatest biomass production (Figure 4.5). Substrate inhibition was observed at concentrations of 40 g of glucose L^{-1} and greater.
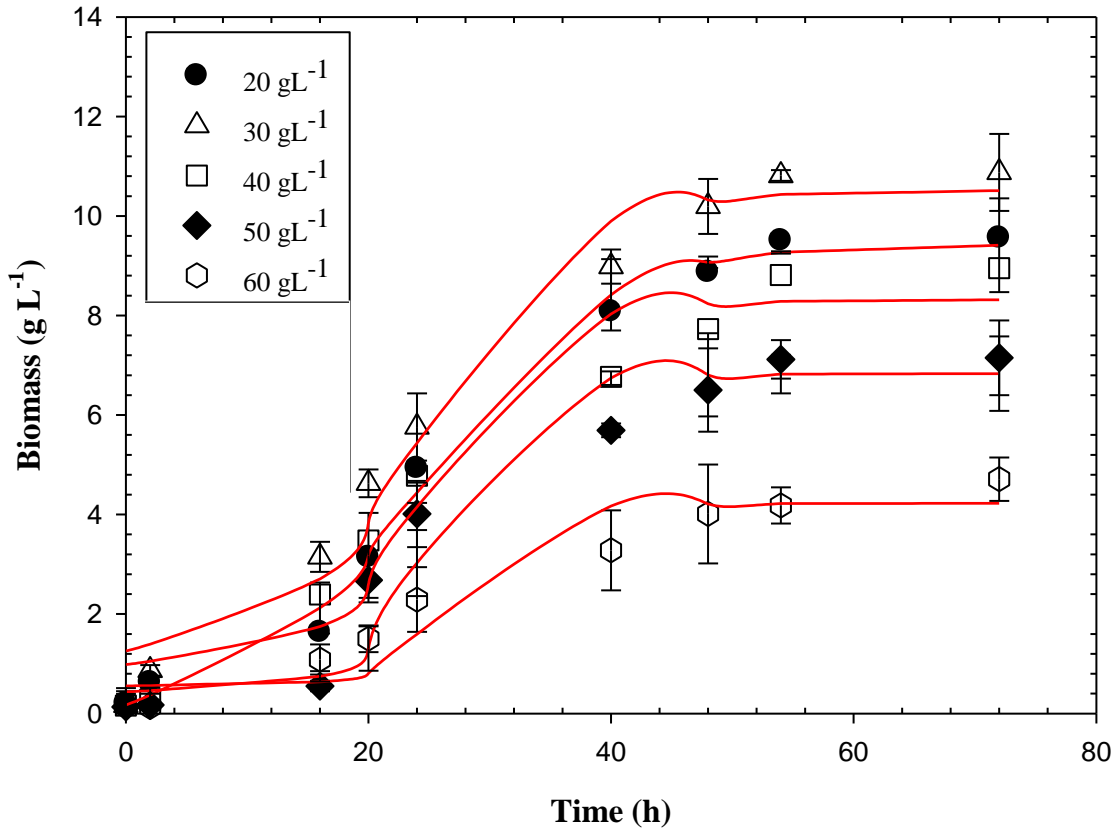
Figure 4.5. Kinetics of *Y. lipolytica* growth at different initial glucose concentration in baffled Erlenmeyer flasks at 200 rpm and 30 °C. Experimental (symbols), logistic (—). Error bars represent the standard deviation of triplicate experiments.

Experimental values of biomass production were adjusted to the integral form of the logistic model [16] using Excel Solver function (Equation 1).

$$X(t) = \frac{Xmax}{1+(C*e^{-\mu t})} \qquad\qquad \text{eq. (1)}$$

Where X is the biomass production calculated at time t (g L^{-1}); X_{max} is the maximum biomass production and it is achieved at the end of the culture (g L^{-1}); μ is specific biomass production rate (h^{-1}) and t is the time (h). Figure 4.5 displays biomass production fitted through logistic model.

In Table 4.1 the kinetic parameters estimated by the diverse growth kinetics are observed.

Table 4.1. Kinetic parameters estimated by the logistic model with different glucose concentrations.

Parameter	20 g L^{-1}	30 g L^{-1}	40 g L^{-1}	50 g L^{-1}	60 g L^{-1}
X_o (g L^{-1})	0.28	1.20	0.58	0.21	0.46
X_{max} (g L^{-1})	9.42	11.78	9.34	7.22	6.09
μ (h^{-1})	0.14	0.45	0.45	0.76	0.48
$Y_{(x/s)}$	0.48	0.43	0.23	0.15	0.08

The maximum concentration of biomass (11.5 g L^{-1}) was obtained using a concentration of 30 g of glucose L^{-1} with a biomass/substrate yield, $Y_{X/S}$, of 0.48. This yield was major than that obtained with 20 g of glucose L^{-1} ($Y_{X/S} = 0.43$). Hence, further experiments were carried out using 30 g of glucose L^{-1}. Tsigie et al. [11] worked with *Y. lipolytica* for maximum biomass production using fermentable sugars as a carbon source in shaken flasks at 150 rpm, 26°C and a pH of 6.5. The authors obtained 8.76 g of biomass L^{-1} using 20 g of sugars L^{-1} after three days, and the concentration of biomass was increased up to 10.75 g of biomass L^{-1} when using 30 g of sugars L^{-1}. Above this concentration of sugars, substrate inhibition was observed. It is well known that glucose has important effects on the regulation of carbon metabolism and other properties in yeast cells. In the presence of glucose, inductors and individual genes are subject to repression.

The effect of biomass concentration on the bioconversion process in the three-phase partitioning bioreactor was observed using 30 g of glucose L^{-1}. Maximum biomass production (11.70 g L^{-1}) was reached at 33 h of growth (Figure 4.6). At this time, orange essential oil (50% v/v) was added to the bioreactor when approximately 10 g of glucose L^{-1} still remained in the culture medium. It was also observed that the percentage of viable cells decreased, reaching 50% after 60 h and 30% at the end of the bioconversion process.
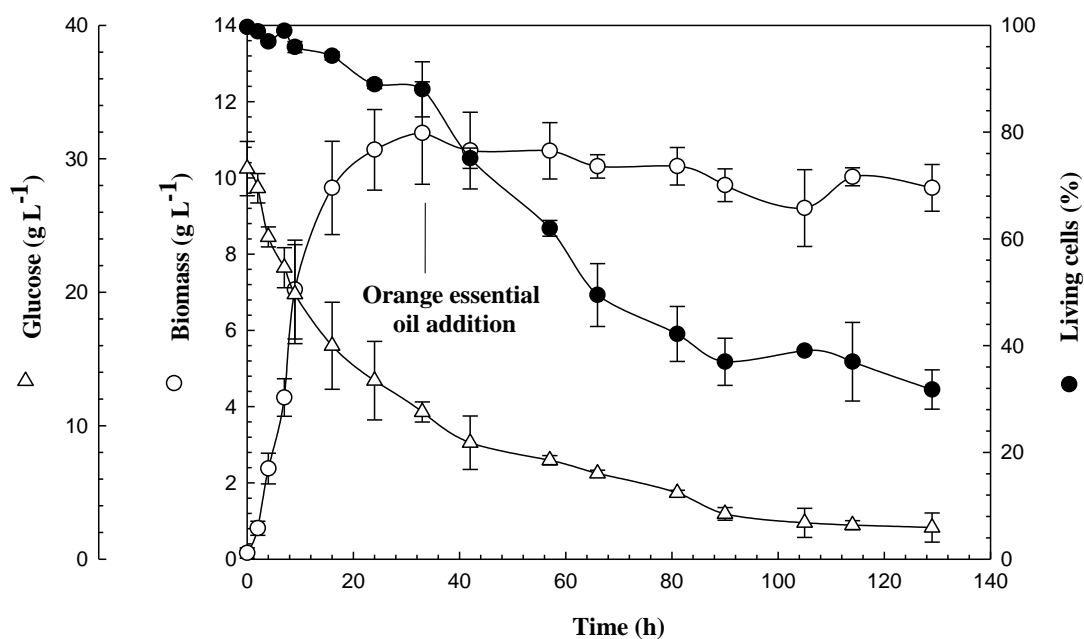
Figure 4.6. Kinetics of cell growth, glucose consumption, and cell viability in the three phasic system. 50% v/v orange essential oil (3.2 g of (+)-valencene L^{-1}) were added for the bioconversión.

Once again, this slight increase in the percentage of viable cells was not statistically different to the value obtained when 20 g of glucose L^{-1} was used. However, the production of (+)-nootkatone (Figure 4.7) peaked at 326.34 mg L^{-1} in the organic phase at 96 h, with a bioconversion of 39.08 %, showing a statistical difference with respect to maximum (+)-nootkatone concentration.
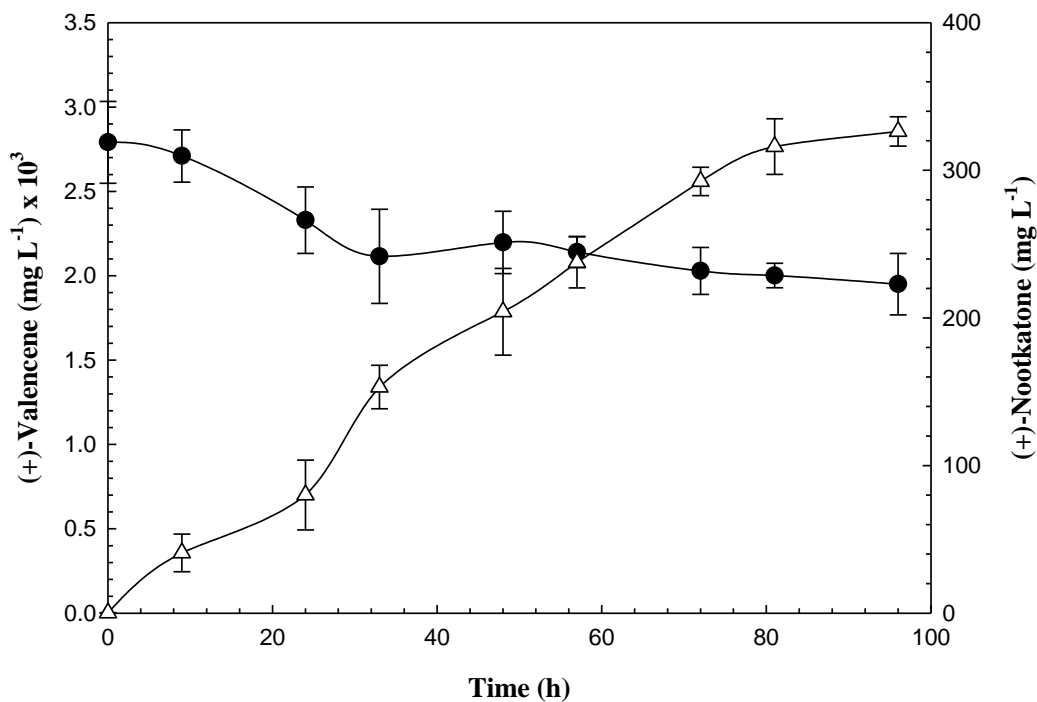
Figure 4.7. Bioconversion of (+)-valencene to (+)-nootkatone in a three phasic system using *Y. lipolytica*. Zero time corresponds to the 33 hours of growth.

Besides, it was observed that cell viability value in the three-phasic system was 10% lower than the value observed in the biphasic system (Figure 4.1); however, there was no statistical difference. Therefore, unknown components in the orange essential oil might not be as toxic as expected at the concentration used.

Specifically, in both biphasic and three-phasic systems studied in this work, after 48 h, the bioconversion process reached a stationary phase, indicating that there was a limiting factor in the production of (+)-nootkatone. Likely due to the loss of cell viability, alteration in the transport mechanism through the cell membrane, accumulation of intermediaries of the bioconversion reaction in the endo-membranes, or excessive product formation to potentially toxic levels. On the other hand, it has been reported that (+)-nootkatone production is inhibited by the intermediary nootkatol, which is an inhibitor of the P450 enzymes [18]. This phenomenon may explain the low efficiency of bioconversion. It was observed a plateau in all bioconversions after 72 h of process. Recently, several authors have reported on microbial

bioconversion of (+)-valencene to (+)-nootkatone using genetically modified yeast strains of *Saccharomyces cerevisiae* [7] and *Pichia pastoris* [19]. Gavira et al. [7] performed optimization of a bioconversion process using a recombinant yeast expressing CYP71D51v2 from tobacco and a P450 reductase from *Arabidopsis*. Yeast cells were cultivated for 24 h in the presence of increasing concentrations of (+)-valencene, β-nootkatol and (+)-nootkatone. The authors claimed that (+)-valencene was tolerated at concentrations up to 4 g L^{-1}, with a recovery of 73% of viable cells after 24 h. On the other hand, β-nootkatol and (+)-nootkatone led to a loss of cell viability at 1 g L^{-1}, with 45% viability already lost at 100 mg L^{-1} in the case of β-nootkatol. The possible reasons for the observed bioconversion efficiency to (+)-nootkatone include: a) the toxicity of products formed, b) accumulation of β-nootkatol in the endo-membranes of yeast, c) inhibition of the CYP71D51v2 hydroxylation reaction by the products, and d) the fact that the formation of nootkatone from β-nootkatol is not dependent from cytochrome P450, but it is catalysed by an unidentified enzyme in yeast [7]. There are few reports characterizing the process in a multiphasic partitioning bioreactor.

Wriessnegger et al. [20] generated by metabolic engineering a strain of *Pichia pastoris* co-expressing the premnaspirodiene oxygenase of *Hyoscyamus muticus* (HPO) and the *Arabidopsis thaliana* cytochrome P450 reductase (CPR) that hydroxylated extracellularly added (+)-valencene. Intracellular production of (+)-valencene by co-expression of valencene synthase from *Callitropsis nootkatensis* resolved the phase-transfer issues of (+)-valencene. Biphasic cultivations of *P. pastoris* resulted in the production of trans-nootkatol, which was oxidized to (+)-nootkatone by an intrinsic *P. pastoris* activity. The over expression of a *P. pastoris* alcohol dehydrogenase and truncated hydroxy-methylglutaryl-CoA reductase (tHmg1p) enhanced, significantly, the (+)-nootkatone yield to 208 mg L^{-1} with 0.5 g L^{-1} of (+)-valencene in fed-batch cultivations after 108 h. The (+)-nootkatone was recovered *in situ* using 10 % (v/v) of n-dodecane as organic phase. Girhard at al. [14] also studied this bioconversion process using a two-phase system with 10% dodecane as the organic phase in a stirred tank bioreactor with a recombinant strain of *Escherichia coli*. An initial concentration of 409 mg of (+)-valencene L^{-1} was used in a relatively short time (8 h), 94.2 mg of (+)-nootkatone L^{-1} was produced with a total bioconversion of 23%. As much as 80% cell death was also observed using other organic solvents, such as isooctane and n-

octane, due to the significant toxicity of these solvents. In contrast, cell viability remained at 90% during the bioconversion process upon use of the organic solvent dodecane.

Fungi extracellular enzymes have been described for the bioconversion of (+)-valencene. Fraatz et al. [4] confirmed the dioxygenase nature of the enzyme of *Pleurotus sapidus* that catalyze (+)-valencene oxidation. The isolation and identification of two allylic (+)-valencene derived hydroperoxides together with homology data from amino acid sequencing suggested a lipoxygenase-like type of enzyme [21].

To our knowledge our research group is the first one reporting a wild type *Y. lipolytica* with the enzymatic machinery to carry out this bioconversion. It has been shown that, the alkane-assimilating yeast *Yarrowia lipolytica* degrades very efficiently hydrophobic substrates such as n-alkanes, fatty acids, fats and oils for which it has specific metabolic pathways [22]. In this regard, cell hydrophobicity must be considered as a key criterion to select effective microorganisms for three-phase partitioning bioreactors devoted to transform hydrophobic substrates [23].

After (+)-valencene bioconversion in biphasic and three-phasic system, analysis by GC revealed several new peaks as well as the substrate peak (Figure 4.8). Based on predicted fragmentation and comparison to authentic reference compounds, three peaks were identified as (+)-valencene (retention time, $t_R = 8.9$ min), nootkatol ($t_R = 10.9$ min) and (+)-nootkatone ($t_R = 12.8$ min). In the chromatogram of the three-phasic system, it is observed that some peaks present in the orange essential oil increased after bioconversion, and new peaks were observed with different retention times to those existing in the orange essential oil, possibly representing products of over-oxidation.

The performance of the bioconversion process in both biphasic and three-phasic systems was analysed in terms of reaction rate, volumetric productivity and product/biomass yield, and nootkatone maximum concentration (Table 4.2). At similar biomass concentrations using 20 g of glucose L^{-1}, there was a non-significant statistical difference for all of the values obtained. The highest mean values were obtained in the biphasic system when non-inhibitory

initial concentrations of (+)-valencene were used. Girhard et al. [14] observed a similar trend in bioconversion per cent and volumetric productivity; for instance: for the biphasic system, 24.7% and 12.97 mg of nootkatone L^{-1} h^{-1}, and for the triphasic system, 23% and 11.77 mg of nootkatone L^{-1} h^{-1}, respectively. These volumetric productivity values are as much as 4-fold greater than those observed in the current work. However, volumetric productivity reported by Girhard et al. [14] accounted for the production of both nootkatol and (+)-nootkatone, while the current study only investigated (+)-nootkatone concentration.

In the three-phasic system, volumetric productivity (3.58 mg L^{-1} h^{-1}) was only 15% lower than that of the biphasic system (4.19 mg L^{-1} h^{-1}). Nevertheless, adding 50% v/v of orange essential oil with an initial 30 g of glucose L^{-1}, resulted in a 7.3% reduction in volumetric productivity after 96 h of bioconversion due to the increase in *Y. lipolytica* 2.2ab biomass.

Alcohol and ketone sesquiterpenes differ from acids, such as artemisinic acid, which get trapped in the outer surface of the cell wall of yeast under conditions of acid fermentation [24], in that they do not accumulate in the endo-membranes or exhibit considerable toxicity to yeast [18]. Accumulation of β-nootkatol in yeast membranes and binding to the active site of the enzyme CYP71D51v2 by differential spectrophotometry strongly support that inhibition of the enzyme during the process of bioconversion [7].

Comparing both three-phasic systems (20 and 30 g of glucose L^{-1}), it can be observed that by using an initial amount of 30 g of glucose L^{-1}, an increment of 16.44% in volumetric productivity after 96 h of bioconversion was evident due to the increase in *Y. lipolytica* biomass. A similar tendency was observed with maximum nootkatone concentration obtained. Despite the fact that the nootkatone/biomass yield was lower in the three-phasic system using 30 g of glucose L^{-1} (27.89 mg g^{-1}), this yield was 21.8 times higher than that (1.28 mg g^{-1}) observed by Girhard et al. [14] under optimized conditions using recombinant *E. coli* with 10% dodecane and 409 mg of (+)-valencene L^{-1}. A primary drawback mentioned by the authors was the accumulation of high concentrations of β-nootkatol in cellular membranes, leading to alterations in membrane permeability, as well as the function of integral proteins, similar to that which has been observed with other hydrocarbons that are concentrated in membranes [25,26]. Accumulation of products, in particular β-nootkatol is thus likely to be an important factor limiting rates and yields during the process of bioconversion [7].
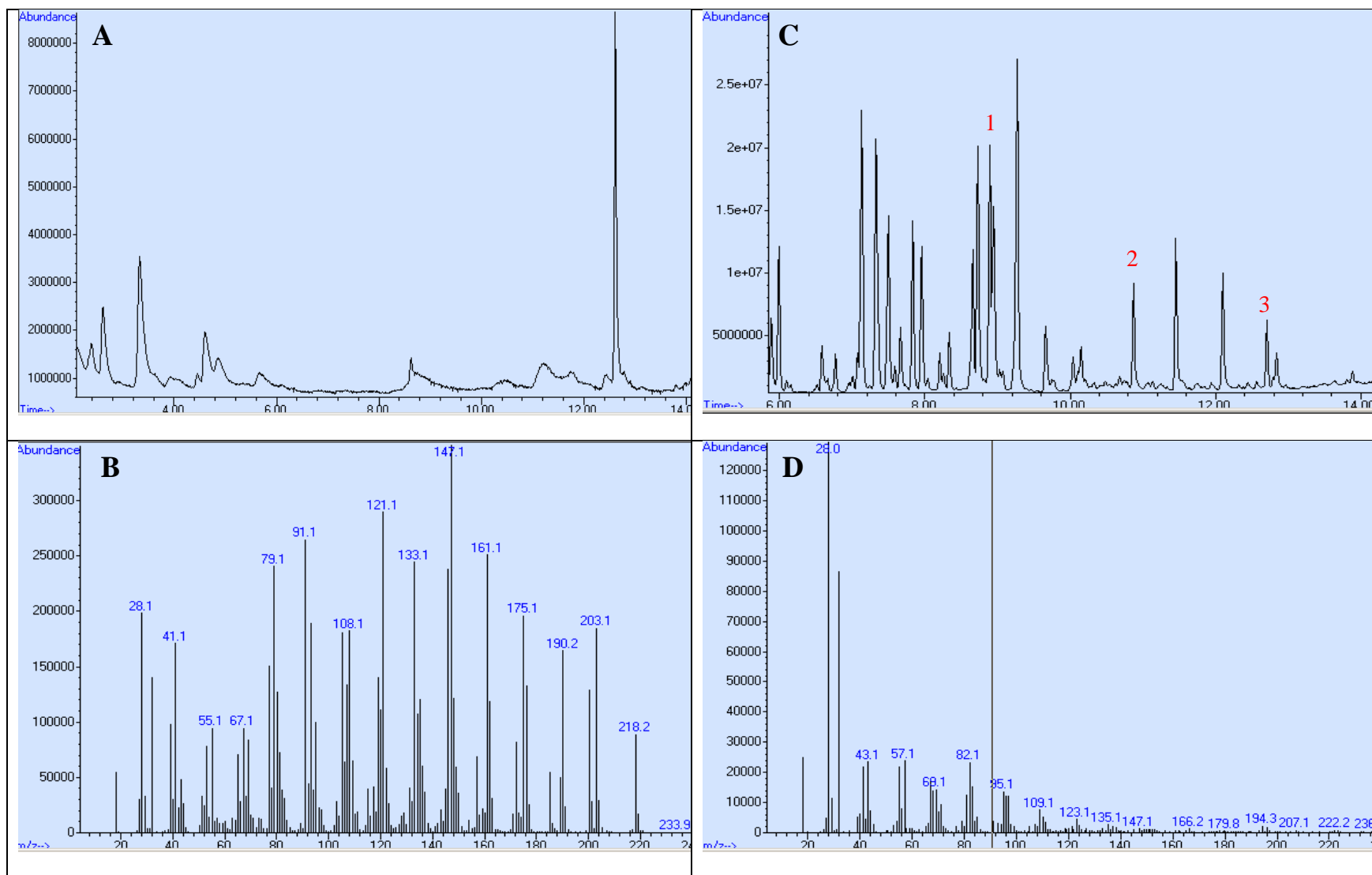
Figure 4.8. GC-MS chromatograms (A and C) for standards and (+)-valencene bioconversion using *Y. lipolytica* a three-phasic system, respectively. And mass fragmentation patterns (B and D) for (+)-nootkatone as a standard and after the bioconversion. (+)-valencene (1), (+)-nootkatone (2), nootkatol (3).

Table 4.2. Bioconversion performance of (+)-valencene to (+)-nootkatone by *Y. lipolytica* at 30°C

System	Biomass (g L^{-1})	Reaction rate (mg nootkatone L^{-1} h^{-1})[iii]	Bioconversion (%)[ii]	Volumetric productivity (mg L^{-1} h^{-1})[iii]	Nootkatone/Biomass Yield (mg g^{-1})	Maximum Nootkatone (mg L^{-1})[iv]
Biphasic (20 g L^{-1})[i,v]	9.36 ± 0.18 (b)	6.74 ± 1.27 (a)	32.7 ± 3.0 (a)	3.15 ± 0.15 (a)	32.32 ± 1.1 (a)	302.7 ± 14.4 (a,b)
Biphasic (30 g L^{-1})[i,v]	11.40 ± 0.98 (a)	3.90 ± 0.20 (b)	35.67 ± 6.77 (a,b)	3.63 ± 0.13 (a,b)	30.63 ± 1.3 (a,b)	348.5± 13.06 (a)
Three-phasic (20 g L^{-1})[i,v]	9.28 ± 0.06 (b)	5.23 ± 1.90 (a)	31.93 ± 18.0 (a,b)	2.92 ± 0.14 (a)	30.20 ± 1.6 (a,b)	280.3 ± 13.7 (b)
Three-phasic (30 g L^{-1})[i,v]	11.70 ± 1.41 (a)	4.99 ± 1.75 (a)	39.08 ± 12.23 (a)	3.40 ± 0.10 (a)	27.89 ± 3.1 (b)	326.3 ± 9.9 (a)

[i] Concentration of initial glucose in the growth medium of *Y. lipolytica.*

[ii] Percentage of (+)-nootkatone produced relative to (+)-valencene consumed based on molecular masses.

[iii] mg of nootkatone L^{-1} produced between 24 and 48 h.

[iv] mg of maximum nootkatone L^{-1} produced after 96 h.

[v] values represent mean ± standard deviation.

Same letters in the same column represent no significant differences (p ≤ 0.05; Tukey, SAS System Version 9).

Based on the aforesaid, some strategies to improve the bioconversion process have been revised and could be studied in future investigations. Some of them include expressing a specific dehydrogenase in *Y. lipolytica* to oxidize nootkatol to nootkatone, thus regenerating NADH, which can also reduce metabolic stress in the strain [7]. Another alternative is to increase the permeability of the cell membrane using a solvent and a selective transporter of nootkatol and (+)-nootkatone in order to avoid toxic product accumulation in the endo-membranes.

4.4. CONCLUSIONS

The addition of a second liquid phase (orange essential oil) for the oxidation of (+)-valencene to (+)-nootkatone by the yeast *Y. lipolytica* 2.2ab in a stirred tank partitioning bioreactor represents a potential application for *in situ* extraction of (+)-nootkatone. A higher concentration of biomass (11.5 g L^{-1}) and final cell viability (30%) was achieved by using a concentration of 30 g of glucose L^{-1} as carbon source. Under this conditions, a (+)-nootkatone production of 285 mg L^{-1} with 33.6% of bioconversion, was observed. The three phase bioconversion system used provides easy control and scalability, presenting an attractive approach for production and *in situ* recovery of (+)-nootkatone.

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CHAPTER V

WHOLE CELL BIOCONVERSION OF (+)-VALENCENE TO (+)-NOOTKATONE BY *YARROWIA LIPOLYTICA* USING A THREE PHASE PARTITIONING BIOREACTOR

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WHOLE CELL BIOCONVERSION OF (+)-VALENCENE TO (+)-NOOTKATONE BY *YARROWIA LIPOLYTICA* USING A THREE PHASE PARTITIONING BIOREACTOR

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ABSTRACT

Low permeability of substrates across the cell membrane, cofactor regeneration and product inhibition are some drawbacks during (+)-nootkatone bioconversion. The aim of this work was to evaluate and enhance the bioconversion of (+)-valencene to (+)-nootkatone with *Yarrowia lipolytica* in a partitioning bioreactor using orange essential oil as the dispersed phase. Preliminary experiments in shake flasks allowed enhancing (+)-nootkatone bioconversion to obtain favorable operating conditions (0.2 % w/v of CTAB and 2.0 mM of niacin and 11.5 g L^{-1} of biomass) to produce 420.9 mg L^{-1}. Bioreactor experiments in a two-phase system using 0.2 % (w/v) of CTAB, 2.0 mM of niacin and 22.5 g L^{-1} of biomass produced a maximum (+)-nootkatone concentration of 619.8 mg L^{-1} which was around the product inhibition concentration. Nevertheless, the partitioning three-phase system using orange essential oil, overcome product inhibition obtaining concentrations of 852.3 mg L^{-1}. This is the first report of a wild type *Y. lipolytica* with the enzymatic machinery to carry out this bioconversion. Multiphase partitioning bioreactor conception seems to have a good potential in enhancing the productivity of (+)-nootkatone. The bioconversion approach presents an attractive way to produce and recover (+)-nootkatone *in situ* using a natural (+)-valencene source.

Keywords: Bioconversion, Partitioning bioreactor, (+)-nootkatone, orange essential oil, Yeast, Product inhibition

5.1. INTRODUCTION

The compound (+)-nootkatone is a sesquiterpenoid which possesses an intense grapefruit-like taste and other valuable properties that are exploited by the fragrance and flavour industries.[1] The production of (+)-nootkatone is performed via chemical synthesis, mainly from the sesquiterpene (+)-valencene, which is readily available from the orange industry and also through the use of environmentally unfriendly oxidising agents, such as tert-butyl peracetate[2] and tert-butyl hydroperoxide in combination with catalytic metal supported on silica.[3] However, the resulting (+)-nootkatone produced via chemical synthesis cannot be marketed as a "natural" product and does not satisfy increasing market demands for natural aromatic compounds. In order to meet this demand, many efforts have been focussed on the use of biotechnological processes with bacteria, fungi or plants.[4] Some researchers have investigated genetically modified microorganisms, and several published reviews[5,6] have provided an overview of recently acquired knowledge about whole cell bioconversions and future industrial applications. Particularly, industry and academia are interested on a biocatalytic process using whole cells which involves the selection of an optimal cellular enzyme, reaction engineering, product recovery and scaling-up.

Gavira et al.[7] reported a bioconversion process for (+)-nootkatone production using plant enzymes expressed in *Saccharomyces cerevisiae*. Nevertheless, the process was inhibited by both the substrate (>4 g L^{-1}) and the product (1.0 g L^{-1}) as well over-oxidation of (+)-valencene. Wriessnegger et al.[8] resolved the phase-transfer issues caused by the external addition of (+)-valencene using intracellular production of (+)-valencene by co-expression of valencene synthase from *Callitropsis nootkatensis* through two-phase cultivations of *Pichia pastoris*. Oppermann et al.[9] reviewed the use of biocompatible organic solvents in two-phase systems and their application in purification, extraction and bioconversion. However, it must be noted that bioconversion productivity of sesquiterpenes in a multi-phase system can be limited by low mass transfer rates at interfaces (gas-liquid or liquid-liquid). Partitioning three-phase bioreactors (gas-liquid-liquid) for oxidative bioconversions[10] have shown significant potential for improving the productivity of many biological processes by overcoming problems related to low solubility or the toxicity of substrates and products.[11] In this regard, essential oils can be used as both substrate reservoir (dispersed phase) and *in situ*

extraction agent in a partitioning bioreactor, thereby increasing the efficiency of the process.[12] Essential oils are dominated by monoterpene hydrocarbons, which are regarded as process waste, mainly because of their low sensory activity, low water solubility and tendency to autoxidise and polymerise;[13] which turns terpene hydrocarbons, such as limonenes, pinenes and sesquiterpinenes, into ideal starting materials for microbial transformations. Maróstica and Pastore[14] previously used industrial waste orange essential oil as a source of R-(+)-limonene, in the biotransformation of R-(+)-α-terpineol with *Fusarium oxysporum*, obtaining a final yield of 450 mg L^{-1}.

This work is aimed to evaluate the enhancement of (+)-nootkatone production from (+)-valencene on a non-previously studied *Yarrowia lipolytica* yeast in a three-phase (aqueous-organic-air) partitioning bioreactor using orange essential oil as a cheap source of (+)-valencene. As a first evaluation of the process, experiments using 1.0 L partitioning bioreactor were conducted in a three-phase system using orange essential oil as disperse phase. To enhance the bioconversion, experiments using baffled Erlenmeyer flasks were conducted to study the effect of cell membrane permeability and NADPH cofactor regeneration on (+)-nootkatone production. Then, the three-phase partitioning bioreactor was once more operated but at optimal operating conditions with the aim of elucidating the potential role of the essential oil overcoming bioconversion inhibition from either (+)-nootkatone or (+)-valencene. Finally, in order to highlight results obtained from the three phase partitioning technology, (+)-nootkatone production is compared to that obtained from a conventional two-phase (aqueous-air) reactor wherein yeast inhibition takes place.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals

(+)-valencene (CAS 75-05-6) and (+)-nootkatone (CAS 93-78-5) with a purity >70% and >85%, respectively were purchased from Fluka (Switzerland) and used as standards. Ethyl acetate (99.5%) used for sesquiterpene extraction was purchased from Quimex (México). Analytical grade orange essential oil (*Citrus aurantium*, var. Amara) was purchased from a local drugstore in Mexico DF) and used as an organic dispersed phase to carry out three-phase bioconversion studies.

5.2.2. Microorganism, culture media and inoculum

Yarrowia lipolytica was previously selected for its capability to convert (+)-valencene to (+)-nootkatone from a screening among 6 selected strains (fungi and yeasts). *Y. lipolytica* was isolated from copra meal that was provided by the Laboratory of Bio-Process of the University of Guadalajara (U de G), Mexico. To the best of our knowledge, *Y. lipolytica* is used for the first time to study this bioconversion. This strain was previously sequenced in the Laboratory of Industrial Biotechnology of the National Polytechnic Institute (CBG-IPN), Mexico. The access in the National Center for Biotechnology Information (NCBI) is EF643594.1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence, identities 94 % with *Y. lipolytica* strain LN-13. The strain was maintained in potato dextrose agar (PDA) at 4 °C, and its cultivation was propagated in 125-mL Erlenmeyer conical flasks containing 30 mL of PDA before being incubated at 30 °C for 4 days. Cells were harvested with 25 mL 0.01% Tween-80 and used as inoculum during bioconversion experiments (stock solution). Culture medium for *Y. lipolytica* was composed of (g L^{-1}): Glucose, 30; Na_2MoO_4, 0.2; $MnSO_4$, 0.4; $FeCl_3$, 0.2; KI, 0.1; $CuSO_4$, 0.04; H_2BO_3, 0.5; $CaCl_2$, 0.1; NaCl, 0.1; $MgSO_4*7H_2O$, 0.5; KH_2PO_4, 1; $ZnSO_4$, 0.4; $(NH_4)_2SO_4$, 5; and yeast extract, 4.0.[15] Prior to sterilization, the pH of culture medium was adjusted to 5.5 using 2 M HCl. For experiments in baffled conical flasks, 100 µL of *Y. lipolytica* cell suspension ($1x10^6$ cells mL^{-1}) was added as inoculum to 100 mL of culture medium. Flasks were incubated at 30°C for 4 days on a rotary shaker at 200 rpm. For bioreactor experiments, 700 µL of inoculum was added to 350 mL of sterile culture medium.

5.2.3. Inhibition experiments

To study the inhibition effect of (+)-valencene and (+)-nootkatone on the bioconversion process, experiments were carried out using baffled Erlenmeyer flasks at different initial concentrations of these components.

5.2.3.1. Substrate inhibition

Baffled Erlenmeyer flasks containing 100 mL of culture media were used during inhibition experiments. Each baffled Erlenmeyer flask was inoculated with $1x10^6$ cells mL^{-1} and

incubated for 4 days at 30 °C. After these days, flasks were supplied with different concentrations of (+)-valencene (0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 3.2, 4.5 and 6 g L^{-1}) then incubated at 30 °C for 4 days to allow time for the bioconversion process to occur. Every day, three baffled Erlenmeyer flasks were taken and centrifuged at 5000 rpm for 10 min. All the experiments were conducted in triplicate. From the supernatant, sesquiterpene extraction was carried out by adding 500 µL of ethyl acetate to 500 µL of sample. The mixture was vortexed twice for 20 s in a vortex, and settled until phase separation occurred, after that 1 µL of the ethyl phase was injected to Gas Chromatography (GC) in order to determine the concentration of (+)-valencene and (+)-nootkatone.[16,17]

5.2.3.2. Product inhibition

Baffled Erlenmeyer flasks containing 100 mL of culture media were used during inhibition experiments. Each baffled Erlenmeyer flask was inoculated with $1x10^6$ cells mL^{-1} and incubated for 4 days at 30 °C. After 4 days, flasks were supplemented with different initial concentrations of (+)-nootkatone: 50, 100, 200, 300, 400, 600 and 1000 mg L^{-1}. The flasks were incubated for 4 days to carry out the bioconversion process. Control experiments containing sterile broth without inoculum were also conducted. Samples (2.0 mL) were taken every 24 h, and the concentrations of (+)-valencene and (+)-nootkatone were determined. All the experiments were conducted in triplicate. Every day, three Erlenmeyer flasks were taken and centrifuged at 5000 rpm for 10 min. From the supernatant, (+)-valencene and (+)-nootkatone were extracted and analysed as explained above.

5.2.4. Cell membrane permeabilization and cofactor regeneration

Two types of experiments were considered to enhance bioconversion and bio-selectivity of the desired product. On one hand, experiments of cell membrane permeabilization using Triton X-100 (0.05-1.0% v/v),[18] Cetyl trimethylammonium bromide (CTAB) (0.1-0.4% w/v),[19] and by sonication (0.25-1.5 h)[20] were conducted in 250 mL Erlenmeyer flasks. On the other hand, experiments for the cofactor NADPH regeneration adding glycerol (0.05-1.0% v/v)[21] and vitamin B_3 niacin (0.5-10 mM)[22] were conducted in Erlenmeyer flasks. Besides, two addition times were studied: when *Y. lipolytica* was inoculated and after 3 days of growth. The bioconversion began when 1.5 g L^{-1} of (+)-valencene (CAS 75-05-6) was

added. Each experiment was performed in triplicate, with their respective positive control only adding (+)-valencene. The results were analyzed by the Tukey´s HSD (Honestly Significant Difference) test (p ≤ 0.05) using the SAS software (Version 9). A qualitative analysis was performed by GC/MS to determine the % of the intermediate (+)-nootkatol excreted to the reaction medium during the bioconversion.

5.2.5. Analytical techniques

5.2.5.1. Biomass determination

Biomass determination was carried out by measuring the dry weight of cells. Samples of 5.0 mL were dried to a constant weight in metal plates which had been previously weighed after being left in an oven at 90 °C overnight.

5.2.5.2. Glucose consumption

Glucose consumption of *Y. lipolytica* was determined by analyzing the glucose concentration in the medium using the dinitrosalicylic acid method described by Miller et al [23] for reducing sugar, and a UV spectrophotometer at a wavelength of 550 nm.

5.2.5.3. Cell viability

Cell viability during process including growth was determined by measuring the concentration of living cells using the methylene blue (MB) dye, as described by Bonora and Mares.[24] Culture samples (around 5 mL) were aseptically centrifuged at 8,000 rpm for 10 min. Cells were suspended and centrifuged in an equal amount of phosphate buffered saline pH 8 (PBS). PBS was used as a diluent. The MB dye was added to sample during 6 min and suspended again in PBS in the same conditions. The living cells were calculated using time course measurement mode at 640 nm.

5.2.5.4. Protein quantification

Protein concentration released into the medium due to cell lysis was determined according to the procedure of Bradford,[25] using bovine albumin serum as the standard. Maximum protein concentration was quantified via a cell rupture mechanical method as follow: a 5 mL stainless steel shaking flask (Sartorius Göttingen, Germany) containing 0.5 g of biomass with 0.2 g of

glass pearls of 0.25 mm diameter was placed in a Mikro-Dismembrator U (Sartorius 37070 Göttingen, Germany) and agitation was kept for 10 min, according to the technique described in Becerra et al. [26]

All the analyses were performed in triplicate

5.2.5.5. Sesquiterpene analysis

The concentrations of (+)-valencene and (+)-nootkatone were determined by gas chromatography (GC-FID) using a Perkin Elmer Auto System XL Gas Chromatograph equipped with a flame ionization detector (Cyclosil-B capillary column; 30 m x 0.32 mm x 0.25 μm; J-&-W Scientific Inc., CA). The temperatures of the injector and detector were constant at 250 °C to 270 °C, respectively. During the analysis 1 μL of sample was automatically injected with a split ratio of 1:4, using helium gas as a carrier. The oven temperature was initially set to 120 °C for 4 min then ramped up to 250 °C at a rate of 10 °C per min and maintained at 250 °C for 5 min, using the technique reported by Girhard et al.[16] Analyses were performed in triplicate, and concentrations were determined using external calibration curves of (+)-valencene and (+)-nootkatone. GC/MS spectra were obtained using a GC/MS-HP6890 (Agilent) equipped with a HP-5 column (30 m x 250 μm x 0.25 μm, Agilent). The same conditions of separation as for the GC-FID analysis were used. Compounds in the samples were identified by comparison of mass spectra with that of pure compounds.

5.2.6. Stirred tank bioreactor for two-phase and three-phase bioconversions

A glass 1.0 L stirred tank bioreactor (model ADI 1025, Applikon) was used for bioconversion processes. The bioreactor had an inside diameter of 9.5 cm and an operation volume of 0.7 L ($H_L/D_T = 0.96$). The bioreactor was equipped with a single Rushton turbine with 6 paddles, $D_i = 4.53$ cm (Di/DT = 0.46), located 4.53 cm from the base of the container. The bioreactor had 2 equidistant deflectors of 1.0 cm in width to improve liquid-liquid mixing. Agitation for yeast growth and bioconversion were maintained at 300 and 200 rpm, respectively. Temperature was maintained at 30°C. The pH was monitored using an AppLiSens Z001023511 pH electrode (Applikon) and controlled at 5.5 using 2 M HCl. Atmospheric air entering the system was filtered through a sterile 0.22 μm filter. From previous experiments,

the air flow rate in the bioreactor for yeast growth and bioconversion were maintained at 1.0 and 0.5 vvm, respectively, through a perforated "L" tube with seven holes of 1.0 mm in diameter. A refrigerant was located at the air exit to avoid sesquiterpenes evaporation from the bioreactor.

5.2.7. Two-phase (aqueous-air) and three-phase (aqueous-organic-air) bioconversions
Bioconversion studies were conducted after the growth phase of *Y. lipolytica* (33 h), by (a) adding a concentrated solution of (+)-valencene to reach a final concentration of 1.5 g L^{-1} or (b) adding 350 mL of orange essential oil (phase ratio of 1:1 v/v) to the culture medium to reach by partition a final concentration of 2.7 g of (+)-valencene L^{-1} in the organic phase and 0.4 g of (+)-valencene L^{-1} in the aqueous phase. Similar phase ratio was used during the screening in serological flasks. A control without the addition of (+)-valencene was included using 30 g of glucose L^{-1}, which was performed in duplicate, in order to determine the effect of adding valencene and other components of orange essential oil to the culture medium. Every day, 5 mL of sample from the bioconversion media was taken and centrifuged at 5000 rpm for 10 min. Then, sesquiterpene extraction was carried out using 500 µL of sample from either aqueous or organic phase and 500 µL of ethyl acetate. The mixture was vortexed twice for 20 s in a vortex, and settled until phase separation occurred, after that 1 µL of the ethyl phase was injected to GC.[16,17]

5.2.8. Determination of partition coefficients
Partition coefficients of (+)-valencene and (+)-nootkatone between a buffer solution (aqueous phase) and orange essential oil (organic phase) were determined taking into account the phase ratio (1:1 v/v) and the initial (+)-valencene concentration conditions used in the three-phase partitioning bioreactor. Therefore, the aqueous phase was prepared as follows: 3.2 g L^{-1} of nootkatone was dissolved in a 0.1 M phosphate buffer adjusted to pH 7.5. Also, the organic phase was orange essential oil containing 3.2 g L^{-1} of (+)-valencene. Then equal amounts of both solutions were mixed, yielding a final solution with a total volume of 6 mL. The mixture was subjected to mechanical agitation for 6 h at 30 °C in order to reach thermodynamic equilibrium. The phases were then separated by centrifugation. (+)-valencene and (+)-nootkatone were extracted from the aqueous and organic phases,

respectively using 500 μL of ethyl acetate. Both phases were analysed quantitatively by GC and the partition coefficient (K_p) was subsequently determined. The partition coefficient was calculated as the ratio of the solute concentration in the organic phase divided by the solute concentration in the aqueous phase at equilibrium.

5.3. RESULTS AND DISCUSSION

5.3.1. Sesquiterpenes in orange essential oil (*Citrus aurantium*, var. amara)

GC-MS analysis were performed for (+)-valencene and (+)-nootkatone standards and orange essential oil to verify the presence of (+)-valencene in orange essential oil. Concentrations of (+)-valencene and (+)-nootkatone in orange essential oil were determined to be 3.2 g L^{-1} and 0.94 mg L^{-1}, respectively. The (+)-valencene concentration obtained was similar to that reported by Del Rio et al.[27] being 2.99 g L^{-1} for the same variety of orange oil. Depending on the harvest of the fruit and its specific variety, the concentration of sesquiterpenes varies for different species of the *Citrus* genus.[28] The highest concentration reported for (+)-valencene was 6 g L^{-1} for the species *sinensis*.[29]

5.3.2. Partition coefficients

The (+)-valencene partition coefficient (K_p) obtained was 6.37, which was 3.5 times lower than the partition coefficient obtained for (+)-nootkatone. During the bioconversion process, (+)-valencene is transferred from the organic phase to the aqueous phase based solely on partition and metabolic demands of the cells transferring the substrate to the cell at sub-inhibitory levels. On the other hand, (+)-nootkatone is transferred from the aqueous phase to the organic phase. The high (+)-nootkatone K_p value implies a good affinity of (+)-nootkatone for orange essential oil (organic phase), which is advantageous for *in situ* recovery in a three-phase partitioning bioreactor and hence avoiding (+)-nootkatone inhibition.

5.3.3. Inhibition experiments

5.3.3.1. Substrate inhibition

Recently, Liu et al.[30] showed that (+)-valencene contained in sweet orange essential oil inhibited the growth of five different microorganisms. The inhibition effect of initial (+)-

valencene concentration on the bioconversion process was evaluated in baffled Erlenmeyer flasks (Figure 5.1A). Maximum final (+)-nootkatone concentration was observed using 1.5 g L^{-1} of (+)-valencene. Above this concentration, an inhibitory effect was evident. In order to determine the maximum (+)-valencene concentration per gram of biomass to be under non-inhibitory conditions during the bioconversion process in the bioreactor, the biomass concentration in the Erlenmeyer flasks was determined. The average concentration obtained was 10.5 g L^{-1} of biomass. This leads to a value of 0.14 g of (+)-valencene per g of biomass, which was similar to the concentrations used in the stirred thank bioreactor experiments. In orange essential oil, there are a large number of compounds used in the food and beverage industry as antimicrobial agents.[31] Limonene, a terpene with the highest antimicrobial activity, is the most abundant compound. Besides, it has been reported that sesquiterpenes have antifungal activity,[32,33] and their biosynthesis is often induced after fungal infection.[34,35,36]

5.3.3.2. Product inhibition

In order to determine product inhibition, further experiments were carried out by adding different amounts of concentrated solution of (+)-nootkatone to culture medium, resulting in different initial concentrations. Figure 5.1B shows a decrease in (+)-nootkatone produced when initial (+)-nootkatone added was above 400 mg L^{-1}, indicating that *Y. lipolytica* present product inhibition above 34.8 mg of (+)-nootkatone (g of biomass)$^{-1}$. Studies performed by Gavira et al.[7] with *S. cerevisiae* show that a concentration of 1.0 g of (+)-nootkatone L^{-1} results in product inhibition and loss of cell viability. Nevertheless, as observed, the studied yeast starts to present product inhibition at a lower (+)-nootkatone concentration. Therefore, further studies in partitioning system are necessary in order to avoid both substrate and product inhibition during the bioconversion process.
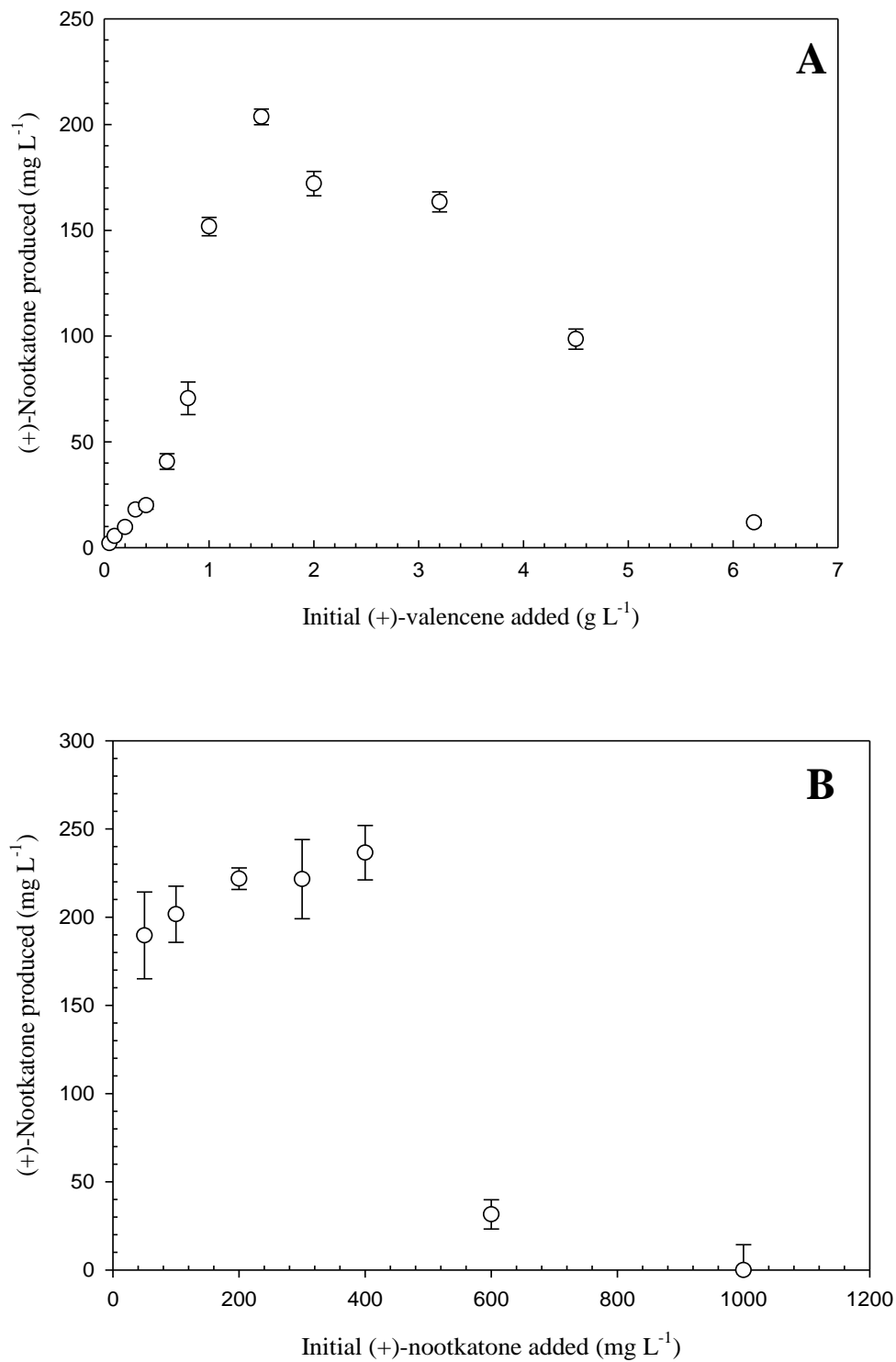
Figure 5.1. A) Effect of initial (+)-valencene concentration during the bioconversion process of *Y. lipolytica*. B) Effect of initial (+)-nootkatone concentration during the bioconversion process of *Y. lipolytica*.

5.3.4. Bioconversion of (+)-valencene in a three-phase system

Firstly, in order to investigate the possible effect of reaction media on viable cells a control experiment without the addition of (+)-valencene or orange essential oil was carried out in the bioreactor. Biomass, substrate concentrations and cell viability were monitored for 129 h (Figure 5.2.). After 48 h of growth, *Y. lipolytica* remained in the stationary phase. Loss of cell viability was observed just as the stationary phase began dropping to 40 %, probably due to the nearly complete depletion of total glucose and cell lysis. Cell lysis is due to older cells that become weaker and less active with age, and cellular membranes eventually deteriorate and begin to release proteolytic enzymes.[37] To evaluate cell lysis of *Y. lipolytica*, soluble protein released into culture medium was measured relative to the concentration of protein released after complete cell lysis via sonication and mechanical rupture.[38] Results indicated that approximately 60 % of protein was released into the culture medium during the bioconversion process. Becerra et al.[26] previously reported that cell lysis of *Kluyveromyces lactis* resulted in the release of 52 % of total protein.
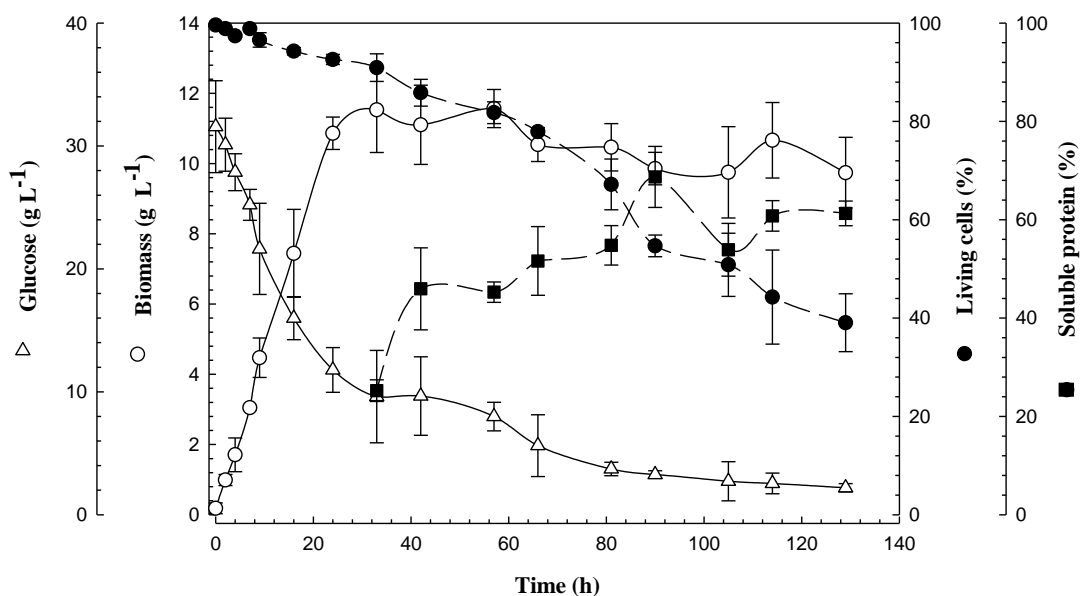
Figure 5.2. Kinetics of cell growth, glucose consumption, cell viability and soluble protein during the bioprocess. *Y. lipolytica* was grown and incubated for 129 h without the addition of (+)-valencene, maintaining the same conditions of the bioconversion experiments.

Secondly, bioconversion experiments were then conducted in a three-phase system using orange essential oil as a source of (+)-valencene for bioconversion to (+)-nootkatone. In this type of experiments *Y. lipolytica* was grown using 30 g L^{-1} of glucose. Maximum biomass production (11.7 g L^{-1}) was reached at 33 h of growth (Figure 5.3.). At this time, orange essential oil (50 % v/v) was added to the bioreactor when approximately 10 g L^{-1} of glucose still remained in the culture medium. It was also observed that the percentage of viable cells decreased, reaching 50 % after 60 h and 30% at the end of the bioconversion process (96 h).
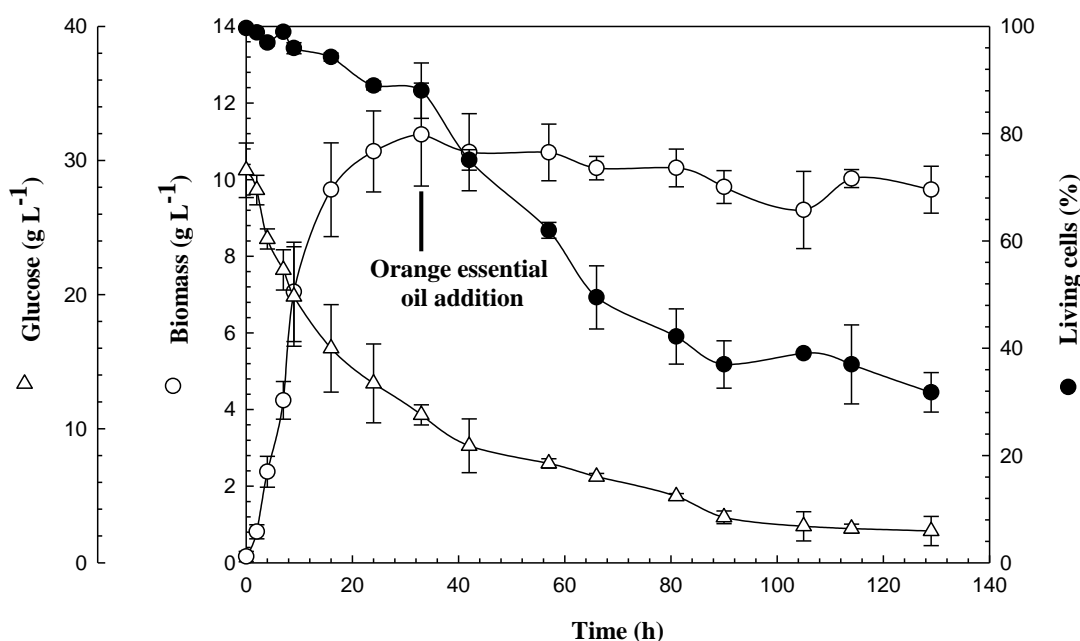

Figure 5.3. Kinetics of cell growth, glucose consumption, and cell viability in the tri-phasic system. Up to 50% v/v orange essential oil (3.2 g L^{-1} of (+)-valencene) were added to initiate the bioconversion process.

The production of (+)-nootkatone (Figure 5.4.) peaked at 326.3 mg L^{-1} in the organic phase at 96 h, with a bioconversion of 39.1 %. In comparison, Gavira et al.[7] cultured for 24 h using 4 g L^{-1} of (+)-valencene and reported production of (+)-nootkatone not more than 10 mg L^{-1}. Papanikolaou et al.[39] investigated the effect of citrus essential oil addition on the physiological behaviour of *Y. lipolytica*. Sugar uptake rate and maximum specific growth rate were not affected by oil addition. However, a loss of cell viability up to 50% was

observed when oil concentration increased up to 1.5 mL L^{-1}. Our results show that cells of *Y. lipolytica* rapidly lost viability even if no orange essential oil was added, suggesting that our strain was sensitive to mechanical and/or oxidative stress.[40,41] Besides, it was observed that cell viability in three-phase system was 10 % lower than that observed in the control system; although, there was no significant statistical difference (p=0.279). Therefore, unknown components in orange essential oil might not be as toxic as expected at the concentration used. Figure 5.4 also depicts that after 48 h, the bioconversion process reached a stationary phase, indicating that there was a limiting factor in the production of (+)-nootkatone.
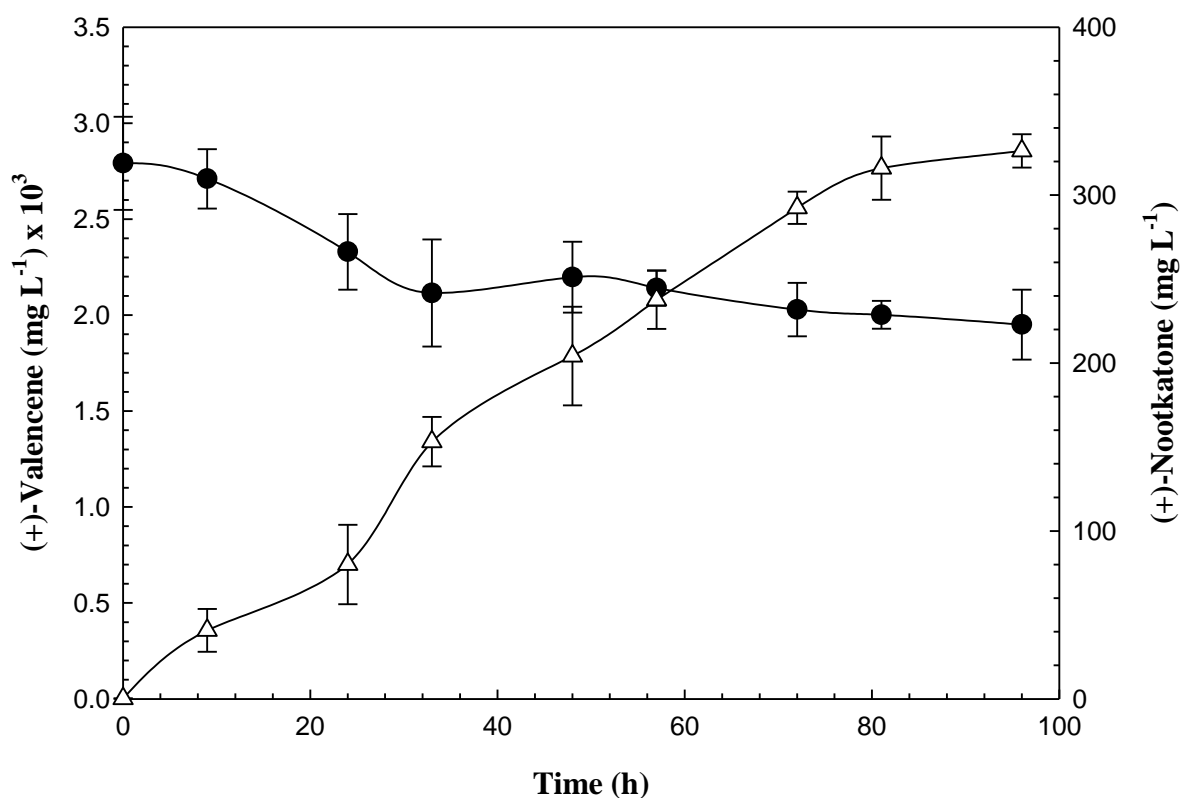
Figure 5.4. Bioconversion of (+)-valencene to (+)-nootkatone in a tri-phasic system using *Y. lipolytica*. Zero time corresponds to 33 h of growth.

Gavira et al.[7] performed optimization of a bioconversion process using a recombinant yeast expressing CYP71D51v2 from tobacco and a P450 reductase from *Arabidopsis*. Yeast cells were cultivated for 24 h in the presence of increasing concentrations of (+)-valencene, β-nootkatol and (+)-nootkatone. The authors claimed that (+)-valencene was tolerated at

concentrations up to 4 g L^{-1}, with a recovery of 73 % of viable cells after 24 h. On the other hand, β-nootkatol and (+)-nootkatone led to a loss of cell viability at 1 g L^{-1}, with 45 % viability already lost at 100 mg L^{-1} in the case of β-nootkatol. The possible reasons for the observed bioconversion efficiency to (+)-nootkatone include: a) product toxicity, b) accumulation of β-nootkatol in the endo-membranes of yeast, c) inhibition of the CYP71D51v2 hydroxylation reaction by the products, and d) the fact that the formation of nootkatone from β-nootkatol is not dependent on cytochrome P450, but is catalysed by an unidentified enzyme in yeast.[7] Fraatz et al.[4] confirmed the dioxygenase nature of the enzyme of *Pleurotus sapidus* that catalyze (+)-valencene oxidation. The isolation and identification of two allylic (+)-valencene derived hydroperoxides together with homology data from amino acid sequencing suggested a lipoxygenase-like type of enzyme.[42] To our knowledge, this report is the first one using a wild type *Y. lipolytica* with the enzymatic machinery to carry out the bioconversion.

The nootkatone/biomass yield in the three-phase system using 30 g L^{-1} of glucose was 27.9 mg g^{-1}, this yield was 21.8 times higher than that (1.28 mg g^{-1}) observed by Girhard et al.[16] under optimized conditions using recombinant *E. coli* with 10% dodecane and 409 mg L^{-1} of (+)-valencene. A primary drawback mentioned by the authors was the accumulation of high concentrations of β-nootkatol in cellular membranes, leading to alterations in membrane permeability, as well as the function of integral proteins, similar to that which has been observed with other hydrocarbons that are concentrated in membranes.[43,44] Accumulation of products, in particular β-nootkatol, is thus likely to be an important factor limiting rates and yields during the process of bioconversion.[7] As such some strategies to improve the bioconversion process was revised by the authors such as increasing the permeability of cell membrane using a solvent and a selective transporter of nootkatol and nootkatone in order to avoid toxic product accumulation in the endo-membranes.

5.3.5. Effect of cellular membrane permeabilization on the bioconversion of (+)-valencene to (+)-nootkatone

5.3.5.1 Membrane permeabilization experiments with Triton X-100

Cellular membrane permeabilization experiments were conducted with Triton X-100 using washed and unwashed cells. A final concentration of 347.7 mg L^{-1} of (+)-nootkatone was reached at a concentration of 0.1% (v/v) of Triton X-100. However, this effect was not statistically significant with respect to that obtained by the control without the addition of Triton (p=0.183). Above 0.1% (v/v), it was observed that the bioconversion decreased, probably due to the high concentrations of this compound. It has been reported for *Y. lipolytica*[45] that the increment in cell membrane permeability depends on the detergent concentration: a greater concentration of Triton indicated greater permeability.

5.3.5.2. Membrane permeabilization experiments with CTAB

Cellular membrane permeabilization experiments with CTAB were carried out. A statistically significant difference was observed at a concentration of 0.2 % (w/v) (see Figure A1 in Annexes), obtaining 420.9 mg L^{-1} of nootkatone and 55.1±18.9 % of bioconversion at 96 h using washed cells. Similar results were achieved by Naina et al.[46] as they observed greater substrate, product and cofactors transport across the cell membranes of *S. cerevisiae* and *Kluyveromyces fragilis* using a concentration of 0.2% (w/v) of this cationic surfactant. The authors reported that yeast cells can replace the purified ADH and GGPDH for the production of NADH and NADPH, respectively. However, the cells require membrane permeabilization to increase cell ADH and the GGPDH achieved through the treatment with CTAB. In this study, CTAB was used to help the cells of *Y. lipolytica* to expel the intermediary nootkatol culture medium during the bioconversion process.

5.3.5.3.Membrane permeabilization experiments by sonication

Finally, using sonication as a mechanical method, with the equipment and operation conditions tested, there was no statistically significant difference of bioconversion compared to the control without sonication, in both cells washed and unwashed (p=0.147).

5.3.6. Effect of cofactor regeneration (NADPH) on the bioconversion of (+)-valencene to (+)-nootkatone using glycerol and Niacin (vitamin B$_3$)

5.3.6.1 Cofactor regeneration experiments using glycerol

It was observed that the addition of glycerol either when the culture medium was inoculated or after 3 days of growth, did not present a statistically significant increase in (+)-nootkatone, resulting in a maximum concentration of 337.6±19.2 mg L^{-1}. It was also observed that the bioconversion using glycerol with washed cells was not statistically significant (p=0.259) in comparison to cells without washing.

5.3.6.2. Cofactor regeneration experiments using niacin (vitamin B$_3$)

In experiments using niacin (vitamin B$_3$) for the regeneration of the cofactor NADPH$^+$, significant differences on (+)-nootkatone production were observed (see Figure A2 in Annexes) when niacin at a concentration of 2 mM was added after 3 days of *Y. lipolytica* growth. Under these conditions a (+)-nootkatone production of 380.4 mg L^{-1} with a 51.5 % of bioconversion was reached. This (+)-nootkatone concentration was higher than that observed when niacin was added at the same time when culture media was inoculated. In spite of several authors reporting bioconversion of (+)-valencene to (+)-nootkatone, very few reports on the improvement of this reaction are available.[7,8,16,47]

5.3.7. Identification of the intermediate nootkatol by GC-MS

The presence of the intermediary nootkatol during the bioconversion was analyzed by GC-MS using percentages of abundance in the control, 2 mM of niacin and 0.2% (w/v) of CTAB (Figure 5.5.).

An increment of nootkatol was observed when CTAB was added confirming that cells were permeabilized. Table 5.1 shows our results compared to those obtained by other authors using other microorganisms. At optimal CTAB concentration the intermediary was expelled to the culture medium obtaining an improvement in (+)-nootkatone production. In addition, the control results show similarities with other authors (see Table 5.1.) in terms of the percentage found of the intermediary.
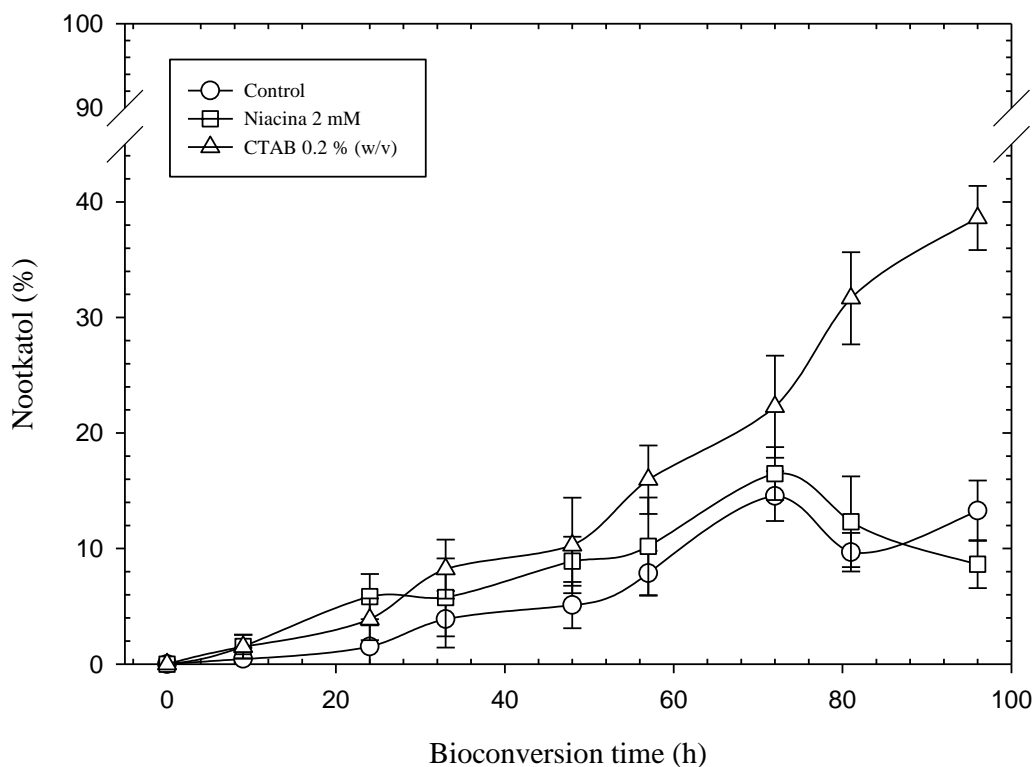
Figure 5.5. Nootkatol release during to the bioconversion process using *Y. lipolytica*.

Table 5.1. Presence of the intermediary nootkatol during the (+)-valencene bioconversion using *Y. lipolytica* in a monophasic system adding 2 mM of niacin and 0.2 % (w/v) of CTAB.

System	(+)-Valencene (g L^{-1})	Nootkatol (%)	(+)-Nootkatone (mg L^{-1})	Reference
Monophasic (Control)	1.50	13.27 ± 2.61	325.3 ±17.3	This work
Monophasic + 2.0 mM Niacin	1.50	8.65 ± 2.07	380.4 ± 31.1	This work
Monophasic + 0.2% w/v CTAB	1.50	38.61 ± 2.77	420.9 ± 33.4	This work
Monophasic	0.46	4.00 ± nr	170.1 ± nr	Kaspera et al.[17]
Biphasic	0.40	12.90 ± nr	5.65± nr	Sowden et al.[47]
Monophasic	0.40	10.00 ± nr	15.00 ± nr	Furusawa et al.[48]
Biphasic	0.40	7.40 ± nr	94.20 ± 1.8	Girhard et al.[16]

nr = Data not reported

However, it was observed that the results using Niacin showed a similarity with respect to the results of the control. This demonstrates that this compound was only used as a cofactor regenerator during the bioconversion. Our results were particularly improved by membrane permeabilization with CTAB causing expel of intermediary nootkatol, cofactor NADH regeneration by the vitamin niacin.

5.3.8. Bioconversion of (+)-valencene to (+)-nootkatone in a two-phase bioreactor using *Y. lipolytica* with CTAB and Niacin

After 24 h of growth 11.5 g L^{-1} of additional biomass, washed and permeabilized using 0.2% (w/v) de CTAB, and 2.0 mM of niacin were added to the bioreactor. Then a concentration of 1.5 g L^{-1} of valencene was also added to start the bioconversion. Cell viability decreased up to 40 % at 96 h of the bioconversion process was observed. A nootkatone production of 619.8 mg L^{-1} was obtained at 120 h, with an approximate consumption of 0.8 g L^{-1} of (+)-valencene (Figure 5.6).
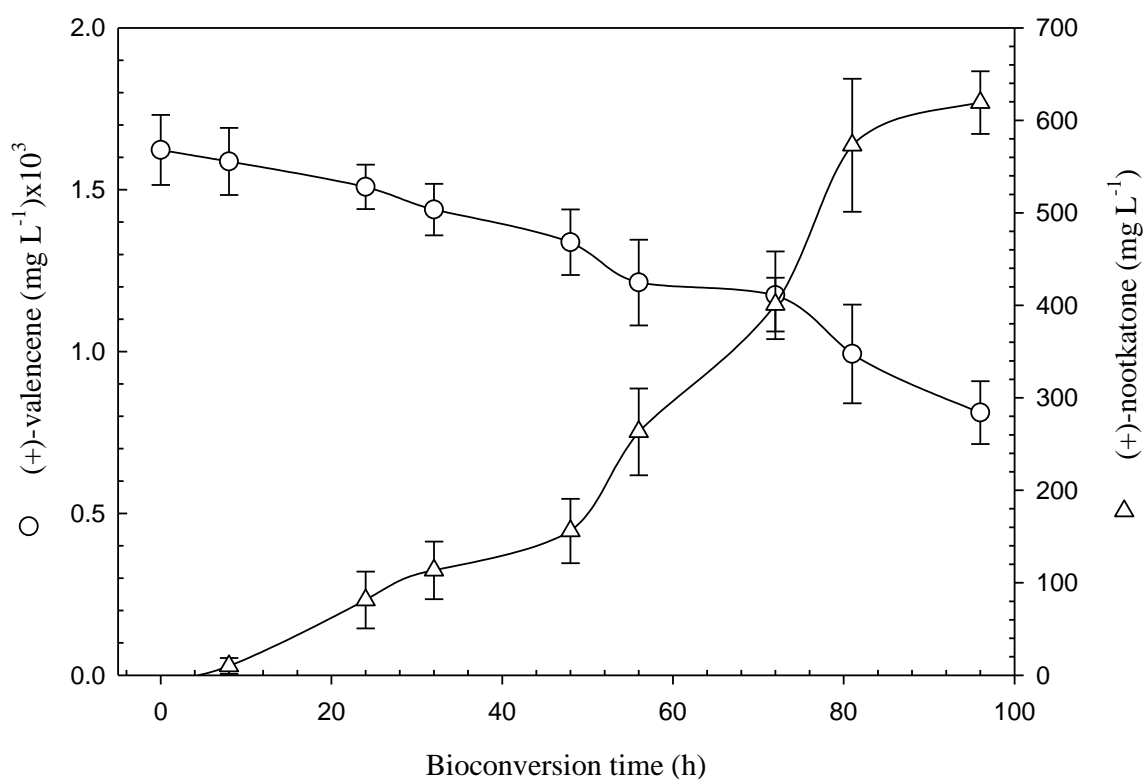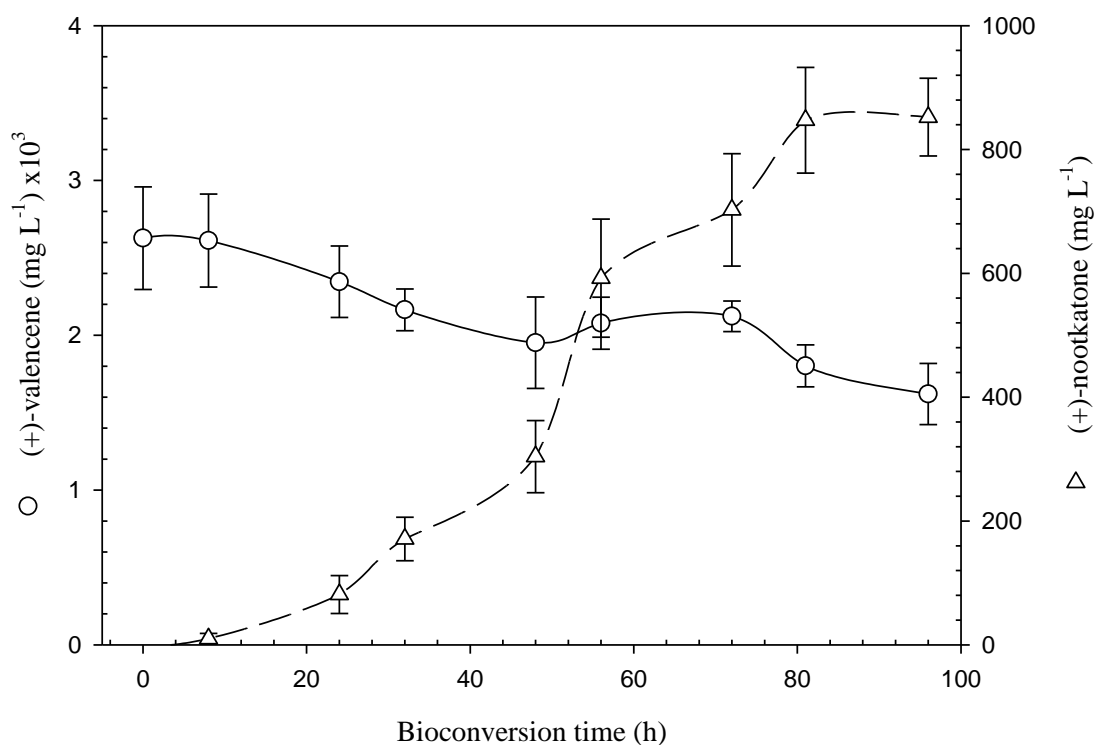

Figure 5.6. Bioconversion of (+)-valencene to (+)-nootkatone in a two-phase system using *Y. lipolytica*. Zero time corresponds to 33 h of growth.

After this time, a limitation of (+)-nootkatone production was observed, since the product concentration reached above the product inhibition determined previously (see Figure 5.1B). Fraatz et al.[4] obtained a final concentration of 600 mg of nootkatone L^{-1}, with the strain of *Pleurotus sapidus* in a fed-batch system, adding valencene pulses up to a concentration of 11 g L^{-1}.

5.3.9. Bioconversion of (+)-valencene to (+)-nootkatone in a three-phase partitioning bioreactor using *Y. lipolytica* with CTAB and Niacin

Experiments in a three-phase system were carried out to obtain higher production. After 24 h of growth, additional biomass up to 22 g L^{-1} washed and permeabilized using 0.2 % (w/v) de CTAB and 2.0 mM of Niacin were added to the bioreactor. After 30 minutes, 50% (v/v) of orange essential oil was added. Cell viability decreased to 30 % at 96 h of the bioconversion process. A (+)-nootkatone production of 852.3 mg L^{-1} in the organic phase was obtained at 120 h (Figure 5.7).

Figure 5.7. Bioconversion of (+)-valencene to (+)-nootkatone in a three-phase system using *Y. lipolytica*. Zero time corresponds to 33 h of growth.

Wriessnegger et al.[8] generated a strain of *P. pastoris* co-expressing the premnaspirodiene oxygenase of *Hyoscyamus muticus* (HPO) and the *Arabidopsis thaliana* cytochrome P450 reductase (CPR) that hydroxylated extracellularly added (+)-valencene. Two-phase cultivations of *P. pastoris* resulted in the production of trans-nootkatol, which was oxidized to (+)-nootkatone by an intrinsic microbial activity. Additional over expression significantly enhanced the (+)-nootkatone yield to 208 mg L^{-1} cell culture with 0.5 g L^{-1} of (+)-valencene in bioreactor cultivations fed-batch in 108 h of bioconversion of final time, the (+)-nootkatone is recovered *in situ* with 10 % (v/v) of n-dodecane as the organic phase. Girhard et al.[16] also studied this bioconversion process using a two-phase system with 10 % dodecane as the organic phase with a recombinant strain of *Escherichia coli*. An initial concentration of 409 mg L^{-1} of (+)-valencene was used in a relatively short time (8 h), 94.2 mg L^{-1} of (+)-nootkatone was produced with a total bioconversion of 23 %. As much as 80 % cell death was observed using other organic solvents such as isooctane and n-octane, due to the significant toxicity of these solvents. In contrast, cell viability remained at 90 % during the bioconversion process upon use of organic solvent dodecane. Comparing the results of our three-phase system using a partitioning bioreactor with those of Fraatz et al.[4] and those obtained in our study using a two-phase system, it is evident that partitioning bioreactor is a promising technology for scaled up in the production of (+)-nootkatone on *Y. lipolytica*. This study highlighted the advantage of using three-phase partitioning bioreactor to enhance productivity of (+)-nootkatone overcoming its inhibition effects. Moreover, a non-previously studied wild-type *Y. lipolytica* strain was used for the production of an important flavor/fragrance compound, namely (+)-nootkatone. Nowadays, *Y. lipolytica* is one of the most extensively studied yeasts[49], capable of producing important metabolites and with an interesting enzymatic machinery, among them Cytochrome P450, which is one of the enzymes involved in the biotransformation process.[7]

Furthermore, experiments were carried out to find the effect of biomass increment on the bioconversion process. The biomass increased up to 32.41 g L^{-1} and the results are summarized in Table 5.2.

Table 5.2. Biomass concentration effect on the (+)-nootkatone bioconversion using *Y. lipolytica* in a three-phase partitioning bioreactor at 30 °C, 200 rpm and 0.5 vvm.

Initial biomass (g L^{-1})	Reaction rate (mg nootkatone L^{-1} h^{-1})	Bioconversion (%)	Volumetric productivity (mg L^{-1} h^{-1})	Yield Nootkatone/biomass (mg g^{-1})	Final nootkatone concentration (mg L^{-1})
11.7 ± 1.4	4.9 ± 1.7	38.8 ± 13.1	3.4 ± 0.1	27.8 ± 3.1	326.3 ± 9.9
22.0 ± 0.5	12.9 ± 1.9	77.9 ± 19.4	8.8 ± 0.6	38.2 ± 3.2	852.2 ± 62.8
32.4 ± 1.3	7.6 ± 0.7	64.9 ± 6.4	6.2 ± 0.3	18.4 ± 1.0	599.6 ± 33.6

As expected initial biomass has an important effect on bioconversion variables, nevertheless, those initial biomass values 11.7 and 32.41 g L^{-1} leaded to lower reaction rate, bioconversion, volumetric productivity, yield and final nootkatone concentration than when 22 g L^{-1} of initial biomass was used during the bioconversion.

5.4. CONCLUSIONS

This work reported a natural method of synthesis to convert (+)-valencene to (+)-nootkatone using cells of a non-previously studied *Y. lipolytica* in a stirred tank partitioning bioreactor. Cell membrane permeabilization with CTAB and cofactor NADH regeneration with Niacin enhanced the bioconversion. Three phase partitioning technology (organic- aqueous-air) is compared to two-phase (aqueous-air) technology. The addition of organic phase, i.e., orange essential oil containing a cheap source of (+)-valencene, overcame bioconversion inhibition from (+)-nootkatone or (+)-valencene observed in the two-phase technology. From these results, the three phase portioning reactor is a potential technology for industrial production and *in situ* extraction of (+)-nootkatone.

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CHAPTER VI

EFFECT OF HYDRODYNAMIC STRESS AND BIOMASS CONCENTRATION ON THE BIOCONVERSION OF (+)-VALENCENE TO (+)-NOOTKATONE WITH *YARROWIA LIPOLYTICA*

EFFECT OF HYDRODYNAMIC STRESS AND BIOMASS CONCENTRATION ON THE BIOCONVERSION OF (+)-VALENCENE TO (+)-NOOTKATONE WITH *YARROWIA LIPOLYTICA*

D.M. Palmerín-Carreño, O.M. Rutiaga-Quiñones, J.R. Verde Calvo, S. Huerta-Ochoa[*]

Abstract

In whole cell bioconversion processes, cell viability and cell concentration are important factors. Process performance might be affected by the hydrodynamic stress caused by the conditions of aeration and agitation on cells growing in stirred bioreactors. The aim of this work was to evaluate the effect of hydrodynamic stress and biomass concentration on the bioconversion of (+)-nootkatone of yeast *Y. lipolytica* 2.2ab in a stirred tank partitioning bioreactor. Orange essential oil was used as a source of (+)-valencene, for the production of (+)-nootkatone. The bioconversion was enhanced using 2X initial biomass concentration reaching a (+)-nootkatone concentration of 803.1 mg L^{-1} and 67.1% of bioconversion at 0.25 vvm and 100 rpm. Reducing hydrodynamic stress loss of cell viability decreased; however, (+)-nootkatone yield was not improved compared to that observed at 200 rpm and 0.5 vvm.

Keywords: hydrodynamic stress; increase biomass bioconversion; partition bioreactor

6.1. Introduction

The nootkatone is a natural ingredient of high value for the flavor and fragrance industry because of its flavor/aroma of grapefruit, low sensory threshold and low availability. However, the microbial sesquiterpene allylic oxidation of (+)-valencene (Figure 6.1) offers an attractive route for obtaining this coveted fragrance [1].

In the whole cell bioconversion processes product yield is affected by stress caused by the hydrodynamic conditions of aeration and agitation of the cells cultured in stirred bioreactors [3]. Another important factor in bioconversion processes is the effect of biomass concentration on bioconversion.

The aim of this work was to evaluate the effect of hydrodynamic stress and biomass concentration on the bioconversion of (+)-valencene to (+)-nootkatone using yeast *Y. lipolytica* 2.2ab in a stirred tank partitioning bioreactor.
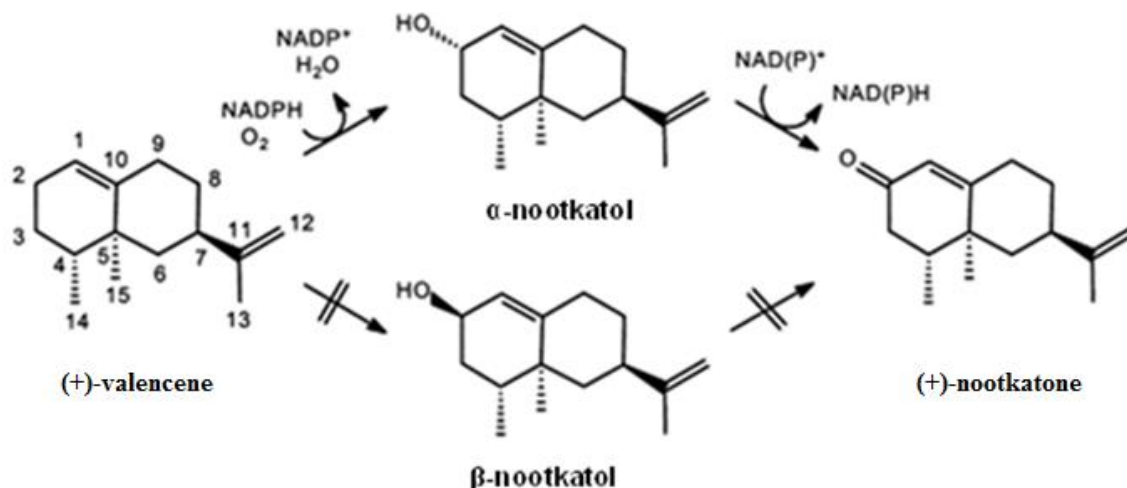
Fig. 6.1. Bioconversion of (+)-valencene to (+)-nootkatone [2].

6.2. Materials and Methods

The growth of *Y. lipolytica* 2.2ab was performed by inoculating a final concentration of 1×10^6 cells mL^{-1} to 350 mL of liquid medium containing (g L^{-1}): Na_2MoO_4, 0.2; $MnSO_4$, 0.4; $FeCl_3$, 0.2; KI, 0.1; $CuSO_4$, 0.04; H_2BO_3, 0.5; $CaCl_2$, 0.1; NaCl, 0.1; $MgSO_4*7H_2O$, 0.5; KH_2PO_4, 1; $ZnSO_4$, 0.4; $(NH_4)_2SO_4$, 5; Yeast extract, 4004 [4], 30 g L^{-1} glucose; with an initial pH of 5.5 at 30°C in a stirred tank reactor final volume of 1 L; with an agitation of 100 and 200 rpm and aeration of 0.5 vvm and 0.25 respectively.

After 33 hours of growth, biomass concentration 2X (22 g L^{-1}) and 3X (34 g L^{-1}), previously treated with 2mM Niacin concentration and 0.2% w/v CTAB to regenerate the cofactor $NADPH^+$ and Membrane permeabilization, respectively were added [5], to the bioconversion media containing 350 mL orange essential oil [6]. Subsequently, kinetics of glucose [7], cell viability [8], (+)-valencene and (+)-nootkatone concentration in the organic phase were obtained by gas chromatography [2]. Each experiment was performed in triplicate. The results obtained were analyzed by the Tukey HSD ($p \leq 0.05$) method with the SAS System package (Version 9).

6.3. Results and Discussions

Hydrodynamic stress experiments were carried out without the addition of orange essential oil as control. Figure 6.2 shows that at lower agitation and aeration (100 rpm, and 0.25 vvm)

the loss of cell viability decreases reaching 60% of viable cells,-: Under these operation conditions, the cell viability was improved compared to the operating conditions of 200 rpm and 0.5 vvm. These results suggest that *Y. lipolytica* 2.2ab strain might be sensitive to oxidative or mechanical stress [3].
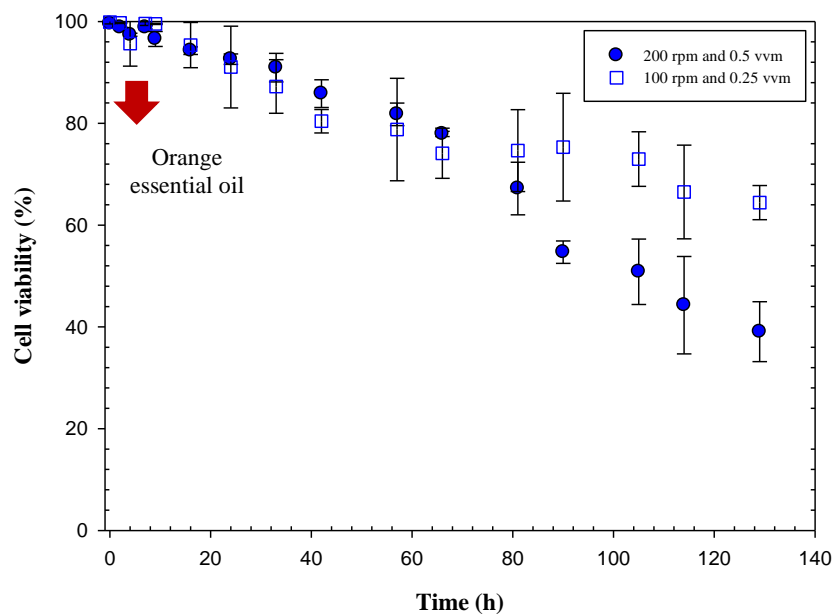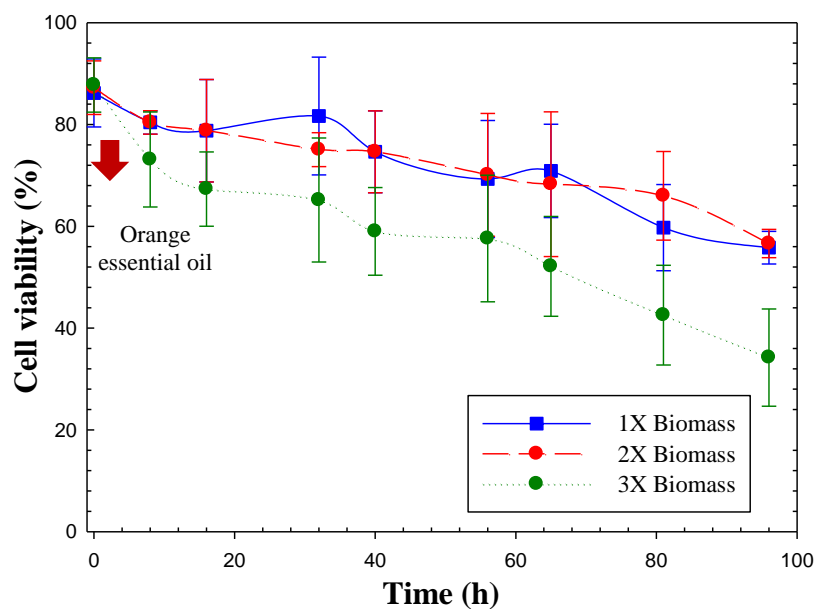

Fig. 6.2. Cell viability kinetics of *Y. lipolytica* 2.2ab incubated without the addition of (+)-valencene at two operation conditions (200 rpm and 0.5 vvm; and 0.25 vvm and 100 rpm).

Another strategy to increase (+)-nootkatone production is increasing the amount of biomass during bioconversion. Then experiments increasing biomass concentration at 100 rpm and 0.25 vvm, in a three phase bioconversion system using orange essential oil (found 1:1 v/v) as dispersed phase were conducted. Figure 6.3 shows bioconversion kinetics using different biomass concentrations 1X, 2X and 3X. At a biomass concentration of 2X, the highest (+)-nootkatone concentration, 803.1 ± 70.1 mg L^{-1}, with a bioconversion of 67.1% and reaction rate of 12.5 mg L^{-1} h^{-1} were reached.
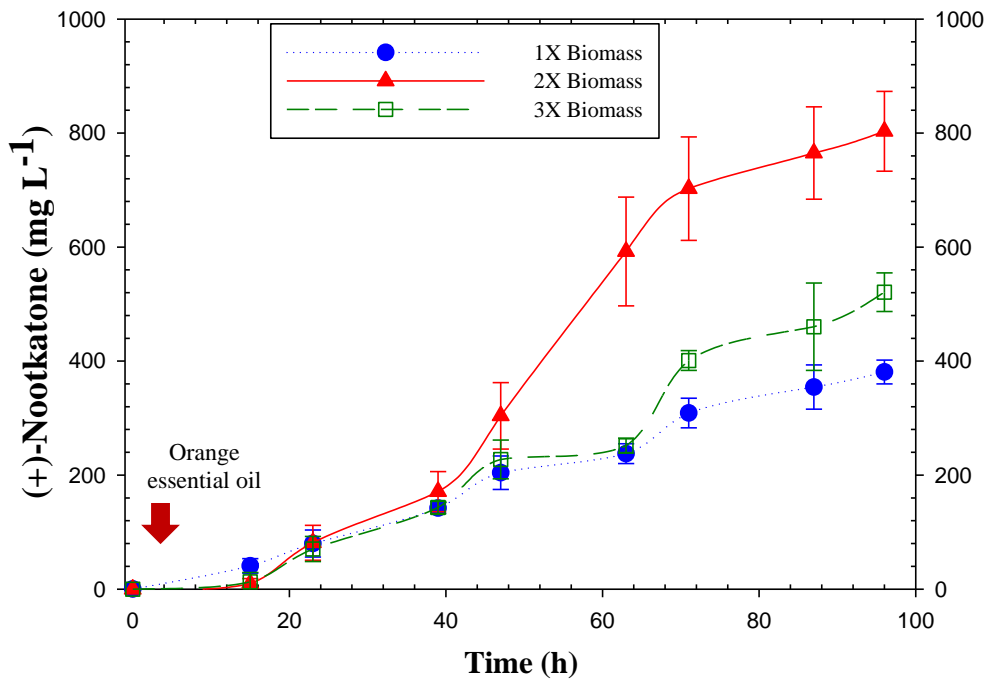
Fig. 6.3. Kinetic of (+)-nootkatone bioconversion at different initial biomass concentrations in a three phase system using *Y. lipolytica* 2.2ab at 100 rpm and 0.25 vvm.

No statistical difference was observed on cell viability using 1X and 2X biomass concentration (Figure 6.4); however, using a biomass concentration of 3X, the (+)-nootkatone concentration decreased due to a reduction in cell viability, which reached a value of 40% after 96 hours of bioconversion. The highest loss of cell viability might be due the increment in oxygen demand as shown in Figure 6.4. Previous results in our laboratory showed an increment of (+)-nootkatone up to 852.3 mg L^{-1} at 200 rpm and 0.5 vvm with 2X biomass [5]. A stationary phase was observed after 72 h of bioconversion. At this point, non-higher concentrations of (+)-nootkatone were reached probably due to an enzymatic deactivation process. Under these operating conditions a greater supply of oxygen was observed, so a bioconversion experiment was performed with the same operating conditions but using a biomass concentration of 3X in order to observe a possible increase in the concentration of (+)-nootkatone. Despite of the increment of dissolved oxygen and biomass concentration, similar results in (+)-nootkatone production were observed.

Fig. 6.4. Cell viability kinetics of *Y. lipolytica* 2.2ab with 1X, 2X and 3X biomass at 100 rpm and 0.25 vvm.
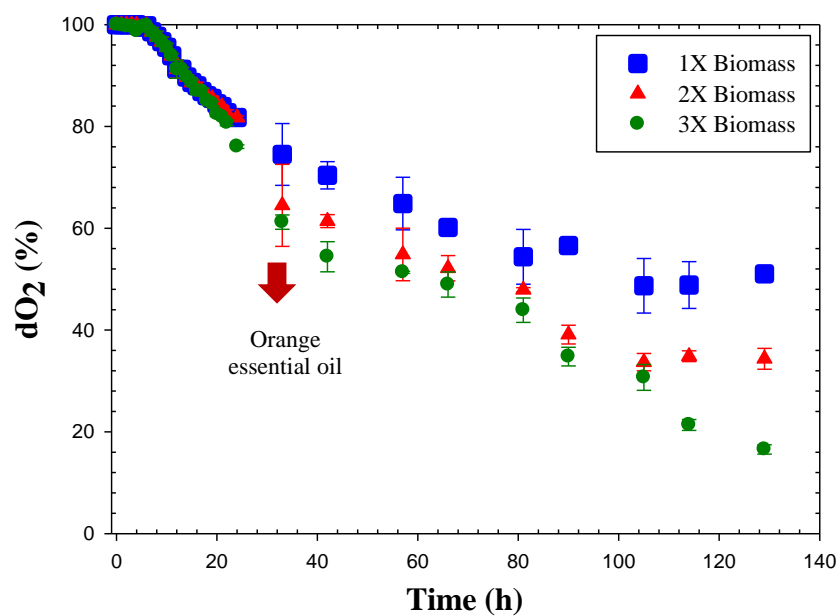
Fig. 6.5. Dissolved oxygen kinetics of *Y. lipolytica* 2.2ab with 1X, 2X and 3X biomass at 100 rpm and 0.25 vvm.

Table 6.1. Bioconversions in a three-phase system at 100 rpm and 0.25 vvm.

Biomass (g L^{-1})[i]	Reaction rate (mg Nootkatone L^{-1} h^{-1})[iii]	Bioconversion (%)[ii]	Volumetric productivity (mg L^{-1} h^{-1})[iii]	Nootkatone/ biomass Yield (mg g^{-1})	Maximum Nootkatone (mg L^{-1})[iv]
11.4 ± 0.8 (c)	4.76 ± 0.4 (c)	31.7 ± 3.8 (c)	3.96 ± 0.21(c)	33.52 ± 4.55 (a)	380.91 ± 20.9 (c)
22.0 ± 0.5 (b)	12.5±2.1 (a)	67.1±15.6(a)	8.36±0.72 (a)	36.77 ± 3.96 (a)	803.14 ± 70.0 (a)
33.4 ± 0.6 (a)	7.1 ± 0.9 (b)	46.0± 7.1 (b)	5.42 ± 0.35 (b)	15.34 ± 0.99 (b)	520.88 ± 33.8 (b)

[i] Concentration of initial biomass in the growth medium of *Y. lipolytica.*
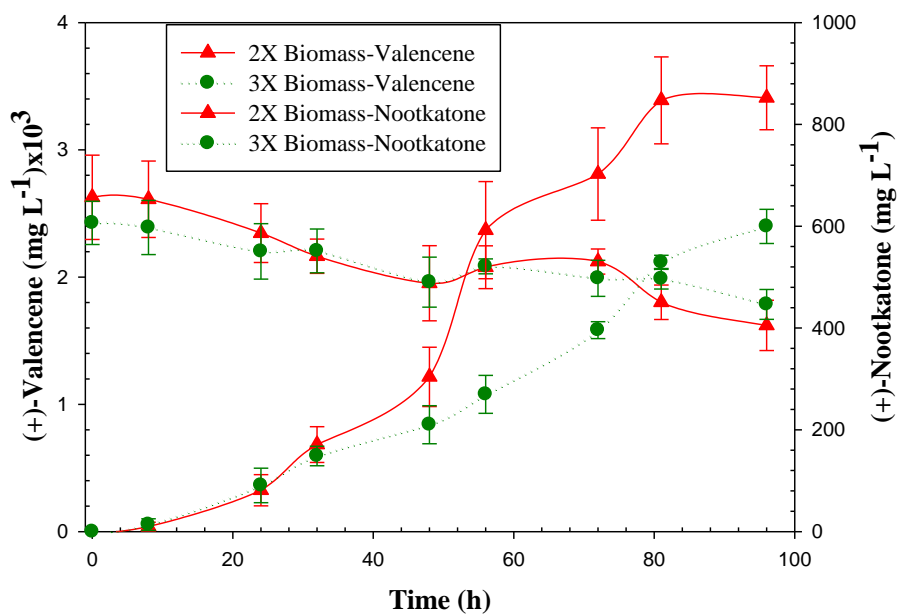[ii] Percentage of (+)-nootkatone produced relative to (+)-valencene consumed based on molecular masses.
[iii] mg of nootkatone L^{-1} produced between 24 and 48 h.
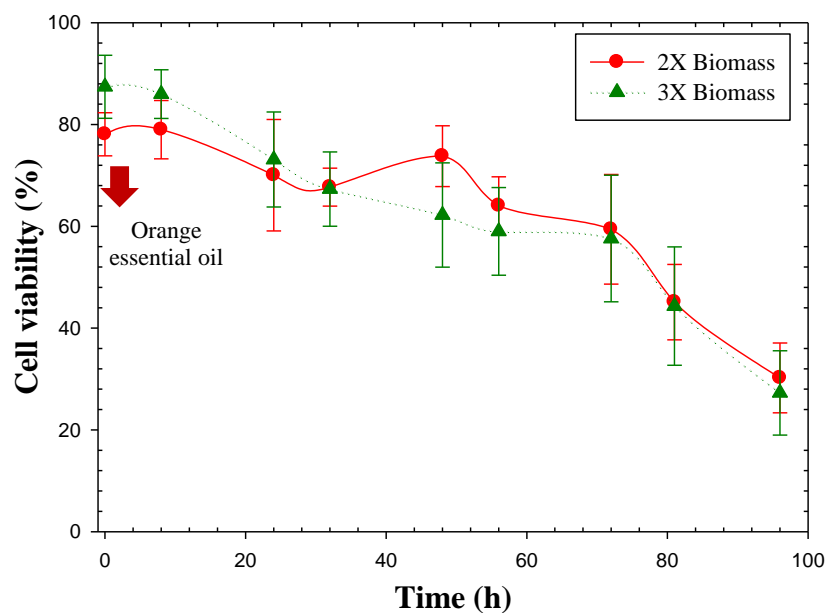[iv] mg of maximum nootkatone L^{-1} produced after 96 h.
[v] values represent mean ± standard deviation.
Same letters represent no significant differences (p ≤ 0.05; Tukey, SAS System Version 9).

Fig. 6.6. Bioconversion of (+)-valencene to (+)-nootkatone in a three phase system using *Y. lipolytica* 2.2ab at 200 rpm and 0.5 vvm.

Fig. 6.7. Cell viability kinetics of *Y. lipolytica* 2.2ab with 2X and 3X biomass at 0.5 vvm and 200 rpm.
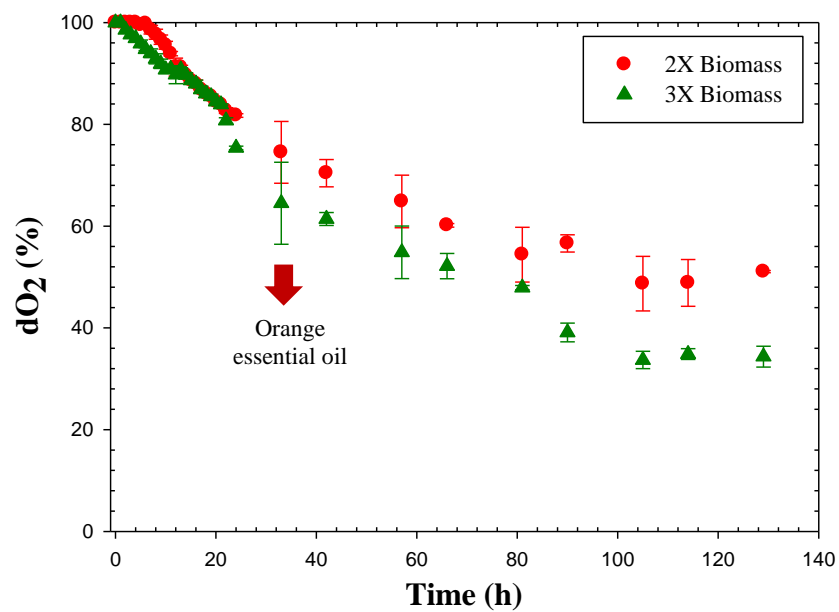
Fig. 6.8. Dissolved oxygen kinetics of *Y. lipolytica* 2.2ab with 2X and 3X biomass at 200 rpm and 0.5 vvm.

In Table 6.2, the results obtained from three-phase experiments are shown. Significantly, at a concentration of 22 g L^{-1} of biomass the maximum bioconversion was reached, this fact confirmed that there is a demand of oxygen by increasing the biomass concentration, which inhibited the bioconversion of substrate. Hsieh et al., [9] used olive oil for the production of biomass and polysaccharides at *Grifola frondosa* at high levels of aeration at reactor level, they observed a decrease of dissolved oxygen up to 10% with 30 g L^{-1} of initial biomass. In our study, a similarity decay of dissolved oxygen up to 20% at the end of the bioconversion with 34 g L^{-1} of initial biomass, the excess of biomass during the bioconversion limits oxygen supply.

Table 6.2. Bioconversions in a three-phase system at 200 rpm and 0.5 vvm.

Biomass (g L^{-1})	Reaction rate (mg Nootkatone L^{-1} h^{-1})[iii]	Bioconversion (%)[ii]	Volumetric productivity (mg L^{-1} h^{-1})[iii]	Nootkatone/ Biomass Yield(mg g^{-1})	Maximum Nootkatone (mg L^{-1})[iv]
22.0 ± 0.5 (b)[i,v]	12.9 ± 2.0 (a)	78.0 ± 19.5 (b)	8.87 ± 0.65 (a)	38.23 ± 3.25 (a)	852.3 ± 62.8 (a)
32.4 ± 1.4 (a)[i,v]	7.7 ± 0.8 (b)	64.9 ± 6.5 (a)	6.24 ± 6.46 (b)	18.5 ± 1.0 (b)	599.6 ± 33.7 (b)

[i] Concentration of initial biomass in the growth medium of *Y. lipolytica*.
[ii] Percentage of (+)-nootkatone produced relative to (+)-valencene consumed based on molecular masses.
[iii] mg of nootkatone L^{-1} produced between 24 and 48 h.
[iv] mg of maximum nootkatone L^{-1} produced after 96 h.
[v] values represent mean ± standard deviation.
Same letters represent no significant differences (p ≤ 0.05; Tukey, SAS System Version 9).

6.4. Conclusions

The study of the effect of biomass concentration on the bioconversion process led to-obtain a maximum production of (+)-nootkatone at 2X, 200 rpm and 0.5 vvm. It was observed that using lower agitation and aeration rates the hydrodynamic stress was reduced, which was observed by decreasing the loss of cell viability; however, the product yields were not improved. These results provide the basis to establish operating windows for oxidative bioconversions.

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CHAPTER VII

WHOLE CELL (+)-NOOTKATONE BIOCONVERSION IN AN ORGANIC PHASE (ORANGE ESSENTIAL OIL) SYSTEM USING *YARROWIA LIPOLYTICA*

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WHOLE CELL (+)-NOOTKATONE BIOCONVERSION IN AN ORGANIC PHASE (ORANGE ESSENTIAL OIL) SYSTEM USING *YARROWIA LIPOLYTICA*

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Abstract

The aim of this work was to assess whole cell bioconversion of (+)-valencene to (+)-nootkatone in an organic phase (orange essential oil) using a stirred tank bioreactor. *Yarrowia lipolytica* 2.2ab was used to carry out the bioconversion experiments; 700 mL of orange essential oil were inoculated with 22.5 g L^{-1} of permeabilized biomass using 0.2 % (w/v) of CTAB, and enriched with 2.0 mM of niacin. Experiments were conducted at 200 rpm, 0.5 vvm and 30 °C. The highest (+)-nootkatone production was 773 mg L^{-1} after 4 days of conversion. It was observed that there was no statistical difference to the value reported previously using a three-phase partitioning bioreactor. However, bioconversion percent and volumetric productivity was enhanced to 82.3 % and 9.5 mg L^{-1} h^{-1}, respectively.

Key words: Bioconversion; orange essential oil; (+)-valencene; (+)-nootkatone; *Yarrowia lipolytica*

7.1. INTRODUCTION

The sesquiterpene (+)-nootkatone has a fruity, typically grapefruit odour and is one of the most commonly compounds used in fragrance industry. This compound is mainly produced by chemical processes and thus, commercially available at a relatively high price. Besides, this compound can also be produced via biological processes and, thus, can be sold as natural product [1,2]. Different efforts have been focused to enhance whole cell bioconversion of (+)-valencene to (+)-nootkatone. Classical genetics approach [3] or metabolic engineering [4] have been reported. Moreover, due to this bioconversion presents substrate and product inhibition [5], the addition of a second liquid phase (organic) has been extensively studied [5,6]. Bioconversion of (+)-valencene to (+)-nootkatone has been described, using a wide

range of microorganisms as catalysts: *Botryodiplodia theobromae* 1368 [7]; *Yarrowia lipolytica* 2.2ab [5]; a recombinant *P. pastoris* co-expressing the premnaspirodiene oxygenase of *Hyoscyamus muticus* (HPO) and the *Arabidopsis thaliana* cytochrome P450 reductase (CPR) that hydroxylated extracellularly added (+)-valencene [4]; a recombinant strain of *E. coli* [6], plant enzymes expressed in *Saccharomyces cerevisiae* [8], *Pleurotus sapidus* [9], *Chaetomium globosum* [10], a dioxygenase of *Pleurotus sapidus* [11] among others.

In recent years, there has been an increasing trend towards valorization of components from essential oils. Amongst these studies, the production of R-(+)-α-terpineol by *Penicillium* sp. 2025, *Aspergillus* sp. 2038, *Fusarium oxysporum* 152B from orange essential oil [12], biotransformation of limonene by fungi and red yeast from *citrus* [13], production of α-terpineol by *Penicillium digitatum* NRRL 1202 from orange peel oil [14], increase bioavailability of orally administered pharmaceutical compounds from essential oils [15], production of peach flavour and lactones by *Yarrowia lipolytica* from ricinoleic acid [16,17].

The use of whole cells in organic-media biocatalysis provides a way to regenerate cofactors and carry out bioconversions or fermentations requiring multi-step metabolic pathways. [18]. This type of bioprocesses overcome the problem of the low volumetric productivity of the reaction system; besides, the organic phase has a positive effect on the stability of the biocatalyst [19,20]. Furthermore, the more hydrophobic solvents manifest less toxicity to whole cells [21].

The aim of this work was to evaluate the performance of *Y. lipolytica* 2.2ab during the bioconversion of (+)-nootkatone in an organic phase (orange essential oil) comparing its volumetric productivity using this organic phase to those reported previously.

7.2. MATERIALS AND METHODS
7.2.1. Chemicals

(+)-Valencene (CAS 75-05-6) and (+)-nootkatone (CAS 93-78-5) with a purity >70% and >85%, respectively were purchased from Fluka (Switzerland) and used as standards.

Analytical grade orange essential oil (*Citrus aurantium*, var. Amara) was purchased from a local drugstore in Mexico City) and used as an organic phase to carry out bioconversion studies.

7.2.2. Microorganism, culture media and inoculum

The yeast *Yarrowia lipolytica* 2.2ab was selected for its potential in the bioconversion of (+)-valencene to (+)-nootkatone under different bioconversion conditions [22]. The strain was maintained in potato dextrose agar (PDA, Bioxon-Dickinson, México) at 30 °C. *Y. lipolytica* 2.2ab was propagated in PDA and incubated at 30 °C for 3-7 days. Cells were harvested with 25 mL 0.01% Tween-80 and counted using a Neubauer chamber. 100 µL of *Y. lipolytica* cell suspension (1×10^6 cells mL^{-1}) were used to inoculate 250 mL conical flask containing 100 mL of culture medium. Flasks were incubated at 30 °C for 4 days on a rotary shaker at 200 rpm. Later biomass was washed and permeabilized using 0.2% (w/v) of CTAB [5].

7.2.3. Stirred tank bioreactor for organic phase bioconversions

A of 1.0 L glass stirred tank bioreactor (model ADI 1025, Applikon) was used for bioconversion processes. Agitation and the air flow rate for the bioconversion were maintained at 200 rpm and 0.5 vvm, respectively. Temperature was maintained at 30 °C. The pH was monitored and controlled at 5.5 using 2 M HCl. A refrigerant was located at the air exit to avoid sesquiterpenes evaporation from the bioreactor [5]. Bioconversion studies were conducted adding 22.5 g L^{-1} of permeabilized biomass enriched with NADPH$^+$ as a cofactor regeneration to 700 mL of orange essential oil (3.2 g L^{-1} of (+)-valencene) to the bioreactor [5]. Every day, 5 mL of sample from the bioconversion media was taken and centrifuged at 5000 rpm for 10 min. Then, sesquiterpene extraction was carried out using 500 µL of sample and 500 µL of ethyl acetate. The mixture was vortexed twice for 20 s in a vortex, and settled until phase separation occurred, after that 1 µL of the ethyl phase was injected to CG [5].

7.2.4. Analytical techniques

Biomass determination was carried out by measuring the dry weight of cells. Samples of 5.0 mL were dried to a constant weight in metal plates which had been previously weighed after being left in an oven at 90 °C overnight.

Glucose consumption of *Y. lipolytica* was determined by analysing the glucose concentration in the medium using the dinitrosalicylic acid method described by Miller et al [23] for reducing sugar, and a UV spectrophotometer at a wavelength of 550 nm.

Cell viability during process was determined by measuring the concentration of living cells using the methylene blue (MB) dye, as described by Bonora and Mares [24]. Culture samples (around 5 mL) were aseptically centrifuged at 8,000 rpm for 10 min. Cells were suspended and centrifuged in an equal amount of phosphate buffered saline pH 8 (PBS). PBS was used as a diluent. The MB dye was added to sample during 6 min and suspended again in PBS at the same conditions. The living cells were calculated using time course measurement mode at 640 nm.

The concentrations of (+)-valencene and (+)-nootkatone were determined by gas chromatography (GC-FID) using a Perkin Elmer Auto System XL Gas Chromatograph equipped with a flame ionization detector (Cyclosil-b capillary column; 30 m x 0.32 mm x 0.25 μm; J-&-W Scientific Inc., CA), using the chromatographic method described previously [5]. Compounds in the samples were identified by comparison of mass spectra with that of pure compounds.

7.3. RESULTS AND DISCUSSION

Kinetics of biomass, glucose consumption and cell viability were obtained during the bioconversion in the organic-phase system using orange essential oil (Figure 7.1). It was observed a decrease of cell viability up to 30 % at 96 h of the bioconversion. Similar results were observed by Palmerín-Carreño et al. [5] in a three-phase system using 50 % (v/v) of orange essential oil as dispersed phase. The prolonged productivity in the organic system depends on cell viability, in this sense *Y. lipolytica* 2.2ab shows acceptable tolerance to orange essential oil. Some parameters used for correlation with solvent toxicity are associated with their physicochemical properties such as: the dielectric constant (ε), dipole moment (μ), and polarizability (α), all related to the polarity of the solvent; however, available data are not enough to test their validity [25].

A progressively decreases of microorganism activity after 8 h to 10 % was also observed by Cabral et al., [20] using *Gluconobacter oxydans* which is able to convert isoamyl alcohol to isovalver aldehyde from isooctane. This decrement was possibly due to shear molecular toxicity of the organic phase or the intrinsic deactivation. Viable cells of *Rhodococcus erythropolis* DCL14 were naturally capable of regenerating the cofactor using n-dodecane as organic phase in order to convert carveol to carvone [2]. Brault et al. [26] reported a whole-cell biocatalyst using an *E. coli* showing excellent tolerance to alcohol and short-chain fatty acid denaturation in a micro-aqueous media during the synthesis of a short-chain ester flavour.
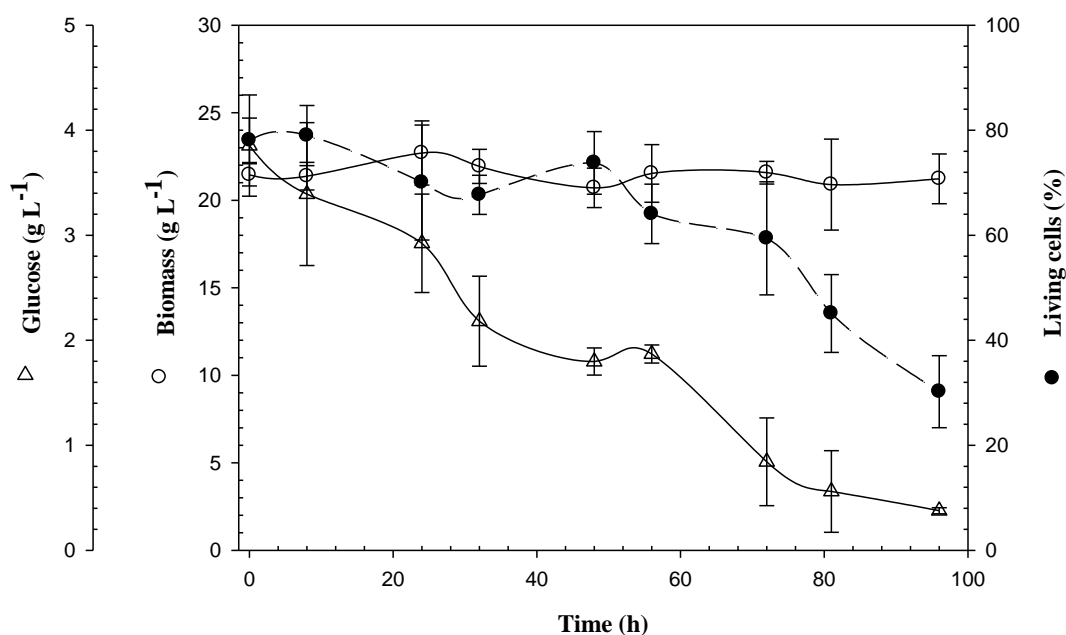
Figure 7.1. Kinetics of biomass (○), glucose consumption (Δ) and cell viability (●) during the bioconversion in an organic-phase system using orange essential oil containing 3.2 g L^{-1} of (+)-valencene.

The kinetics of (+)-valencene consumption and (+)-nootkatone production during the bioconversion in the organic-phase system are plotted in Figure 7.2. A nootkatone production of 774 mg L^{-1} was obtained at 96 h, with an approximate consumption of 0.9 g L^{-1} of (+)-valencene. A product/substrate yield of 0.86 g (+)-nootkatone per g of (+)-valencene was achieved. This value was 60 % larger than that obtained in a three-phase system [5].

Maróstica and Pastore [12] used *Fusarium oxysporum* 152B, to convert R-(+)-limonene to R-(+)-α-terpineol, yielding nearly 450 mg L^{-1} after 3 days of transformation in the presence of a solution of 1.0 % orange essential oil in 250 ml conical flasks containing 50 ml of liquid medium. *Pseudomona putida* cells exposed to very high concentrations of phenol led to an increase in the trans acid [27]. They found that the bioconversion ratio increased around ten-fold both for 16:1 and 18:1 fatty acids.
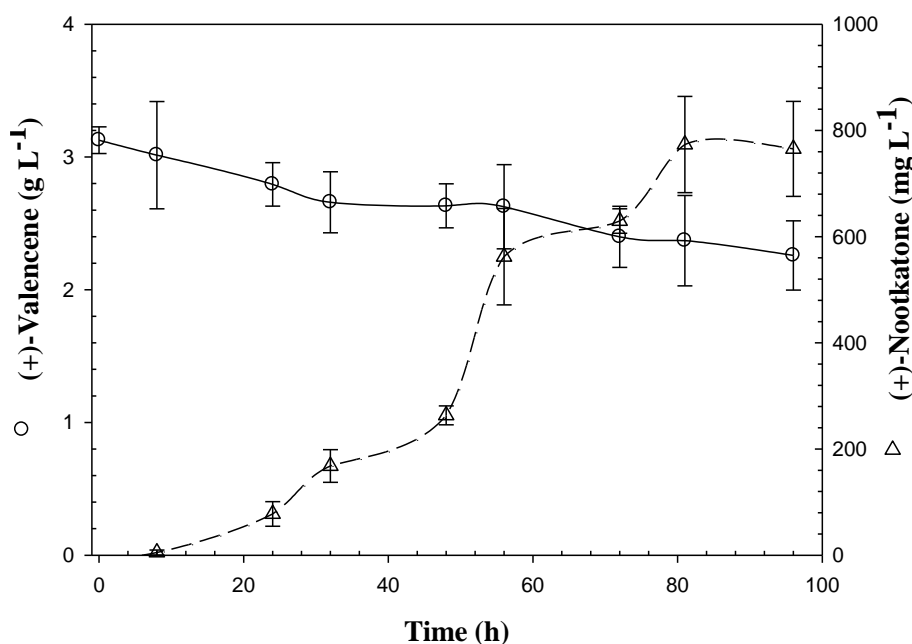
Figure 7.2. Kinetics of (+)-valencene consumption (○) and (+)-nootkatone production (Δ) during the bioconversion in an organic-phase system using *Y. lipolytica* 2.2ab.

In Table 7.1, bioconversion results are compared to those previously reported in two and three-phase systems [5]. Authors demonstrated that using a two-phase system bioconversion was inhibited by product. However, when a three-phase system was used 852 mg (+)-nootkatone L^{-1} were obtained in the organic phase, 50% v/v. The (+)-nootkatone concentration in the organic phase system is slightly lower than those obtained by Palmerín-Carreño et al. [5] using 50 % (v/v) of orange essential oil; however, overall volumetric productivity is higher. This result demonstrated that the substrate concentration in a saturated organic phase was not inhibitory for *Y. lipolytica*. One possible hypothesis is that this event presents two parallel *Y. lipolytica* metabolism for (+)-valencene distribution, one could lead

to an intermediary bioconversion to produce energy and another pathway could lead to the formation of (+)-nootkatone. This has previously been reported in some strains of the genus *Pseudomonas* during the production of terpineol [26].

Tabla 7.1. Overall volumetric productivity in two-phase, three-phase and organic phase systems

System	Bioconversion (%)[i]	Final nootkatone concentration $(mg\ L^{-1})$[ii]	Overall volumetric productivity $(mg\ L^{-1}\ h^{-1})$[iii]	Reference
Aqueous-phase	77.2 ± 11.3 (b)	619.8 ± 33.9 (c)	6.46 (b)	Palmerín-Carreño et al. [5]
Organic-phase	82.3 ± 19.3 (a)	773.5 ± 32.6 (a,b)	8.06 (a)	This work
Three-phase	77.9 ± 19.4 (c)	852.2 ± 62.8 (a)	4.44 (a)	Palmerín-Carreño et al. [5]

[i] Percentage of (+)-nootkatone produced relative to (+)-valencene consumed based on molecular masses.

[ii] mg of maximum nootkatone L^{-1} produced after 96 h.

[iii] mg of nootkatone per liter of bioreactor per hour

Same letters represent no significant differences ($p \leq 0.05$; Tukey, SAS System Version 9).

7.4. CONCLUSIONS

Finally, in this paper a method for converting (+)-valencene into high concentrations of (+)-nootkatone was presented. We report for the first time the (+)-valencene to (+)-nootkatone bioconversion in an organic phase (orange essential oil) using *Y. lipolytica*. It was demonstrated that the use of vegetable oils as organic phase is especially useful in the aforementioned reactions since it inhibited the concurrent oxidative metabolism, enabling to obtain higher (+) nootkatone yield (0.86 g (+)-nootkatone per g of (+)-valencene) at a bioconversion close to 82 % than that observed from conventional two-phase and three phase reaction systems.

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CHAPTER VIII

HYDRODYNAMIC AND OXYGEN MASS TRANSFER STUDIES IN A THREE-PHASE (AIR–WATER–ORANGE ESSENTIAL OIL) STIRRED TANK BIOREACTOR FOR THE BIOCONVERSION OF (+)-VALENCENE WITH *YARROWIA LIPOLYTICA*

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Abstract

The productivity of the bioconversion of (+)-valencene to (+)-nootkatone by *Yarrowia lipolytica* 2.2ab in a multiphase system can be limited by mass transport. The aim of this work was to characterize the hydrodynamic and oxygen mass transfer in a three phase (gas–water–orange essential oil) partitioning bioreactor. Firstly, a central composite experimental design was used to investigate the interaction of three operational factors i.e. agitation (300, 400, 500 rpm), aeration rates (0.5, 0.75, 1.0 vvm), and orange essential oil volume fraction (20, 35, 50%), on two response variables i.e., the *Sauter* mean droplet diameter (d_{32}) and the volumetric oxygen transfer coefficient (k_La). The hydrodynamic and mass transfer studies led to k_La and d_{32} values of 53.76 h^{-1} and 10.09 μm, respectively. Secondly, the substrate (k_S) and product (k_P) global mass transfer coefficients were determined in a modified Lewis cell. The maximum k_S and k_P values obtained were 3.02x10^{-5} and 2.81x10^{-5} m s^{-1}, respectively. The volumetric mass transfer coefficients (k_SA and k_PA) were calculated using interfacial areas (A) of the dispersed phase (orange essential oil) estimated from the "Sauter" mean drop diameter (d_{32}) in one liter stirred tank partitioning bioreactor. Finally, from a regime analysis it was observed that the characteristic time obtained for oxygen uptake rate (94 s^{-1}) was among the characteristic times determined for oxygen transfer (67 s^{-1}) at200 rpm and 0.5 vvm as operating conditions, suggesting that the oxygen transfer rate under certain operating conditions could be a limiting step in the bioconversion process.

Keywords: Multiphase bioreactor; hydrodynamics; oxygen mass transfer; (+)-nootkatone; *Yarrowia lipolytica*

8.1. INTRODUCTION

The compound (+)-nootkatone is a valuable sesquiterpenoid which possesses an intense grapefruit-like taste that are exploited by the fragrance and flavour industries (Ladaniya, 2010). Industry and academia are interested on this biocatalytic process using whole cells, which involves the selection of an optimal cellular enzyme, reaction engineering, product recovery and scaling-up. Different efforts have been focused to enhance whole cell bioconversion of (+)-valencene to (+)-nootkatone. Classical genetics approach (Omarini et al., 2014) or metabolic engineering (Wriessnegger et al., 2014) have been reported. Girhard et al. (2009) described the conversion of (+)-valencene by recombinant *E. coli* expressing CYP109B1 from *Bacillus subtilis* in a two-liquid-phase system. Recently, in previous work the bioconversion of (+)-valencene to (+)-nootkatone with *Yarrowia lipolytica* in a partitioning bioreactor using orange essential oil as the dispersed phase was successfully evaluated (Palmerín-Carreño et al., 2015). The addition of orange essential oil as second liquid phase as a substrate reservoir and in situ product extracting agent overcame substrate and product inhibition increasing process performance. Orange essential oil used in this type of bioprocess has concentrations of (+)-valencene and (+)-nootkatone of 3.2 g L^{-1} and 0.94 mg L^{-1}, respectively (Palmerín-Carreño et al., 2015). These concentrations are known to be dependent from the harvest and fruit species.

Multiphase partitioning bioreactors conception seems to have a great potential in enhancing the productivity of many bioprocesses by overcoming issues of poor substrate solubility and toxicity (Melgarejo-Torres et al., 2015). However, the productivity of oxidative bioconversions in a multiphase system (Doig et al., 2002) could be limited by low mass transfer rates across the inter-phases (gas–liquid and/or liquid–liquid). Substrate, product and oxygen transfer rates will also depend on the fluid physicochemical properties, temperature, pressure, media composition, agitation conditions, oxygen superficial gas velocity and the configuration of the bioreactor. Physicochemical properties influence thermodynamic equilibrium of substrate and product in the interface observed in the partition coefficient. The high (+)-nootkatone partition coefficient (22.5) reported by Palmerín-Carreño et al. (2015) implies a good affinity of (+)-nootkatone for orange essential oil (organic phase), which is advantageous for *in situ* recovery in the partitioning bioreactor.

Mass transfer rates in a multiphase system are functions of the *Sauter* mean drop size diameter (d_{32}), oxygen volumetric mass transfer coefficient (k_La), substrate partition coefficient, and the oxygen solubility into the medium. For a specific partitioning bioreactor and medium, it is normally possible to increase the liquid–liquid interfacial area and k_La, and consequently mass transfer rates, using high agitation and aeration rates. However, this causes high power consumption and significantly increasing operation costs. High stirring rates also present a limitation when applied to cells that are sensitive to hydrodynamic stress and can promote stable emulsion formation leading to downstream processing problems. Hydrodynamic studies in two-phase partitioning bioreactors have focused on elucidation of the interfacial area available form ass transfer in order that substrate supply (normally from the non-aqueous phase) does not become the rate limiting step of the process. The interfacial area has previously been correlated to the dispersed phase hold-up fraction and the Weber number (Quadros and Baptista, 2003). The interfacial area available for mass transfer (a) is given by:

$$a = \frac{6\phi}{d_{32}} \tag{1}$$

where d_{32} is the *Sauter* mean drop diameter and ϕ is the volume fraction of the dispersed phase. Accurate knowledge of the effect of bioreactor operating conditions on d_{32} is therefore very important. Besides, knowledge of d_{32} can give an early indication of the stability of the liquid–liquid dispersion created. The physicochemical properties of the media can influence both mean drop size and drop size distribution. If dispersed-phase viscosity, μ_d, is low, the force resisting drop break-up comes only from surface tension (Nienow, 2004). The majority of studies have used organic solvents as the second phase; however, these pose additional toxicity and flammability problems. Recently, the use of essential oils has been reported as a safe alternative for the bioconversion of pharmaceutical and terpenes (Krings and Berger, 2010).

The aim of this work was to characterize the hydrodynamic and oxygen mass transfer in a three phase gas–water–orange essential oil dispersion and determine substrate and product global mass transfer coefficients in three biphasic water–orange essential oil system. This

work will be useful for the purposes of bioreactor design and scale-up and will inform our ongoing studies of oxidative bioconversions in multiphase systems.

8.2. MATERIALS AND METHODS

8.2.1. Chemicals

(+)-Valencene (CAS 75-05-6) and (+)-nootkatone (CAS 93-78-5) with a purity >70% and >85%, respectively were purchased from Fluka (Switzerland) and used as standards. Ethyl acetate (99.5%) used for sesquiterpene extraction was purchased from Quimex (Mexico). Analytical grade orange essential oil (*Citrus aurantium*, var. Amara) was purchased from a local drugstore in Mexico DF, and used as an organic dispersed phase.

8.2.2. Measurement of physicochemical properties of two-phase system

8.2.2.1. Determination of viscosity

The apparent viscosity of orange essential oil was measured using a modular compact rheometer Physica MCR 300 (Brookfield Engineering Laboratories, Germany). Samples of 20 mL were placed in a PP glass, doing the reading with a LV2 geometry. Temperature was controlled with a Peltier (TEL 150 P-C) at 30 °C. Measurements were performed in triplicate.

8.2.2.2. Measurement of the density of orange essential oil

A hydrometer DMA 35 (Anton Paar, Austria) was used to determine the amount of mass contained in a certain volume of orange essential oil.

8.2.2.3. Measurement of interfacial tension

Interfacial tension was measured using a Tensiometer (Fisher, Model 20) at 30° C. A sample of 20 mL of mineral medium were placed in a clean container glass (4 cm diameter) and 4 cm in height, and 20 mL of orange essential oil was added carefully, taking care not to disturb the interface between two fluids. Then, platinum ring (0.6 mm diameter) was placed below the interface. Then, the ring was elevated at a constant speed until a thin interface film was formed; tension measurements were taken until the thin film was broken. Calibration is performed using distilled water as reference. Measurements were performed in triplicate.

8.2.3. Stirred tank bioreactor description

A 1.0 L glass stirred tank bioreactor (model ADI 1025, Applikon) was used for hydrodynamic and mass transfer characterization. The bioreactor had an inside diameter of 9.5 cm and an operation volume of 0.7 L ($H_L/D_T = 0.96$). The bioreactor was equipped with a single Rushton turbine with 6 paddles, $D_i = 4.53$ cm ($D_i / D_T = 0.46$), located 4.53 cm from the base of the container. The bioreactor had 2 equidistant deflectors of 1.0 cm in width to improve liquid-liquid mixing. Temperature and agitation were maintained at 30°C and 300 rpm, respectively. The pH was monitored using an AppLiSens Z001023511 pH electrode (Applikon) and controlled at 5.5 using 2M NaOH. Atmospheric air entering the system was filtered through a sterile 0.22 μm filter. The air flow rate in the bioreactor was 0.5 vvm through a perforated "L" tube with seven holes of 1.0 mm in diameter.

8.2.4. Aerated power consumption (P_g)

Aerated power consumption was measured using the methodology reported by Ascanio et al. (2004). The method is based on electrical measurements (Watts and amperes) carried out directly on the motor shaft of the bioreactor impeller. To consider the losses occurring in the agitation system, electrical measurements were also obtained when the bioreactor was empty. All measurements were performed in triplicate.

8.2.5. Determination of oxygen mass transfer coefficient (k_La)

Oxygen mass transfer coefficient (k_La) was determined according to the dynamic method reported by Koizumi and Aiba (1984) using dissolved oxygen electrode (AppLiSens Z010032520, Applikon). Oxygen electrode was attached to a controller Bio IDA 1010 (Applikon). Dissolved oxygen concentrations were taken every 5 seconds. The effect of operating conditions: agitation rate (300, 400, and 500 rpm), aeration rate (0.5, 0.75 and 1.0 vvm), and orange essential oil fraction (20, 35 and 50% v/v) on the volumetric oxygen transfer coefficient was studied.

8.2.6. Measurement of Sauter mean drop diameter (d_{32})

The *Sauter* mean drop diameter (d_{32}) was measured using a particle Analyzer, Optical Reflectance Meter (3D ORM, MTS, Messtechnic Schwartz) (Jaradat et al., 2010; Ahosseini

et al., 2010). Two thousand automatic determinations of the drop diameter (d_i) for each measurement were taken, that allows the calculation of d_{32}, as defined in equation (2):

$$d_{32} = \frac{\Sigma d_i^3 n_i}{\Sigma d_i^2 n_i} \qquad (2)$$

Mean drop diameter and drop diameter distribution were obtained *in situ* and *on-line* in all experiments. Since the 3D-ORM equipment measures the drop size by optical reflectance of a laser beam and the fact that gas bubbles do not have reflectance properties, it was assumed that gas bubbles did not interfere with the measures of the drop size of orange essential oil. This was confirmed measuring the size of ion exchange beads at different agitation and aeration rates as a positive control. Therefore, droplet size measurements were taken directly from the three-phase system. With the aim that the drop size distribution reach the equilibrium, measurements were taken 15 minutes after agitation had begun.

8.2.7. Lewis cell

A modified Lewis cell was used, consisting of a jacketed glass vessel 11.8 cm high and with an internal diameter of 6.7 cm (Melgarejo-Torres *et al.,* 2012). Each phase had a volume of 182 mL. Two independent marine propellers (d_i= 3.5 cm), each one located inside a suction glass tube (internal diameter 4 cm and height 3.5 cm) supported by six baffles producing a radial and tangential flow motion to a constant interfacial area (a_{cell}) of 18.5 m^2 m^{-3}. Temperature of both liquids inside the Lewis cell was kept at 30 °C by passing water through the cell's jacket from a water bath. Different agitation rates (40, 60 and 80 rpm) in the organic phase were settled, allowing different liquid velocities (V_S) in each phase near the interface. The aqueous phase was constant (60 rpm). To determine V_S, small bead particles were made from silicon and epoxy resin with the same fluid density. Time required for the small bead to go around the internal tube at different agitation rates in each phase was measured and an arithmetic average was obtained, and this value was used to calculated fluid velocity (Lizardi-Jiménez and Gutiérrez-Rojas, 2011). Then, global mass transfer coefficients could be estimated at liquid velocity values close to those obtained in a dispersion system.

8.2.8. Experiments in the Lewis cell

In the experiments using orange essential oil, only the organic phase was agitated. Aqueous phase agitation rate was 60 rpm for the water-orange essential oil system. Therefore, the aqueous phase was prepared as follow: 3.2 g L^{-1} of nootkatone was dissolved in a 0.1 M phosphate buffer adjusted to pH 7.5. Also, the organic phase was orange essential oil containing 3.2 g L^{-1} of (+)-valencene. Samples of 1.0 mL were taken from the organic phase at different time intervals from t = 0 to 3 h; this time was necessary to reach equilibrium. The samples were analysed by gas chromatography (GC) and a standard curve was used to determine substrate and product concentration in the organic phase. Substrate and product concentrations in the aqueous phase were calculated from mass balance.

8.2.9. Sesquiterpene analysis

The concentrations of (+)-valencene and (+)-nootkatone were determined by gas chromatography (GC-FID) using a Perkin Elmer Auto System XL Gas Chromatograph equipped with a flame ionisation detector (Cyclosil-B capillary column; 30 m x 0.32 mm x 0.25 µm; J-&-W Scientific Inc., CA). The temperatures of the injector and detector were constant at 250 °C to 270 °C, respectively. During the analysis 1 µL of sample was automatically injected with a split ratio of 1:4, using helium gas as a carrier. The oven temperature was initially set to 120 °C for 4 min then ramped up to 250 °C at a rate of 10 °C per min and maintained at 250 °C for 5 min, using the technique reported by Girhard et al.(2009). Analyses were performed in triplicate, and concentrations were determined using external calibration curves of (+)-valencene and (+)-nootkatone.

8.2.10. Estimation of mass transfer coefficients

Solute transfer rate through the interface water-ionic liquid can be expressed in a general form by the equation (2):

$$\frac{dS}{dt} = -ka_{Cell}(S - S_f) \tag{3}$$

where: dS/dt is the mass transfer rate in the interface, k is the global mass transfer coefficient, a_{Cell} is the interfacial area, S is the substrate or product concentration in the bulk aqueous

phase, and S_f is the solute equilibrium concentration in the aqueous phase. Solving Equation (1) at $t = 0$ $S = S_0$ and at $t = t$ $S = S$:

$$\ln \frac{(S - S_f)}{(S_0 - S_f)} = -ka_{Cell}t \tag{4}$$

The concentration S_f can be expressed as a function of the initial solute concentration, S_0, and its partition coefficient, K_p:

$$S_f = \frac{S_o}{K_p + 1} \tag{5}$$

8.2.11. Experimental design of response surface

To study the effect of three operation variables (aeration and agitation rates, and the dispersed volume fraction) on two response variables (k_La and d_{32}), a central composite design (Montgomery, 2005) was used. Experiments were carried on in the bioreactor with three independent factors, each one with three levels, three central points and 11 replications. A second order polynomial model provided the best fit to the experimental data. For response surface methodology (RSM), design of experiments software "Fusion Pro" (version 6.7.0, USA) was used for the regression analysis and the graphs of the experimental results.

8.3. RESULTS AND DISCUSIONS

8.3.1. Physicochemical properties of orange essential oil and biocompatibility

The physicochemical properties and biochemical characteristic of the orange essential oil evaluated in this work (*Citrus aurantium* var. amara) are shown in Table 8.1.

Table 8.1. Physicochemical properties and biochemical characteristic of the orange essential oil used.

Property	Value
Density (Kg m^{-3})	836
Dynamic Viscosity (Kg m^{-1} s^{-1})	0.12
Interfacial tension (Kg s^{-2})[a]	0.0059
(+)-valencene partition coefficient [b]	6.37

(+)-nootkatone partition coefficient [b]	22.50

[a] *Saturated with growth medium for Y. lipolytica at 30°C.*

[b] *Saturated with phosphate buffer solution 0.1M at pH 7.5.*

8.3.2. Power consumption and Sauter mean drop diameter (d_{32})

The results obtained for the hydrodynamic experiments using orange essential oil are shown in the Table 8.2. All experiments were considered to be within the turbulent flow regime based on the calculated Reynolds numbers and visual observation of the fluid flow. The Reynolds numbers calculated were based on the viscosity of the continuous phase and ranged among 6,000 and 10,000. For the orange essential oil, a positive effect of increasing agitation rate on P_g/V value was observed as expected. On the other hand, P_g/V values decreased as aeration rates increased as would be expected for a single liquid phase. In the range studied, there was not observed any effect of dispersed volume fraction on P_g/V values. Despite these similar patterns for the three fractions of dispersed orange essential oil phases, it was observed that experimental P_g/V values were affected by the physicochemical properties of the oil used (Table 8.2). The order of magnitude of P_g/V values was similar to those obtained by Nielsen et al. (2003) using a 2-L New Brunswick Bioflo I bioreactor ($H_L/D_T= 0.96$; $D_i/D_T= 0.45$) with a working volume of 1 L and two phase liquid dispersions comprising water and *n*-hexadecane when agitated with two six-blade Rushton turbine impellers.

The relationship between inertial forces and cohesion forces, as represented by the Weber number (N_{We}), was among $390<N_{We}<10,000$ for the orange essential oil. The differences in σ and oil viscosity probably led to a different balance in droplet break-up and coalescence phenomena for dispersed orange essential oil phase. The relationships between the measured d_{32} values for oil as a function of agitation and aeration rate are shown in the Table 8.2. The values of d_{32} for the different fractions of orange essential oil are lower when stirring and the fraction of dispersed phase increases. The maximum value of$d_{32}$was observed for 0.20 disperse phase fraction (Table 8.2). For both fractions 0.35 and 0.50 a general decrease in d_{32} values was observed with increasing dispersed phase volume fraction. The physicochemical properties of the dispersed phase modified the relationship of inertial and cohesion forces which controls break-up of the droplets. Dispersed phase viscosity has a profound influence on both mean drop size and drop size distribution (Maaß *et al.,* 2012). Torres-Martínez *et al.*

(2009) found that the viscosity of the various ionic liquid phases appeared to be the most critical physical parameter governing mass transfer rate in a multiphase bioreactor system, while density and interfacial tension were found to be of lesser importance.

8.3.3. Oxygen mass transfer coefficient (k_La)

The effect of bioreactor operating conditions and dispersed phase volume fraction on experimentally determined k_La values for the orange essential oil is shown in the Table 8.2. For three oil fractions k_La values generally increased as agitation and aeration rates increased as expected from conventional biphasic gas–liquid system studies. k_La values were around $26\,h^{-1}$ at a dispersed phase volume fraction of 35%, which was significantly lower value than that k_La obtained at a dispersed phase volume fraction of 50%, which was $>116\,h^{-1}$. For all three fractions, the measured k_La values were observed to increase as the oil volume fraction increased. Nielsen et al. (2003) examined the effect of increasing volume fraction on k_La using n-hexadecane as the second liquid phase. These authors found that oxygen mass transfer rates actually increased when the organic phase volume fraction was increased. This was attributed to the addition of a second immiscible phase with a much higher solubility of oxygen compared to water, increasing the general level of oxygen solubility in the system.

In a revised approach (Nielsen et al., 2005) considering the same aqueous-organic phase system, the authors using a more fundamental two-phase mass transfer model, it was demonstrated the potential for oxygen mass transfer enhancement by organic phase addition which was one of the motivations for employing a distinct second phase in a partitioning bioreactor. Gomes et al. (2007) claimed that the presence of oil in the medium increases the overall k_La value of the system, but this effect is opposite when the medium contains surfactant (Tween 80). On the other hand, Quijano et al. (2009) worked with a three-phase system (gas-water-silicone oil) and found to silicone oil had a positive effect on the overall oxygen transfer.

8.3.4. Substrate and product global mass transfer coefficients obtained in the Lewis cell

The substrate and product global mass transfer coefficients (k_S and k_P, respectively) were estimated from the resulting slopes, early defined in (Eq. (3)), of the logarithmic profile of

solute concentration against removal time divided by mass transfer area ($18.5\ \mathrm{m^2\,m^{-3}}$) of the Lewis cell. The k_S and k_P plotted against the superficial velocity (V_S) of organic phase in the water-orange essential oil system are shown in Figure 8.1.

Table 8.2. Operational variables and experimental results obtained for orange essential oil.

| ϕ	rpm (min^{-1})	vvm (min^{-1})	P_g/V (W m^{-3})	d_{32} (µm)	k_La (h^{-1})	N_{We}	N_{Re}
0.20	300	0.50	1784±0.20	13.05±0.32	47.27±0.42	392	5746
	400	1.00	2429±0.08	13.49±0.21	53.04±0.33	697	7653
	500	0.75	3225±0.12	14.10±0.15	59.83±0.21	1090	9573
0.35	300	0.75	1828±0.30	18.93±0.30	24.65±0.13	392	5746
	400	0.50	2431±0.04	14.75±0.05	16.63±0.51	697	7653
	400	0.75	2490±0.11	14.25±0.13	27.45±0.34	697	7653
	400	0.75	2437±0.15	15.49±0.08	24.65±0.22	697	7653
	500	1.00	3055±0.06	12.35±0.21	47.92±0.15	1090	9573
0.50	300	1.00	1829±0.05	8.27±0.11	62.21±0.72	392	5746
	400	0.75	2540±0.12	9.26±0.08	88.51±0.51	697	7653
	500	0.50	3433±0.32	7.98±0.20	116.44±0.44	1090	9573

Global mass transfer coefficients are influenced by hydrodynamics near the interface. It can be seen in Figure 8.1 that the increase in the V_S of organic phase increased the k_S value. Where k_S values showed a direct linear relationship to V_S of the aqueous phase. The k_S values increased almost 10 fold up to $3.02\mathrm{x}10^{-5}\ \mathrm{m\ s^{-1}}$ when the V_S increased 4 fold (from 0.006 to $0.025\ \mathrm{m\ s^{-1}}$).

The increment of the inertial forces decreased the thickness of the boundary layers of the liquids, probably diminishing resistance to the mass transport. It can also be seen in the same Figure that the highest values of k_P were obtained with the lower V_S. Variations in the k_P values in the range tested did not let us to observe a tendency with respect to V_S of the organic

phase, the maximum k_P value (2.81×10^{-5} m s^{-1}) was obtained at V_S of 0.005 m s^{-1}. On the other hand, the maximum k_P value was 0.21 times lower than that obtained for k_S. Maximum k_S and k_P values of 3.02×10^{-5} and 2.81×10^{-5} m s^{-1}, respectively, were obtained. Molecular diffusivities for the substrate and the product (8.33×10^{-10} and 7.99×10^{-10} cm s^{-1}, respectively) in the organic phase were estimated using Wilke-Chang correlations (Bird et al., 2010). On the other hand, analyzing the role of operating condition on mass transfer during liquid–liquid extraction through dimensional analysis (Tudose and Lisa, 2003) it was showed the effect of density and viscosity of the orange essential oil on mass transfer coefficients.

Mass transfer coefficients obtained in this work were on the same order of magnitude as those reported in similar Lewis cells. For example, Melgarejo-Torres et al., (2012) obtained k values of $1.2 \times 10^{-5} - 4.3 \times 10^{-5}$ m s^{-1} for a bicyclic cetone (*Bicyclo[3.2.0]hept-2-en-6-ona*) transferred from an organic phase ([MeBuPyrr][BTA]) to an aqueous phase composed of phosphate buffer. The agitation rates in the organic phase were 0–205 rpm. Srivastava et al. (2000) reported k values of 1.1×10^{-5} and 1.2×10^{-5} m s^{-1} for a limonene/water/heptanoic acid system, where heptanoic acid was the molecule transferred from the aqueous phase to the organic phase (limonene). Baldascini et al. (2001) obtained k values of 2.3×10^{-5} m s^{-1} for epoxide hydrolase transferred from its own organic phase to an aqueous phase composed of phosphate buffer with n-octane. The agitation rates in both phases were 150–200 rpm. These studies noted that by increasing the agitation rate in both phases, the thickness of the boundary layer of the interface is reduced, leading to an increase of the mass transfer coefficient.
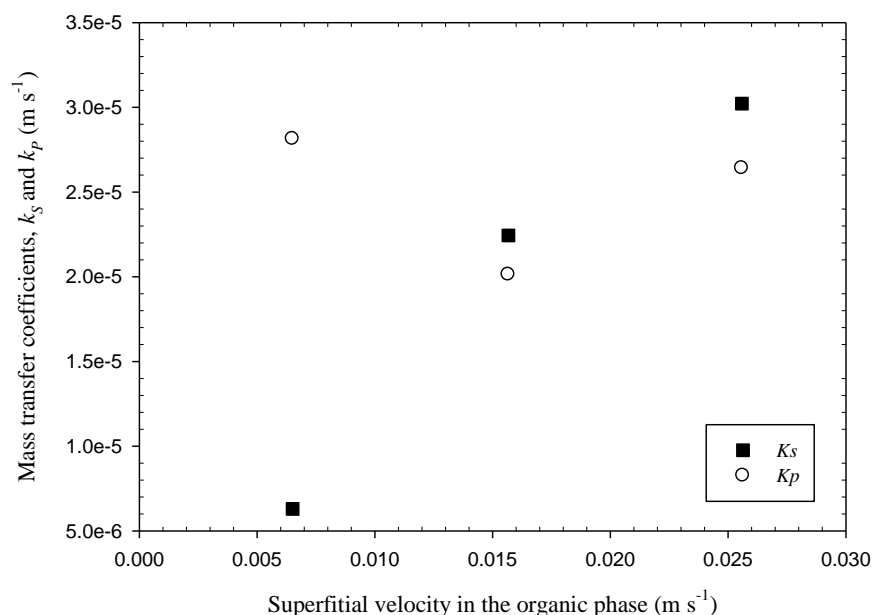
Figure 8.1. Global mass transfer coefficients versus superficial velocity of the organic phase for the phosphates buffer- orange essential oil system: (■) substrate, k_S; and (○) product, k_P.

Global mass transfer coefficients are not only influenced by hydrodynamics near the interface, but also by the thermodynamics. Global mass transfer coefficients values in relation to the physicochemical properties of orange essential oil (interfacial tension, viscosity, density) and the partition coefficients of the compounds extracted (Table 8.1) were analysed.

It was observed that the viscosity of the oil showed a direct linear relationship to k_S. The increment of mass transfer coefficients due to the increment in viscosity of the organic phase (tri-n-butyl phosphate) has also been reported by Bandyopadhyay et al. (1996) for the extraction of tellurium (IV) using a biphasic system. Interfacial tension also showed a direct linear relation-ship to k_S. However, the influence of interfacial tension has mainly been related to droplet breakage and coalescence mechanisms (Jaradat et al., 2010) rather than mass transfer resistances. More molecules of the orange essential oil dissolved in the aqueous phase probably influence molecular diffusivity through the aqueous phase boundary layer; therefore, mass transfer was reduced. It has been reported the study of a biphasic system using orange essential oil and n-octanol, which are partially soluble. The authors claimed that

concentration influenced phase equilibrium, and interfacial physical properties (Maróstica and Pastore, 2007).

Besides, interfacial tension values of the biphasic system are much lower than the surface tension of pure ionic liquids (Hanczyc et al., 2007). Therefore, these results show an important influence of the physicochemical properties of essential oils phase on mass transfer.

8.3.5. Volumetric mass transfer coefficients in a stirred tank partitioning bioreactor
The lower values k_S and k_P measured in the modified Lewis cell in both biphasic systems were multiplied by the mass transfer volumetric areas (A) of the dispersed orange essential oil phases obtained under different operation conditions (oil fraction, agitation and aeration rates) in a stirred tank partitioning bio-reactor, as reported by Torres-Martínez et al. (2009). In this respect, volumetric mass transfer coefficients for the substrate and the product (k_SA and k_PA)were obtained, since they are useful when studying this type of partitioning bioreactors. The obtained range values were: k_SA (6732 h^{-1}) and k_PA (21492 h^{-1}) in the biphasic system. Melgarejo-Torres et al. (2012) reported a k_SA (7.5–36 h^{-1}) and K_PA (2.8–12 h^{-1}) for the water-[OMA][BTA] system. This difference in several orders of magnitude might be due to Melgarejo et al. (2012) used a dispersed phase fraction of 5 %, ten times lower than those used in this work and also the physicochemical properties of [OMA][BTA].

A regime analysis based on characteristic times of the mass transfer mechanisms involved using parameters obtained in this work and reaction parameters from previous work (Palmerín-Carreño et al., 2015) was carried on (Table 8.3). This analysis has been used to obtain a quantitative estimate of bioreactor performance (Melgarejo-Torres et al., 2011).

It was observed that the characteristic times for substrate and product transfer mechanism were about the same order of magnitude, less than one second, indicating that they were the fastest. This fact was probably due to both the physicochemical characteristic of the orange essential oil and the high volume fraction used allowing high liquid-liquid interfacial areas. On the other hand, the characteristic times for oxygen transfer and oxygen consumption are

about the same order of magnitude, oxygen transfer is almost 40 % faster than oxygen consumption. This means that under the operation conditions oxygen transfer was not limiting the bioconversion process. Finally, it was observed that the characteristic time for substrate consumption was the largest time, so bioconversion was limited by reaction.

Table 8.3. Characteristic times in a three-phase stirred tank bioreactor

Mechanism	Definition	Value (s)
Substrate transfer	$t_S = \dfrac{1}{k_S A}$	0.53
Product transfer	$t_P = \dfrac{1}{k_P A}$	0.16
Oxygen transfer	$t_{O_2} = \dfrac{1}{k_L a}$	66.96
Oxygen consumption*	$t_{\Re_{O_2}} = \dfrac{C_{O_2}}{X \Re_{O_2}}$	94.23
Substrate consumption*	$t_{\Re_S} = \dfrac{[S]_0}{X \Re_S Y_{S/P}}$	85096

*Estimated from data obtained by Palmerín-Carreño et al. (2015)

Melgarejo-Torres et al. (2011) reported a regime analysis of a Baeyer–Villiger bioconversion in a three-phase stirred tank partitioning bioreactor using [MeBuPyrr][BTA] as dispersed phase. The authors found that the characteristic time for oxygen transfer and oxygen consumption were similar in the interval studied and therefore oxygen mass transfer might be the limiting step of the bioconversion.

8.4. CONCLUSIONS

A regime analysis of the mass transfer phenomena and reaction rates allowed us to establish possible limiting steps during the bioconversion process. The characteristic time obtained for oxygen uptake rate (94 s^{-1}) was among the characteristic times determined for oxygen transfer (67 s^{-1}) under 200 rpm and 0.5 vvm operating conditions, suggesting that the oxygen transfer rate under certain operating conditions could be a limiting step in the bioconversion process.

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CHAPTER IX

GENERAL CONCLUSIONS AND PERSPECTIVES

GENERAL CONCLUSIONS

A screening method to evaluate the potential ability of the strains to biotransform (+)-valencene to (+)-nootkatone in different bioconversion systems was developed. Two different physiological and morphological strains (*B. theobromae* 1368 and *Y. lipolytica* 2.2ab) were selected due to they were able to produce higher (+)-nootkatone concentrations in all bioconversion systems. Membrane aerated biofilm reactor (MABR) using *B. theobromae* 1368 showed being an attractive technological alternative; however, further studies of operation and optimization of biofilm reactor are needed to enhance bioconversion. The physiological characterisation of whole cell biocatalysis using *Yarrowia lipolytica* 2.2ab for the bioconversion of (+)-valencene to (+)-nootkatone in a partitioning bioreactor demonstrated that this bioconversion system presents an interesting way to produce and recover (+)-nootkatone. Permeability of cell membrane with CTAB, NADPH cofactor regeneration with Niacin and increasing twice the biomass concentration enhanced the production of (+)-nootkatone up to product inhibition concentrations. The addition of organic phase, i.e., orange essential oil containing a cheap source of (+)-valence in a three-phase partitioning bioreactor, overcame the bioconversion inhibition.Despite of under lower hydrodynamic stress (lower agitation and aeration rates) cell viability loss was diminished; the (+)-nootkatone production yields were not improved.

Finally, whole cell bioconversion of *Y. lipolytica* 2.2ab in an organic phase (orange essential oil) to produce (+)-nootkatone was successfully assessed, as it was observed during the screening method.

This thesis promises to be an important step in the analysis of process performance in bioconversions using a TPPB and useful tool in the understanding and evaluation of the design of bioconversions in this type of bioreactors.

PERSPECTIVES

Identify the enzyme or enzymes involved in the bioconversion.

Modify genetically the *Y. lipolytica* 2.2ab and *B. theobromae* 1368 strains to enhance (+)-nootkatone production.

This work could be a starting point to establish the window of operation for commercial exploitation involving bioconversion of sesquiterpenes in a three phase partitioning bioreactor.

ANNEXES

Figures annexed of Chapter VI.
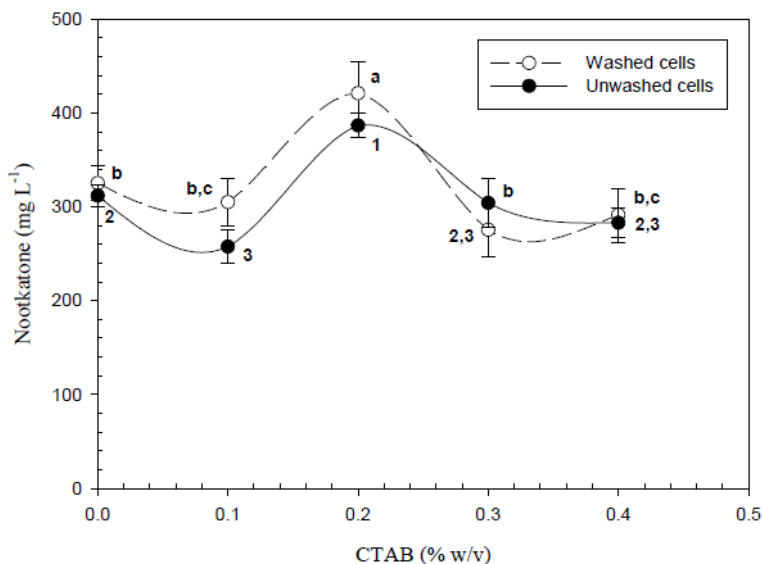
Figure A1. Effect of CTAB concentration on the bioconversion process using washed (○) and unwashed cells (●) of *Y. lipolytica*. Same letters for washed cells represent no significant differences, and same numbers for unwashed cells represent no significant differences (p $\leq$ 0.05; Tukey, SAS System Version 9) (p $\leq$ 0.05; Tukey, SAS System Version 9).
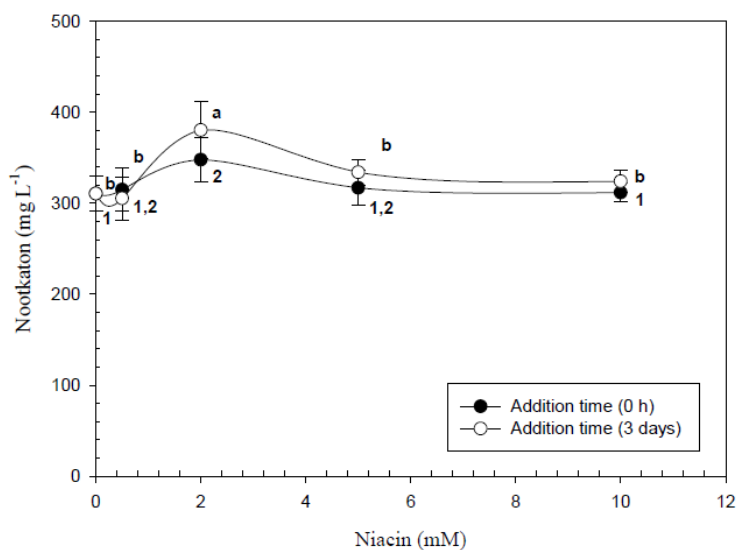
Figure A2. Effect of niacin concentration on the bioconversion process using *Y. lipolytica*. Niacin addition at 0 h (●) and after 3 days (○) of inoculation time. Same letters for addition time after 3 days represent no significant differences, and same numbers for addition time at 0 h represent no significant differences (p $\leq$ 0.05; Tukey, SAS System Version 9) (p $\leq$ 0.05; Tukey, SAS System Version 9).

DERIVATIVE WORKS OF THIS THESIS

INTERNATIONAL SCIENTIFIC INDEXING - (ISI).

1. Palmerín-Carreño D.M., Rutiaga-Quiñones O.M., Verde Calvo J.R., Prado-Barragán A., Huerta-Ochoa S. Screening of microorganisms for bioconversion of (+)-valencene to (+)-nootkatone. LWT- Food Science and Technology, (2015), **64**(2): 788-793.

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4. Palmerín-Carreño D.M., Castillo Araiza C.O., Rutiaga-Quiñones O.M., Verde Calvo J.R., Prado-Barragán A., Huerta-Ochoa S. Whole cell (+)-Nootkatone bioconversionin an organic phase (orange essential oil) system using *Yarrowia lipolytica* (In preparation).

5. Palmerín-Carreño D.M., Castillo-Araiza C.O., Rutiaga-Quiñones O.M., Verde Calvo J.R., Huerta-Ochoa S. Hydrodynamic and oxygen mass transfer studies in a three-phase (air–water–essential orange oil) stirred tank bioreactor for the bioconversion of (+)-valencene with *Yarrowia lipolytica*. (In preparation).

PARTICIPATION IN CONGRESS:

INTERNATIONALS

1. Carlos Castillo-Araiza, Dulce Palmerín-Carreño, Miguel Arellano, Abhishek Dutta, Arely Prado-Barragán and Sergio Huerta-Ochoa. An Approach to Develop a Kinetic Model from a Stirred Tank Reactor accounting for the Role of Hydrodynamics: (+)-Nootkatone Bioconversion, The 9th International Conference on Chemical Kinetics**,** June 28 to 02 July, 2015. Ghent, Belgium. (Modality Oral).

NATIONALS

1. Dulce María Palmerín Carreño, José Ramón Verde Calvo, Olga Miriam Rutiaga Quiñones and Sergio Huerta Ochoa*,* Efecto del estrés hidrodinámico y la concentración de biomasa en la bioconversión de (+)-nootkaton con *Yarrowia lipolytica*, XXXVI Encuentro Nacional, AMIDIQ, Retos de la Ingeniería Química en la Globalización. May 05 to 08, 2015, in Cancún QR, México. (Modality Oral).

2. Dulce María Palmerín Carreño, José Ramón Verde Calvo, Olga Miriam Rutiaga Quiñones and Sergio Huerta Ochoa*,* Mejoramiento de la bioconversión de (+)-valenceno a (+)-nootkaton con *Yarrowia lipolytica*, XXXV Encuentro Nacional, AMIDIQ, La interdisciplinariedad en la Ingeniería Química. May 06 to 09, 2014, in Puerto Vallarta, Jalisco, México. (Modality Oral).

3. Dulce María Palmerín Carreño, José Ramón Verde Calvo, Olga Miriam Rutiaga Quiñones and Sergio Huerta Ochoa*,* Bioconversión microbiana de (+)-valencene a (+)-nootkatone en un biorreactor de partición, XXXIV Encuentro Nacional, AMIDIQ, La Ingeniería Química en la Biotecnología y la Energía. May 07 to 10, 2013, in Mazatlán Sinaloa, México. (Modality Oral).

4. Dulce María Palmerín Carreño, Olga Miriam Rutiaga Quiñones, José Ramón Verde Calvo and Sergio Huerta Ochoa, Screening of selected microorganisms for bioconversion of (+)-valencene to (+)-nootkatone, XV Congreso Nacional de la

SMBB, 2013, June 23-28, in Cancun QR, México. (**Third place at Biotechnology, Modality Poster**).

5. Dulce María Palmerín Carreño, Olga Miriam Rutiaga Quiñones, José Ramón Verde Calvo and Sergio Huerta Ochoa, Hydrodynamic and oxygen mass transfer studies in a three-phase (air-water-orange oil) stirred tank bioreactor, XV Congreso Nacional de la SMBB, 2013, June 23-28, in Cancun QR, México. (Modality Poster).

6. Dulce María Palmerín Carreño, Olga Miriam Rutiaga Quiñones, José Ramón Verde Calvo and Sergio Huerta Ochoa, Bioconversion of (+)-valencene to (+)-nootkatone using a membrane-aerated biofilm reactor, XV Congreso Nacional de la SMBB, 2013, June 23-28, in Cancún QR, México. (Modality Oral).

HUMAN RESOURCES TRAINING

Coach Masters student Hanne De Crits with the thesis: KINETICS OF THE (+)-NOOTKATONE BIOCONVERSION USING WHOLE CELLS OF *YARROWIA LIPOLYTICA*. Chemical Engineering, Faculty of Engineering Technology, Campus Group T Leuven, Belgium.

These annexes shows the published papers from this thesis.

ANNEXE III

Screening of microorganisms for bioconversion of (+)-valencene to (+)-nootkatone

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A B S T R A C T

The production of (+)-nootkatone, highly appreciated by fragrance and flavour industries, can be performed by whole-cell bioconversion from the sesquiterpene (+)-valencene, a compound readily available in orange essential oil. The aim of this work was to screen for microorganisms that convert (+)-valencene to (+)-nootkatone using different bioconversion systems. The screening was conducted using six different microorganisms, and bioconversion experiments were set up on surface culture using aerological flasks containing PDA at 30 °C. It was observed that *Botryodiplodia theobromae* 1368, *Yarrowia lipolytica* 2.2ab, and *Phanerochaete chrysosporium* oxidized (+)-valencene to (+)-nootkatone, reaching (+)-nootkatone concentrations of 231.7 ± 2.1, 216.9 ± 5.8 and 100.8 ± 2.6 mg L^{-1}, respectively. Different bioconversion conditions were also tested—aqueous, organic, and biphasic—all resulting in similar (+)-nootkatone production. Both *B. theobromae* 1368 and *Y. lipolytica* 2.2ab showed substrate inhibition above 4.2×10^{-2} and 0.13 g of (+)-valencene (g of biomass)$^{-1}$, respectively, in aqueous phase experiments. Furthermore, *B. theobromae* 1368 and *Y. lipolytica* 2.2ab showed product inhibition when concentrations reached above 17.02 and 34.78 mg of (+)-nootkatone (g of biomass)$^{-1}$, respectively. The experimental method presented will be useful for ongoing studies on the selection and operation of the proper bioreactor at different bioconversion conditions.

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1. Introduction

Flavours and aromas are highly valued in food, cosmetic, chemical, and pharmaceutical industries. Most of the aromatic compounds available are obtained by chemical synthesis or solvent extraction. However, consumers' growing aversion towards synthetic chemicals added to foods, cosmetics, and other household products has encouraged research efforts towards the production of aromatic and flavouring compounds using biological organisms (Maróstica & Pastore, 2007). The sesquiterpene molecules and their oxygenated derivatives are widely used in the aroma industry. Of particular interest is (+)-valencene ((2R)-8,8,8a-trimethyl-2-prop-1-en-2-yl-1,2,3,4,6,7-hexahydronaphthalene), the main bicyclic sesquiterpene found in orange peel oil (Elston, Lin & Rouseff, 2005). Sesquiterpenes are low-priced compounds commonly used as substrates for the bioconversion of various aromas (Misawa et al., 2011). The production of (+)-nootkatone has been conducted by chemical synthesis mainly from the sesquiterpene (+)-valencene, but also through the use of oxidising agents known to be unsafe to the environment, such as tert-butyl peracetate (Wilson & Shaw, 1978) or tert-butyl hydroperoxide in combination with metal catalysts supported on silica (Salvador & Clark, 2002). As such, the

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ANNEXE IV

BIOCONVERSION OF (+)-NOOTKATONE BY *Botryodiplodia theobromae* USING A MEMBRANE AERATED BIOFILM REACTOR

BIOCONVERSIÓN DE (+)-NOOTKATON POR *Botryodiplodia theobromae* UTILIZANDO UN REACTOR DE BIOPELÍCULA DE MEMBRANA AIREADA

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Abstract

The aim of this work was to evaluate the bioconversion of (+)-valencene to (+)-nootkatone by *B. theobromae* using a membrane aerated biofilm reactor (MABR) in a two liquid phase system with orange essential oil as the organic phase. In the aqueous phase system, a (+)-nootkatone production rate up to 3.98 mg L^{-1} h^{-1} was achieved, obtaining a final product concentration of 398.08 mg L^{-1} with a bioconversion of 62 %. A two liquid phase system, using orange essential oil as the dispersed phase, was also studied and a final (+)-nootkatone concentration of 310.37 mg L^{-1} was achieved in the organic phase, with a bioconversion of 30.5 % and a production rate of 2.46 mg L^{-1} day^{-1}. The lower performance obtained using the two phase system was probably due to mass transfer limitations. The present work is the first report on an MABR for the bioconversion of (+)-valencene to (+)-nootkatone. Further studies on bioconversion products and optimization of biofilm reactor operations are needed to enhance bioconversion.

Keywords: bioconversion, (+)-nootkatone, *Botryodiplodia theobromae*, orange essential oil, membrane aerated biofilm reactor.

Resumen

El objetivo de este trabajo fue evaluar la bioconversión de (+)-valenceno a (+)-nootkaton por *B. theobromae* usando un reactor de biopelícula de membrana aireada (MABR) en un sistema de dos fases líquidas con aceite esencial de naranja como fase orgánica. En el sistema de fase acuosa, se logró una tasa de producción de (+)-nootkaton de hasta 3.98 mg L^{-1} h^{-1}, obteniendo una concentración de producto final de 398.08 mg L^{-1} con una bioconversión de 62 %. También se estudió un sistema de dos fases líquidas, utilizando aceite esencial de naranja como fase dispersa, y se alcanzó una concentración final de (+)-nootkaton en la fase orgánica de 310.37 mg L^{-1}, con una bioconversión de 30.5 % y una tasa de producción de 2.46 mg L^{-1} $día^{-1}$. El menor rendimiento obtenido mediante el sistema de dos fases fue probablemente debido a las limitaciones de transferencia de masa. El presente trabajo es el primer reporte utilizando un MABR para la bioconversión de (+)-valenceno a (+)-nootkaton. Se necesitan estudios adicionales sobre los productos de bioconversión y la optimización de las condiciones de operación del reactor de biopelícula para mejorar la bioconversión.

Palabras clave: bioconversión, (+)-nootkaton, *Botryodiplodia theobromae*, aceite esencial de naranja, reactor de biopelícula de membrana aireada.

1 Introduction

The compound (+)-nootkatone is a sesquiterpenoid which possesses an intense grapefruit-like taste and other valuable properties that are highly appreciated by the fragrance and flavor industries (Ladaniya, 2010). The production of (+)-nootkatone is performed via chemical synthesis, mainly from the sesquiterpene

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ANNEXE V

Research Article

Whole cell bioconversion of (+)-valencene to (+)-nootkatone by *Yarrowia lipolytica* using a three phase partitioning bioreactor

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Abstract

BACKGROUND: Low permeability of substrates across the cell membrane, cofactor regeneration and product inhibition are some drawbacks of (+)-nootkatone bioconversion. The aim of this work was to evaluate and enhance the bioconversion of (+)-valencene to (+)-nootkatone with *Yarrowia lipolytica* in a partitioning bioreactor using orange essential oil as the dispersed phase.

RESULTS: Preliminary experiments in shake flasks allowed enhancing (+)-nootkatone bioconversion to obtain favorable operating conditions (0.2% w/v of CTAB, 2.0 mmol L^{-1} of niacin and 11.5 g L^{-1} of biomass) to produce 420.9 mg L^{-1}. Bioreactor experiments in a two-phase system using 0.2% (w/v) of CTAB, 2.0 mmol L^{-1} of niacin and 22.5 g L^{-1} of biomass produced a maximum (+)-nootkatone concentration of 619.8 mg L^{-1} which was around the product inhibition concentration. Nevertheless, the partitioning three-phase system using orange essential oil overcame product inhibition, obtaining concentrations up to 852.3 mg L^{-1}.

CONCLUSIONS: This is the first report of a wild type *Y. lipolytica* with the enzymatic machinery to carry out this bioconversion. The multiphase partitioning bioreactor concept seems to have good potential for enhancing the productivity of (+)-nootkatone. The bioconversion approach presents an attractive way to produce and recover (+)-nootkatone in situ using a natural (+)-valencene source.
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Keywords: bioconversion; partitioning bioreactor; (+)-nootkatone; orange essential oil; yeast; product inhibition

INTRODUCTION

The compound (+)-nootkatone is a sesquiterpenoid which possesses an intense grapefruit-like taste and other valuable properties that are exploited by the fragrance and flavour industries.[1] The production of (+)-nootkatone is performed via chemical synthesis, mainly from the sesquiterpene (+)-valencene, which is readily available from the orange industry and also through the use of environmentally unfriendly oxidising agents, such as tert-butyl peracetate[2] and tert-butyl hydroperoxide in combination with catalytic metal supported on silica.[3] However, the resulting (+)-nootkatone produced via chemical synthesis cannot be marketed as a 'natural' product and does not satisfy increasing market demands for natural aromatic compounds. In order to meet this demand, efforts have been focused on the use of biotechnological processes with bacteria, fungi or plants.[4] Some researchers have investigated genetically modified microorganisms, and several published reviews[5,6] have provided an overview of recently acquired knowledge about whole cell bioconversions and future industrial applications. In particular, industry and academia are interested in a biocatalytic process using whole cells which involves the selection of an optimal cellular enzyme, reaction engineering, product recovery and scaling-up.

Gavira et al.[7] reported a bioconversion process for (+)-nootkatone production using plant enzymes expressed in *Saccharomyces cerevisiae*. However, the process was inhibited by both the substrate (>4 g L^{-1}) and the product (1.0 g L^{-1}) as well

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