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PRODUCTION OF NATURAL AROMA COMPOUNDS BY BIOCATALYSIS

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1. ABSTRACT

Esters play a significant role in the food industry, they are among the most important and versatile components of natural flavours and fragrances in food, drinks and cosmetics Their preparation starting from natural substrates and using bioprocesses (e.g., fermentation or enzymatic reactions) is appealing, since the final product can be labelled and commercialized in EU and USA as natural. Therefore, new biotechnological approaches for obtaining flavours are highly demanded as long as they are efficient and sustainable. Many flavour/fragrance esters can be enzymatically obtained using lipases that catalyse esterification, transesterification or interesterification reactions.

In this PhD thesis we studied two systems for production of flavours esters:

1) A straightforward biocatalytic method for the enzymatic preparation of different flavour esters starting from primary alcohols (e.g., isoamyl, *n*-hexyl, geranyl, cinnamyl, 2-phenethyl, and benzyl alcohols) and naturally available ethyl esters (e.g., formate, acetate, propionate, and butyrate) was developed. The biotransformations are catalysed by an acyltransferase from *Mycobacterium smegmatis* (MsAcT) and preceded with excellent yields (80-97%) and short reaction times (30-120 minutes), even when high substrate concentrations (up to 0.5 M) were used. This enzymatic strategy represents an efficient alternative to the application of lipases in organic solvents and a significant improvement compared to already known methods in terms of reduced use of organic solvents, paving the way to a sustainable and efficient preparation of natural flavouring agents.

2) Mycelium-bound lipase of dry mycelium of *Aspergillus oryzae* catalysed direct esterification of alcohols and acetic acid in organic solvent, showing high stability towards substrates and products. Water produced during the esterification did not significantly affect the

equilibrium of the reaction, allowing for high conversions. These features were exploited for preparing flavour-active acetate esters (*e.g.*, isoamyl and cinnamyl acetate) in batch and continuous systems. A continuous stirred tank membrane reactor (CST-MR) was developed securing good reactor productivity and high biocatalyst stability.

Both production systems are promising, represent two different alternatives and can be further optimized and scaled up for the interests of the industry.

2. RIASSUNTO

Gli esteri svolgono un ruolo rilevante nell'industria alimentare, sono tra i composti più importanti e versatili di aromi e fragranze naturali in alimenti, bevande e cosmetici. La loro preparazione da substrati naturali e l'utilizzo di bioprocessi (eg. fermentazione o reazioni enzimatiche) è attrattivo, perché il prodotto finale può essere etichettato e commercializzato in UE e USA come naturale. Pertanto, nuovi approcci biotecnologici per ottenere aromi sono desiderati, una volta che siano efficienti e sostenibili. Molti esteri con proprietà aromatiche possono essere ottenuti enzimaticamente usando lipasi che catalizzano reazioni di esterificazione, transesterificazione o interesterificazione.

In questa tesi di dottorato abbiamo sviluppato due sistemi per la produzione di esteri con proprietà aromatiche:

1) Un metodo biocatalitico per la preparazione enzimatica di diversi esteri con proprietà aromatiche da alcoli primari (isoamilico, n-esilico, geranilico, cinnamilico, 2-feniletilico e benzilico) ed esteri etilici naturalmente disponibili (formiato, acetato, propionato e butirrato). Le biotrasformazioni sono catalizzate da un'aciltransferasi di *Mycobacterium smegmatis* (MsAcT) e precedute da eccellenti rese (80-97%) e brevi tempi di reazione (30-120 minuti), anche quando sono state utilizzate concentrazioni di substrato elevate (fino a 0,5 M). Questa strategia enzimatica rappresenta un'alternativa efficace all'applicazione delle lipasi nei solventi organici e un miglioramento significativo rispetto ai metodi già noti in termini di uso ridotto di solventi organici, aprendo la strada a una preparazione sostenibile ed efficiente degli agenti aromatizzanti naturali.

2) Un metodo biocatalitico con la lipasi legata al micelio liofilizzato di *Aspergillus oryzae* che catalizza l'esterificazione diretta di alcoli e acido acetico in solvente organico. Ha mostrato un'elevata stabilità verso

substrati e prodotti. L'acqua prodotta durante l'esterificazione non ha influenzato in modo significativo l'equilibrio della reazione, consentendo conversioni elevate. Queste caratteristiche sono state sfruttate per preparare esteri con proprietà aromatiche dell'acetato (isoamil e cinnamil acetato) in sistemi in *batch* e continui. È stato sviluppato un *continuous stirred tank membrane reactor (CST-MR)* ovvero un reattore continuo a membrana sotto agitazione per garantire una buona produttività e un'elevata stabilità del biocatalizzatore.

Entrambi i sistemi di produzione sono promettenti, rappresentano due diverse alternative e possono essere ulteriormente ottimizzati e scalati per gli interessi del settore.

3. INTRODUCTION

2.1. Flavours, the importance from prehistory until present days

Since the prehistory, some of our ancestors were pleased by the aromas, the one contained in flowers or certain herbs added to the food. They had applications for aromatic plants in the burial sites, as offerings to the gods. Fragrant oils were widely used throughout the Middle East to provide skin care and protection from the hot and dry environment. With the emergence of writing, we have many references to the use of herbs and spices in the Mesopotamia, ancient Egypt and Indus Valley (Pybus et al, 1999; Rowe, 2009). Some proof of this can be found at least as early as 2,000 BC in the '*Enûma Eliš',* the Babylonian creation myth in cuneiform from Mesopotamia, in which there are mentions to the spices and fragrances that were added to wine (Pybus et al, 1999).

Still in the ancient world, in Egypt, as revealed in the 'Book of the Dead', 1,500 BC, incenses and fragrances played a key role in religious rites, and in the embalmment of the deceased. The prayers of the priests were believed to be carried via incense into heaven and to the ears of Osiris and other gods who ruled over the worlds of the dead (Budge, 1960). The most famous queen of Egypt, Cleopatra, performed aromatic baths with flowers, herbs and spices, which had relaxing, antiseptic, and cosmetic properties; besides that, she wore perfumes, with myrrh and cardamom in their compositions (Morris, 1984; Tsoucalas & Sgantzos, 2019).

Subsequently, between 700 and 500 BC, the Greeks, more specifically Rhodian potters, developed vast quantities of perfume bottles, decorated and adorned with animalistic and human motifs, which were shipped around the Mediterranean area (Voudouri & Tesseromatis, 2015).

The ancient Greeks and Romans used perfumes extensively, keeping their clothes in scented chests and incorporating scent bags to add fragrance to the air. They often applied different perfumes to each part of the

body, where mint was preferred for the arms; palm oil for the face and breasts; marjoram extract for the hair and eyebrows; and essence of ivy for the knees and neck (Berguer, 2007). At their feasts, Greek and Roman aristocrats adorned themselves with flowers and scented waxes and added the fragrance of violets, roses, and other flowers to their wines. In the cities there were many perfume shops, for all walks of society. In Greek mythology, the invention of perfumes was attributed to the Immortals; Homer reports that whenever the Olympian gods honoured mortals by visiting them, an ambrosial odour was left, an evidence of their divine nature (Piesse, 1879).

Philosophers such as Aristotle were able to present some of the ideas that can be appropriated today as science, in texts such as *'De Sensu et Sensibilibus'*, in *'Parva Naturalia'*, and in *'Historia Animalium'*, where he reports observations and some concepts of taste and smell, and predilection of certain animals for some flavours and odours (Ross, 1955; Balme & Gotthelf 2002). The perfumes were not universally accepted in ancient Greece; Socrates, for example, objected to them altogether (Morfit, 1847). However, the use of perfumes became so popular that laws were passed in Athens to restrain their use. Although this prohibition, however, their use increased unabated, and the Greeks added considerably to the stock of fragrant plants from East, that composed the core of the perfume industry (Schilling et al, 2010).

Already in the age of Christ, the Bible brings various accounts and passages, either in the New or Old Testament, about the use of spices and herbs in everyday use, such as in the preparation of food, rituals, and perfumes. Christ at his birth was presented by the Three Wise Men with gold, incense and myrrh, these last two are notoriously known for their aromatic properties and symbolized deity and death, respectively (Pybus & Sell, 2007).

After the fall of the West Roman Empire, in Europe, in the Middle Ages the foundations of chemistry were lost into the mysticism of alchemy.

Some techniques remained in use, especially distillation, but this also became a mystical affair. The classical world used distillation to refine a crude material into a more valuable one, such as an aromatic arose into an essential oil from what was merely part of plant, a basic concept that we are still familiar with today (Guenther, 2013). The powerful mediaeval church frowned on the use of fragrance because it was not appropriate, and the herbs were still used for flavours, encouraging earthly vanity. During this time, in Arab world progress was being made, which is reflected in the number of words which origin is Arabic, including alcohol and chemistry itself. The great trading cities of Venice and Genoa controlled much of this trade, with the Silk Road to the East carrying precious spices and oils via Constantinople, the capital of Byzantium, the Eastern Roman Empire (Pybus et al, 1999).

The fall of the Byzantine Roman Empire, which had been the junction between Occident and Orient, had a big impact on trade routes to India and the East Asia, the origin of the most prized spices. This led adventurers, especially from the big trading cities of Genoa and Venice, to search for alternative routes to the East. Briefly, flavours and fragrances drove the Europeans to the discovery of the Americas. The exposure to hitherto unknown cultures and the rediscovery of classical science, often via Arabic texts, drove to a new intellectual flowering in Europe (Pybus & Sell, 2007).

In this period, the Renaissance, there was a greater interest in knowing more about the properties of flavours. Leonardo da Vinci, in the Codex Atlanticus, presented nine diagrams in which he compares the behaviour of light, strength, sound, magnetism, and odour (Kelee, 2014). The Cardinal Gasparo Contarini, an alchemist, wrote about the elements and their combinations, and a volume published posthumously, in 1548, was dedicated to flavours, odours, and colours (Doty, 2015). Contarini was convinced that there were eight flavours or tastes and argued that cooking or conserving fruits could produce flavours not found in nature, and also noted that the names of flavours are often employed to explain the variety of

odours (Doty, 2015). The concepts of science, based on the observation and measurement, were established by Galileo Galilei (1564-1642), resuming Aristotle's adopted practice (Hilliam, 2004).

Later on, in the early 1700s, maybe the single most important event for the flavour and fragrance industry was the invention of the thermometer by Celsius and Fahrenheit (Hopp & Mori, 1993). The essentials of modern chemistry were established by the studies of Lavoisier, Dalton, Newton and others, and the mystical systems from the alchemists were left behind (Rowe, 2009). From the early 1800s, developments in chemistry, mostly in Great Britain and Germany, began to create the flavour and fragrance industry. Purification of natural materials, especially essential oils, led to the identification of aroma-active materials, such as benzaldehyde in the 1834, and posteriorly cinnamaldehyde and vanillin (Morris, 1984). The knowledge of organic chemistry increased, beginning maybe with the discovery of August Wöhler that these isolated materials could be synthesized in the laboratory (Nicolaou, 2014). The cycle of the chemical industry was initiated, with the identification, laboratory synthesis, large-scale synthesis and commercialization resulting in falling costs and price. At this time, important events and discoveries were very frequent, particularly in the area of flavourings. It can cited the synthesis of cinnamaldehyde in 1852 and vanillin in 1872, which alongside with the coumarim were the first synthetic fragrance and flavour compounds made available for use in the food industry. The release of ‘Chanel Nº5’ by Coco Chanel in 1921 is a milestone to this day in the perfume industry; in 1956 it was developed and released the first instrument of nuclear magnetic resonance (NMR); the Gas Chromatography (GC) was developed by James and Martin in 1959 (Morris, 1984; Pybus et al, 1999; Swift, 2012). And after the end of Second World War, the flavour industry has expanded notably.

With the advent of current chemistry, the countless chemical structures were elucidated by sophisticated modern techniques such as mass spectroscopy and NMR, which was followed by their chemical

synthesis (Hopp & Mori, 1993). Currently, the industry uses flavours and fragrances as food additives, cosmetics, perfumes, chemical and pharmaceutical formulations and even in cleaning products and they are mostly produced through artificial means, by chemical synthesis or by extraction from plant and animal sources (Gupta et al, 2015). It is estimated that this industry worth US$26,3 billion (Leffing & Associates, 2019) and that 95% of chemicals used in flavours and fragrances are artificially derived principally from petroleum (Vandamme & Soetaert, 2002). The first classification of flavour chemicals is by volatility or by how easily the compound evaporates. The volatile compounds travel through air into the nose or the oesophageal passageway to arrive at the nasal receptors and elicit a response (Theimer, 2012). The non-volatile compounds must be carried to the taste buds of the tongue or the inner lining of the mouth via food or saliva to elicit a response (Dresow & Böhm, 2009). There are numerous volatile compounds that are responsible for the flavours, such as aldehydes, esters, alcohols, ketones, volatile acids, lactones, furans, phenolic compounds, terpenoids, sulphur compounds (Hadi, 2013; Theimer, 2012). Among these compounds, the ester molecules are responsible for a lot of sensory tastes and are the pillar of any flavour and fragrance industry.

2.2. Flavour Esters

Flavour esters are volatile compounds, particularly found in fruits and vegetables, where these compounds are the secondary products of diverse metabolic pathways (Crouteau & Karp, 1994; Longo & Sanroman, 2016). These molecules act as an interface between the producing organism and its environment, having biological function such as combat infectious diseases, attract pollinators and disincentive or incentive the herbivores to eat it (Crouteau & Karp, 1994; Swift, 2012). The flavour esters with short chain often have pleasurable characteristics, fruity and floral odours, therefore having a great commercial value. Aliphatic esters in diverse

combinations play a major role in many fruit flavours, and may even be species markers. Hexyl acetate contributes to Cox's Orange Pippin apples with their intense characteristic aroma (Bomgardner, 2012). Most of the flavours of Golden Delicious apples come from hexyl 2-methylbutyrate. Methyl and ethyl cinnamates give sweet, honey scent and are related with strawberry flavour (Burdock, 2016; López et al, 2004). Pineapple flavours contain the methyl and ethyl esters of (*Z*)-dec-4-enoic, (*E*)-hex-3-enoic and (*E*) and (*Z*)-oct-4-enoic acids (Burdock, 2016; Fischer & Scott, 2007). Ethyl (*E,Z*)-deca-2,4-dienoate is the characteristic compound of Bartlett pears (Burdock, 2016; Shiota, 1990).

The majority of flavour esters are prepared by chemical synthesis or by extraction from plants and animal sources, but since they occur as complex mixtures with very varied (generally low to ultra-trace concentrations) they could not be viable for extraction (Vandamme & Soetaert, 2002). The chemical synthesis seems to be an immediate alternative, but it is not eco-friendly, and may have some toxic effects to the customer's health, hence the products containing these synthetic flavours cannot carry the label 'natural' (Gupta et al, 2015; Kim, 2005). The non-natural fragrances were created either by emulation of natural structures through systematic studies on the relation between odour and chemical structure, or by accident (John & Tyman, 1995). The use of new biotechnological processes has increased notably in the past decades, with four different major biotechnological methods: through enzymes, microorganisms, plant cell cultures, and plant tissue cultures. Processes employing enzymes are the most frequent techniques (Gupta et al, 2015).

Moreover, the increasing sensitivity of the ecological systems supports the preference for environmentally friendly processes and consumers have developed a choice for 'natural' and 'organic' products, thus prospering a market for flavours of biotechnological origin (Frey & Roussef, 2005; Wackett, 2012).

According to effective European law (EG 1334/2008), a ‘natural flavouring substance’ is a compound ‘obtained by appropriate physical, enzymatic or microbiological processes from material of vegetable, animal or microbiological origin’. Also in the United States of America, the Code of Federal Regulation (CFR - Title 21) of the Food and Drug Administration (FDA) contains a similar definition including the terms ‘enzymolysis’ and ‘fermentation’.

Independently from the current laws and standards that favour biotechnology, providing advantages particularly for the production of volatile flavours, more recent efforts for a biotechnology of flavours are the worldwide focus on a ‘greener chemistry’ and the association of flavours with beneficial health effects (Kerton & Marriot, 2013; Tao & Kazlauskas, 2011).

2.3. Biocatalysis

Within the vast world of biotechnology, biocatalysis is the one that aims to study catalyse reactions carried out with biological catalysts, whether they are whole cells or isolated enzymes. The use of enzymes is considered a valid alternative to conventional synthesis techniques thanks to their peculiar advantages, as well as to the high availability deriving from biological systems and the variety of chemical reactions they catalyse (Krings & Berger, 1998).

The main advantage of enzymes is the chemo regio/stereo selectivity with which they can catalyse a reaction. Enzymes are in fact chemospecific, that is, they are able to act on a specific functional group, without involving others present in the same molecule, which would normally react by chemical catalysis (Nair et al, 2010).

Even more interesting are the region specificity and the stereo specificity that distinguish them. Thanks to their complex three-dimensional structure, they are able to distinguish between identical functional groups located in different regions of the substrate; the possible stereo genic

centres present in the molecule are also recognized (Pawelek et al, 2000). Being the enzymes chiral catalysts formed by L-amino acids, they can selectively react by forming an enzyme-substrate complex with only one enantiomer, or transform a prochiral substrate into an optically active product (Nair et al, 2010).

Enzymes, unlike chemical catalysts, are used under mild reaction conditions in terms of temperature and pH. The main solvent is water (Timson, 2019). This not only makes it easier to set up biotransformations, but also to use them more safely for the operator and for industrial scale-up. There are also no problems related to their disposal or disposal of solvents, wastewaters with extreme pH or containing heavy metals. Enzymes are therefore environmentally sustainable and safe (Kraft, 2005).

Currently, many researchers and industries have moved to biocatalytic flavour synthesis due to consumer's inclination for natural flavours over chemical ones. The volatile flavours, like other agonists, frequently carry stereo centres, and both intensity and quality are generally affected by the stereochemistry (Berger, 2015). Chiral flavours commonly occur in nature as single enantiomers. Because diverse enantiomers or region isomers could show different sensorial properties, their specific synthesis is beneficial (Vandamme & Soetaert, 2002). To perform their physiological functions, volatile flavours have to possess not only physical volatility, but also chemical instability of the structures (thiol groups and aldehyde functions, Z-double bonds, tertiary alcohols, others) (Berger, 2015). Biocatalysis constitute a useful tool in this field, catalysing large numbers of stereo and regio selective chemical preparations that are simply achieved by the less selective classical manipulation These preparations use soft operating conditions, have high specificity with few reactions, and produce high purity compounds by avoiding the costly purification techniques (Tao & Kazlauskas, 2011; Timson, 2019). As most bioprocesses are under ambient conditions, volatile flavours should become interesting in practical point of view for the production in industrial scale.

2.4. The enzyme MsAcT

MsAcT is an acyltransferase isolated from *Mycobacterium smegmatis*, which has wide substrate specificity. Its structure is at the base of the interesting transfer capacity of the acyl group in water. A difference from lipases, which can catalyse the alcoholysis and the aminolysis under anhydrous conditions, is that MsAcT possesses the ability to synthesize esters and amides with a great specificity of substrate in an aqueous medium. Moreover, in the presence of hydrogen peroxide, this enzyme is also able to catalyse the perhydrolysis of esters with the formation of peracids (Leeuw et al, 2018; Wiermans et al, 2013).

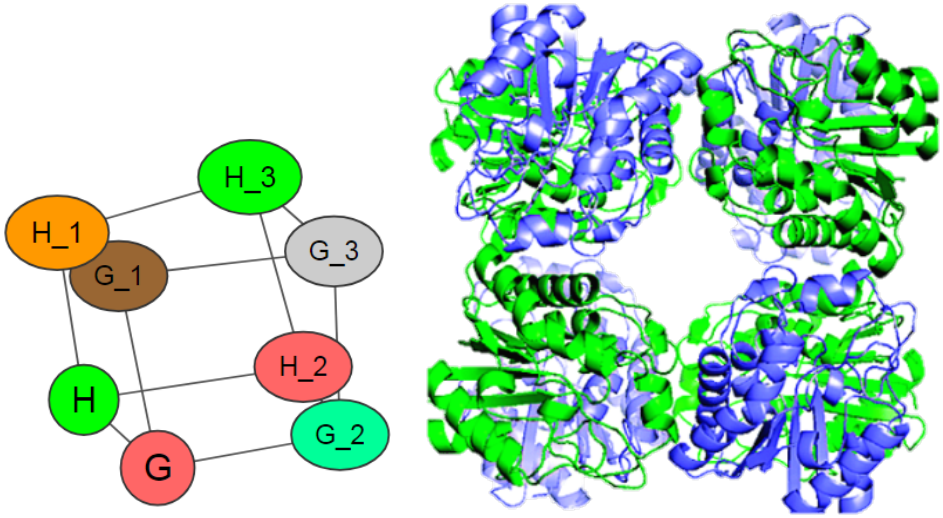

Figure 1. Schematic and three-dimensional representation of the enzyme MsAcT in octamer form. (Mathews et al, 2007)

MsAcT is an octamer. The single structurally identical monomers contain different insertions that contribute to the oligomerization and considerably restrict the shape of the active site, thus limiting its accessibility

(Figure 1). These properties allow obtaining a functional environment for acylation: the presence of a hydrophobic tunnel (Mathews et al, 2007).

By virtue of its peculiar properties, MsAcT is an enzyme potentially able to be used for biotechnological applications in numerous types of industry, for example, the transfer of acyl chains onto a nucleophilic acceptor such as an alcohol is one of the main reactions of interest in the conversion of vegetable oils into high added value products (Kazemi et al, 2019).

Considering acyl chains of reduced length compared to those of fatty acids, their transfer catalysed by MsAcT to an alcohol, with consequent ester formation, can be an efficient biotechnological method for the preparation of aromas, obtaining natural flavours through bioprocesses.

To better understand the reaction mechanism of MsAcT, studies have been carried out on the architecture of its active site. The catalytic triad consists of Serine-Aspartate-Histidine and the "oxyanionhole" in which the tetrahedral intermediate is formed, stabilized by two hydrogen bonds with amino acid residues (Asparagine 192, Alanine 55), characteristics in common with the SGNH Serin superfamily hydrolase (Mathews et al, 2007).

The catalysis begins with the nucleophilic attack on the carbonyl group of the acyl donor by the Ser 11 hydroxyl group, thanks to the decrease of its pKa determined by the particular spatial configuration of the three amino acid residues. In this phase, it forms a tetrahedral intermediate ("oxyanion") stabilized by two hydrogen bonds with the amino residues of Ala55 and Asn94 of the protein structure, in the so-called "oxyanionhole" (Contente et al, 2018).

Subsequently, it is observed the release of the alcoholic part (R'OH) of the acyl donor and the formation of the acyl-enzyme complex, attackable by nucleophilic species such as water, alcohols or amines (acyl group acceptors). The entire catalytic cycle of transesterification uses a primary alcohol as nucleophile. It is divided into two parts (Figure 2): the first half concerns the enzymatic acylation through the transfer of the acyl group from

the donor to the Ser 11, while in the second half the acyl group is transferred from the enzyme to the final acceptor (Leeuw et al, 2018).

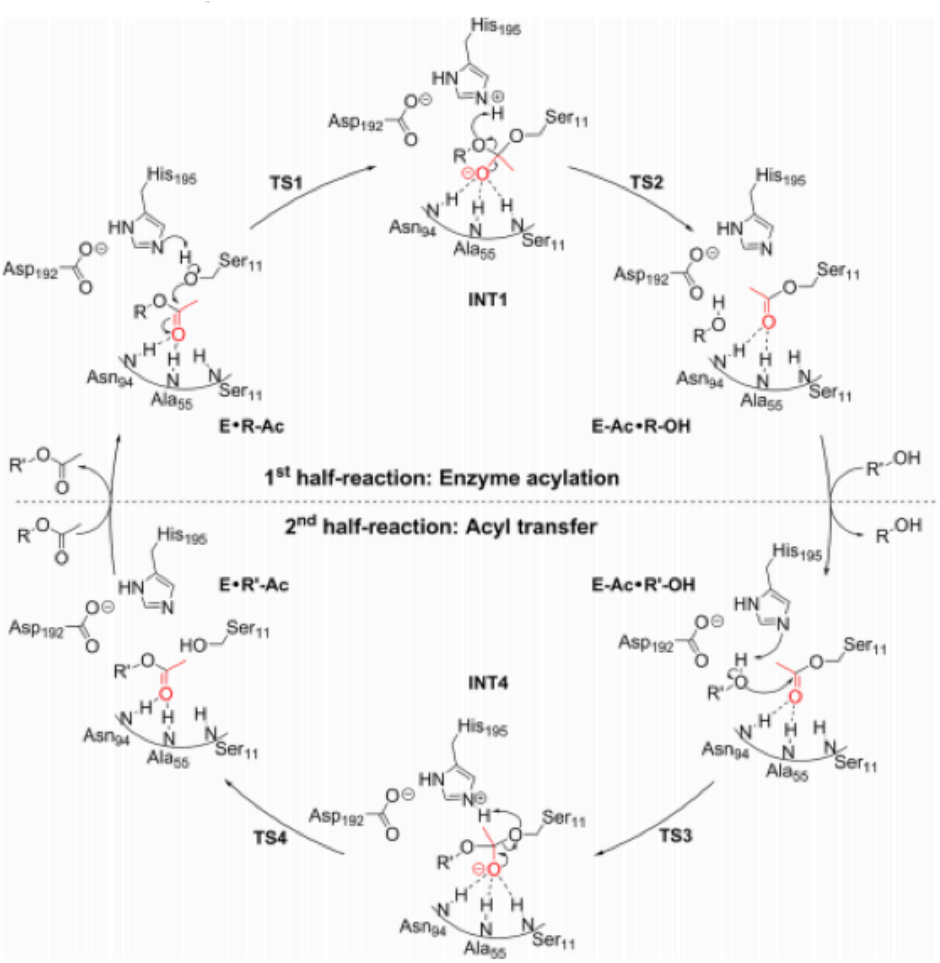

Figure 2. Mechanism of action for transesterification catalysed by MsAcT. The catalytic cycle can be divided into two half-reactions, involving enzyme acylation and acyl transfer to the substrate. (Kazemi et al, 2019)

Despite the high biotransformation efficiency, there is a margin of improvement in the enzymatic performance that consists in eliminating the hydrolysis reaction of the acyl donor and the newly formed product caused by the presence of water. Due to fact that this side reaction is observed in the long term, a reduction in the efficiency of biotransformation and enzyme

inactivation due to the lowering of the pH could occur because of the formation of carboxylic acids derived from the hydrolysis of the acyl donor (Hendil-Forssell et al, 2016).

From a thermodynamic equilibrium point of view it is normal that the hydrolysis reaction occurs, but the condensation reaction is largely favoured. The main reason is the greater affinity of MsAcT for primary alcohol compared to water, as a consequence of the hydrophobic nature of the active site of the enzyme (Leeuw et al, 2018).

2.5. Esterase from *Aspergillus oryzae*

Aspergillus oryzae is a filamentous fungus, firstly isolated from koji by Ahlburd in 1876 and identified as *Eurotium oryzae* and later renamed *Aspergillus oryzae* by Cohn (Machida et al, 2008). This microorganism has the Generally Regarded As Safe (GRAS) status granted by the Food and Drug Administration (Abe et al, 2006) which allows the use of its metabolites in the manufacture of human and animal foods. The use of *Aspergillus oryzae* is also endorsed by the World Health Organization (WHO) (FAO WHO, 1987).

Due to their historical use as a safe source of native enzymes and proven growth in industrial production conditions, *Aspergillus oryzae* and *Aspergillus soye* fungi, called koji molds, have been widely used in the production of fermented Asian food for over a thousand years. Some of the products made from their metabolites are sake (rice wine), shochu (fermented drink), shoyu (soy sauce), miso (soy paste), tofu, spices and vinegar (Abe et al, 2006).

Filamentous fungi are well recognized for their capacity to secrete large quantity of proteins and, among them, the *Aspergillus* species is largely used for the production of industrial enzymes (Demain & Vaishnav, 2009; Pandey et al, 1999) In particular, *Aspergillus oryzae* is often used for the production of enzymes. Some studies show the use and production of

various enzymes, among which a lipase capable of catalysing acylation with free acetic acid, and optimal ester acetate yield may be obtained (Barbesgaard et al, 1997).

Lyophilized mycelium from an isolated strain of *Aspergillus oryzae* showed excellent ability to promote direct acetylation, and was used to show the factors that influence the production of cell-bound carboxyesterases (Converti, 2002; Romano, 2005). Direct acetylation of alcohols is very difficult to achieve through the catalytic enzyme because an enzymatic activity is often inhibited by the presence of free acids, which has been confirmed in previous studies with *Aspergillus oryzae*, whereas the effect of some alcohols was nearly negligible (Romano 2005; Tamborini et al 2013).

As fungal lipases often have several interesting features of biocatalysis, they are usually secreted, as extracellular enzymes, but researches have demonstrated the existence of mycelium-bound enzymes with optimal activities, which can be exploited directly used as whole cells (Panda & Gowrishankar, 2005).

In addition, the use of whole cells for biocatalysis can protect enzymes and their activity better than an immobilization matrix. The high stability of lipases to organic solvents is desirable and more attractive than transesterification and other synthetic reactions of commercial importance (Klibanov 2001).

However, many studies focusing on *Aspergillus oryzae* lipase have failed, and as they were unable to work with high substrate concentrations and did not consider several substrates, these results are unsatisfactory, mainly for industrial purposes.

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4. OBJECTIVES

Considering the importance of aromas in our daily lives, the increasing demand for natural products and their nature, this project had as its general objective the production of natural aromas through biocatalysis.

As specifics objectives can be listed:

- purification, quantification and determination of the activity of the MsAcT by p-nitrophenyl acetate;
- determination of molar conversion several different flavor esters derived from transesterification of primary alcohols with ethyl esters bearing short-chain acyl groups;
- determination of molar conversion of flavor esters derived from direct esterification between primary alcohols with acetic acid;
- preparation of flavour esters using mycelium-bound lipase from *Aspergillus oryzae* in a continuous stirred tank membrane reactor (CST-MR).
- determination of biocatalytic activity and stability of mycelium-bound lipase of *Aspergillus oryzae* on stirred tank membrane reactor (CST-MR)
- determination of reactor productivity of ester on stirred tank membrane reactor (CST-MR)

5. RESULTS CHAPTERS

5.1. Efficient enzymatic preparation of flavour esters in water

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Efficient enzymatic preparation of flavor esters in water

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KEYWORDS: *Flavor esters, enzymatic acylation, biocatalysis, acyltransferase, Mycobacterium smegmatis (MsAcT)*

ABSTRACT: A straightforward biocatalytic method for the enzymatic preparation of different flavor esters starting from primary alcohols (e.g., isoamyl, *n*-hexyl, geranyl, cinnamyl, 2-phenethyl, and benzyl alcohols) and naturally available ethyl esters (e.g., formate, acetate, propionate, and butyrate) was developed. The biotransformations are catalyzed by an acyltransferase from *Mycobacterium smegmatis* (MsAcT) and proceeded with excellent yields (80-97%) and short reaction times (30-120 minutes), even when high substrate concentrations (up to 0.5 M) were used. This enzymatic strategy represents an efficient alternative to the application of lipases in organic solvents and a significant improvement compared to already known methods in terms of reduced use of organic solvents, paving the way to a sustainable and efficient preparation of natural flavoring agents.

INTRODUCTION

Many esters are components of natural flavors and are used as flavor and fragrance in food, drinks and cosmetics.[1] Their preparation starting from natural substrates and using bioprocesses (e.g., fermentation or enzymatic reactions) is appealing, since the final product can be labelled and commercialized in EU and USA as natural.[2, 3] Therefore, new biotechnological approaches for obtaining flavors are highly demanded as long as they are efficient and sustainable.[4, 5] Many flavor/fragrance esters can be enzymatically obtained using lipases that catalyze esterification, transesterification or interesterification reactions in media (e.g., organic solvents) characterized by low water activity.[6-8] The presence of water is critical for the equilibrium of lipase-catalyzed reactions in organic media, strongly limiting the overall yields; in fact water may compete as a nucleophile with the alcohol in the attack of the acyl-enzyme intermediate, thus favoring hydrolysis over transesterification, [9-11] and for this reason water must be avoided or removed during the reaction for achieving high yields. [12,13] Flavor ester biosynthesis in an aqueous system composed of coconut cream and fusel oil was achieved with limited conversion (14.25 mg/g, based on cream weight), after optimization of the process catalyzed by the lipase Palatase.[14] The potential of lipases for flavor ester production has been exploited also using immobilized enzymes[15] and whole microbial cells;[16-18] these systems have been especially employed for setting up robust processes and/or facilitating product recovery. Acetate esters are amongst the most valuable flavor esters and few of them has been produced in hydrophobic solvents (e.g., *n*-pentane, *n*-hexane or *n*-heptane) using different immobilized lipases;[19] in a large scale production of flavor esters, Novozym 435 (35 g/L) was able to give yields ranging between 91 and 95% after 24 h starting from an alcohol concentration of 1.0 M and an excess of acetic acid.[12] Alternatively, the use of *Candida rugosa* lipase, immobilized in calcium alginate gel, allowed for a productivity of 1600-2200 mmol/h/g of biocatalyst for the preparation of isoamyl and butyryl acetate.[20]

An interesting enzymatic alternative for preparing esters is the use of the acyltransferase from *Mycobacterium smegmatis* (MsAcT).[21] MsAcT is characterized by a hydrophobic tunnel leading to the active site, where water access is disfavored;[21,22] moreover, MsAcT is functionally active as a closely aggregated octamer in aqueous solution.[21] This overall architecture means that MsAcT is able to favor transesterification reactions with respect to hydrolysis even in water, unlike lipases that catalyze transesterification only under conditions of low water activity.[22] For these structural reasons, MsAcT has been used for catalyzing transesterification reactions of primary and secondary alcohols with ethyl acetate in aqueous buffers, often with good enantioselectivity.[22-24] The ratio between the catalytic rate constants of the hydrolysis and synthesis of benzyl acetate were compared, showing that MsAcT is indeed an effective acetyltransferase rather than a hydrolytic enzyme.[24] At reasonably high concentrations of the ester, the reaction mixtures of these biotransformations are actually two-liquid phase systems composed of the hydrophobic ester and water, and the reaction occurs in the aqueous phase. The peculiar features of this enzyme makes it also suitable for catalyzing amide synthesis in aqueous media.[25, 26]

In this work, we have explored the potential of MsAcT for flavor ester preparation; the enzyme was able to work in two-liquid phase systems composed of sparingly water-soluble esters and water, accomplishing the reaction in the aqueous phase. Several (24) different flavor esters derived from transesterification of primary alcohols (e.g., isoamyl, *n*-hexyl, geranyl, benzyl, 2-phenylethyl, and cinnamyl) with ethyl esters bearing short-chain acyl groups (e.g., formyl, acetyl, propionyl, and butyryl) were prepared in excellent yields and short reaction times loading high concentration of substrates (up to 500 mM), making the process interesting at industrial level.

MATERIALS AND METHODS

Chemicals. All reagents and solvents were obtained from commercial suppliers and were used without further purification.

Preparation of the acetyltransferase from *Mycobacterium smegmatis* (MsAcT). *E. coli* BL21 star (DE3) was used as host for the production of the recombinant protein; cloning was carried out as described before, starting from a synthetic gene encoding for MsAcT (GenBank accession: ABK70783) from *Mycobacterium smegmatis* str. MC2 155.[26] Recombinant *E. coli* was firstly grown at 37 °C in LB (Luria-Bertani) liquid medium in the presence of 25 μg/mL kanamycin for 16 h; this starting culture was used to inoculate the cultivation medium (Terrific Broth: 12 g/L bacto-tryptone, 24 g/L yeast extract, 4 g/L glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4, 25 μg/mL kanamycin), which was brought to an initial OD_{600nm} of 0.1. Cultivation was carried out at 37 °C in orbital shakers with an agitation speed 110 rpm; cells were grown until an OD_{600nm} of 0.5-0.6. The expression of MsAcT was induced by adding isopropyl-*b*-d-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The culture was further incubated for 16 h at 25 °C. Wet cells (2.0 g) were then harvested by centrifugation (20 min, 9000 rcf at 4 °C) and washed with 20 mM phosphate buffer pH 8.0, resuspended in 10 mL of buffer (100 mM phosphate buffer pH 8.0, 6 mM imidazole, 100 mM NaCl) and sonicated (5 cycles of 1 min on and 1 min off at 4° C). Cell debris were recovered by centrifugation (45 min, 36000 rcf at 4 °C). The enzyme was purified by affinity chromatography with HIS-Select® Nickel Affinity Gel. The fractions showing activity were pooled and dialyzed against phosphate buffer (100 mM, pH 8.0) and stored at 4 °C; 96 mg of pure protein were obtained. Activity was spectrophotometrically evaluated at 400 nm by determining the release of *p*-nitrophenol after hydrolysis of *p*-nitrophenylacetate at 25 °C in a half-microcuvette (volume 1 mL) for 2 min. One unit (U) of activity is defined as the amount of enzyme which catalyzes the consumption of 1 μmol of *p*-nitrophenylacetate per minute. Reference conditions were: 0.1 mg/mL *p*-nitrophenylacetate, 0.1% v/v EtOH, different amounts of MsAcT in 100 mM phosphate buffer, pH 8.0. The specific activity of the purified enzyme was 110 U/mg.

Preparation of flavor esters. Standard transesterification reactions were carried out in 10 mL screw cap tubes: alcohols (250 mM) and ethyl esters (10% v/v) were added to 2.5 mL of phosphate buffer (100 mM, in the range of pH 8.0) containing MsAcT as purified enzyme (0.1-1.5 mg/mL; the reaction was left under magnetic stirring at 25 °C. The reactions were stopped after 24 h and extracted with EtOAc (2x8 mL); the organic phases were collected, dried over Na_2SO_4 and evaporated. Products were purified by flash chromatography (eluent: *n*-hexane/EtOAc) and their structures and purity secured by ^{1}H NMR analysis by comparison with commercially available samples. Optimization of geranyl and cinnamyl acetate synthesis was carried out using 1 mL reaction mixture in phosphate buffer (100 mM, in the range of pH between 5.0 and 8.0), containing geraniol or cinnamyl alcohol (25-500 mM), MsAcT (0.1-1.5 mg/mL), and fixed amount of ethyl acetate (10% v/v) were left under magnetic stirring at different temperatures (20-40 °C). Aliquots (50 µL) were withdrawn at different reaction times, extracted twice with EtOAc (2x100 µL) and the collected organic phase was dried over Na_2SO_4. Preparative biotransformations at multi-milligram scale aimed at synthesizing geranyl acetate and cinnamyl acetate were carried out using geraniol (250 mM) and cinnamyl alcohol (250 mM) dissolved in 9 mL of phosphate buffer (0.1 M, pH 8.0) in the presence of 10 mg of MsAcT and 1 mL of EtOAc at 25 °C. The reactions were stopped after 24 h and extracted with EtOAc (2x8 mL); the organic phases were collected, dried over Na_2SO_4 and evaporated. Products were purified by flash chromatography; 442 mg (90% yield) of geranyl acetate (eluent: *n*-hexane/EtOAc 98:2, chemical purity > 98%) and 410 mg (93% yield) of pure cinnamyl acetate (eluent: *n*-hexane/EtOAc 95:5, chemical purity > 98%) were obtained. Purity of the product was confirmed by ^{1}H NMR and gas-chromatography analysis.

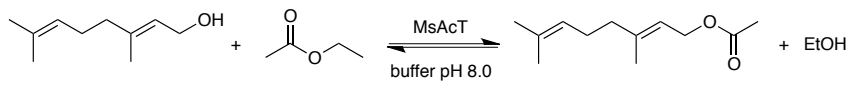
Analysis of the biotransformations. Aliquots of the biotransformation medium (100 µL) were withdrawn at different reaction times, quenched with 5 ml of 0.5 M sulfuric acid and extracted with 100 µL of ethyl acetate. The

amounts of produced flavor esters and the residual quantity of alcohol substrates were determined by gas-chromatographic (GC) analysis. Geraniol, cinnamyl alcohol, *n*-hexanol, benzyl alcohol, 2-phenylethanol and their esters were analysed by gas-GC analysis on a Carlo Erba Fractovap GC equipped with a fused-silica capillary column MEGA-SE30 (100% methyl polysiloxane; 25 m x 0.25 mm i.d.), with the injector temperature at 200 °C. Oven temperatures ranged from 45 to 180 °C. Retention times (temperature gradient: from 80 °C to 180 °C with 5 °C/min): geraniol 7.3 min; geranyl formate 10.2 min; geranyl acetate 10.2 min; geranyl propionate 12.5; geranyl butyrate 14.5 min; Retention times (temperature gradient: at 80 °C for 2 min then from 80 °C to 180 °C with 5 °C/min gradient): cinnamyl alcohol 9.9 min; cinnamyl formate 10.7 min; cinnamyl acetate 13.2 min; cinnamyl propionate 15.5 min; cinnamyl butyrate 17.8 min; Retention times (temperature gradient: at 50 °C for 10 min then from 50 °C to 150 °C with 10 °C/min gradient): *n*-hexanol 5.3 min; *n*-hexyl formate 7.7 min; *n*-hexyl acetate 8.8 min; *n*-hexyl propionate 15.2 min; *n*-hexyl butyrate 16.8 min; Retention times (temperature from 80 °C to 180 °C with 10 °C/min gradient): benzyl alcohol 3.0 min; benzyl formate 3.4 min; benzyl acetate 4.2 min; benzyl propionate 5.0 min; benzyl butyrate 6.5 min; 2-phenylethanol 3.7 min; 2-phenylethyl formate 4.3 min; 2-phenylethyl acetate 5.4 min; 2-phenylethyl propionate 6.6 min; 2-phenylethyl butyrate 7.8 min. Conversions of isoamyl alcohol into its esters were determined by GC analysis on a Carlo Erba Fractovap GC equipped with a fused-silica capillary column MEGA-DEX DMP-Beta (dimethyl pentyl-*b*-cyclodextrin; 25 m x 0.25 mm i.d.), with the injector temperature at 200 °C. Retention times (temperature gradient: from 40 °C to 180 °C with 5 °C/min): isoamyl alcohol 6.7 min; isoamyl formate 5.8 min; isoamyl acetate 7.1 min; isoamyl propionate 9.4 min; isoamyl butyrate 11.5 min. Initial rates were defined as the amount of mmol of ester produced after 30 min per amount of enzyme.

RESULTS and DISCUSSION

The reaction between geraniol and ethyl acetate (EtOAc) using the acyltransferase from *Mycobacterium smegmatis* (MsAcT) as biocatalyst was studied in a two-liquid phase system composed by water and EtOAc; the product (geranyl acetate) presents a green type flavour and a floral type odour.[1] Different parameters (pH, temperature and enzyme concentration) were optimized, while keeping the amount of geraniol (0.065 mM) and the phase ratio between water and EtOAc (9:1) fixed; a Multisimplex optimization design was employed,[27] using initial rate and molar conversions after 30 min as response variables. The best results (corresponding to a conversion >98% after 30 minutes) were obtained using 1.0 mg/mL of MsAcT at pH 8.0 and 25 °C; under these conditions the production of the ester was studied using different initial geraniol concentrations (Table 1).

Table 1. Acetylation of geraniol at different initial concentrations using MsAcT (1.0 mg/mL, 110 U/mL) in the presence of EtOAc (10% v/v) in phosphate buffer (0.1 M, pH 8.0) at 25 °C. [a] As determined by gas chromatography. [b] Time refers to maximum conversion observed.



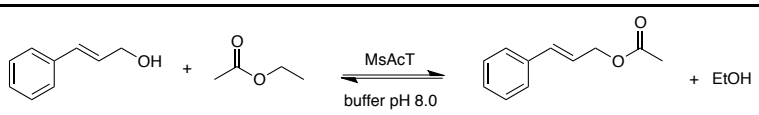
Geraniol (mM)	Initial rate (μmol/mg min)	Maximum conversion (%)[a]	Maximum product yield (mg/mL)	Time (h)[b]
100	2.25	> 98	19.6	2
200	4.56	> 98	39.2	24
250	6.36	98	48.1	24
300	6.57	95	55.9	24
400	7.68	79	62.0	48
500	7.65	68	66.7	48

The highest rates of geranyl acetate synthesis were achieved between 400-500 mM geraniol concentration, while almost quantitative yields were obtained only at alcohol concentrations lower than 300 mM. Possible product inhibition was evaluated by assessing the activity of MsAcT in the presence of increasing concentrations of EtOH and geranyl acetate (0-500 M); the activity of the enzyme was assay (with *p*-nitrophenyl acetate) in the presence of concentrations of EtOH between 250 and 500 mM showed that the enzyme was 40-50% inhibited, while no significant effects were observed with geranyl acetate.

The best compromise between rates and conversion was observed starting from 250 mM geraniol, allowing for 98% conversion (48.1 mg/mL analytical yield) after 24 hours. Notably, good conversion (68%) after 48 hours was achieved even when the biotransformation was carried out starting from 500 mM geraniol, allowing for the accumulation of 66.7 mg/mL of geranyl acetate.

A similar evaluation of the effect of substrate concentration was carried out in the case of the acetylation of cinnamyl alcohol (Table 2). Cinnamyl acetate has a balsamic-floral odour and a pineapple flavor.[1]

Table 2. Acetylation of cinnamyl alcohol at different initial concentrations using MsAcT (1.0 mg/mL, 110 U/mL) in the presence of EtOAc (10% v/v) in phosphate buffer (0.1 M, pH 8.0) at 25 °C. [a] As determined by gas chromatography. [b] Time refers to maximum conversion observed.



Cinnamyl alcohol (mM)	Initial rate (μmol/mg min)	Maximum conversion (%)[a]	Maximum product yield (mg/mL)	Time (h)[b]
100	2.54	> 98	17.6	1
200	5.07	> 98	35.2	3
250	6.32	> 98	44.0	6
300	6.82	92	48.6	24
400	8.53	84	59.2	24
500	8.65	79	69.6	24

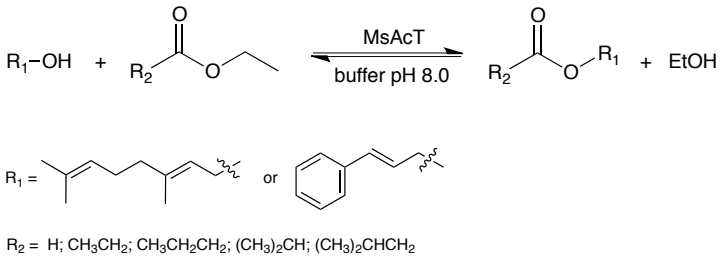
Enzymatic acetylation of cinnamyl alcohol occurred with generally better rates and yields than observed with geraniol, allowing for almost complete conversion of the alcohol up to 250 mM initial concentration after 6 hours. In the case of the reaction carried out with the highest initial concentration of alcohol (500 mM), 79% molar conversion was obtained after 48 hours.

Acetylation of geraniol and cinnamyl alcohol was performed on semipreparative scale (10 mL) starting from 250 mM alcohol concentration; the reactions were stopped and worked-up when conversions reached the maximum yield, allowing for the recovery of 442 mg of geranyl acetate (90% recovered yield) and 410 mg of cinnamyl acetate (93% yield), respectively.

Acylation of geraniol and cinnamyl alcohol were then studied with other acyl donors, which varied in acyl chain length; the results of the biotransformations carried out under optimized conditions (alcohol 250 mM, acyl donor 10% v/v, enzyme 1.0 mg/mL, buffer pH 8.0, 25 °C) are summarized in Table 3.

Table 3. Acylation of geraniol (250 mM) and cinnamyl alcohol (250 mM) using MsAcT (1.0 mg/mL, 110 U/mL) in the presence of different ethyl esters (10% v/v) in

phosphate buffer (0.1 M, pH 8.0) at 25 °C. [a] As determined by gas chromatography. [b] Time refers to maximum conversion observed.

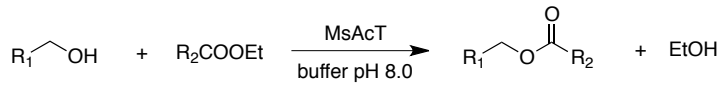


Alcohol	Ester	Ester main flavor property [1]	Initial rate (μmol/mg min)	Conv. (%)[a]	Time (h)[b]
Geraniol	Ethyl formate	rose	7.69	> 98	3
Geraniol	Ethyl propionate	fruity	1.51	86	48
Geraniol	Ethyl butyrate	fruity/apricot	1.15	62	48
Geraniol	Ethyl isobutyrate	rose/apricot	1.42	74	48
Geraniol	Ethyl isovalerate	rose/sweet apple	0.88	38	48
Cinnamyl alcohol	Ethyl formate	fruity/apple	7.72	> 98	1
Cinnamyl alcohol	Ethyl propionate	fruity/woody	2.13	> 98	3
Cinnamyl alcohol	Ethyl butyrate	fruity/honey	1.87	90	24
Cinnamyl alcohol	Ethyl isobutyrate	fruity/apple-banana	1.92	87	24
Cinnamyl alcohol	Ethyl isovalerate	rose/apple	0.91	44	48

The ability of acting as acyl donor decreased by increasing the acyl chain length; geranyl formate was obtained in quantitative yield after 1-3 h, whereas, with geranyl propionate and butyrate, the maximum yields (ranging between 65-98%) were reached only after 1-2 days.

Enzymatic acylation was then studied with different primary alcohols and different acyl donors; all the reactions studied were aimed at preparing esters used as flavor or fragrance components (Table 4). Biotransformations were carried out starting from 100, 250, and 500 mM alcohol concentrations.

Table 4. Acylation of different primary alcohols (starting 100-500 mM concentrations) with short chain ethyl esters using MsAcT (1.0 mg/mL, 110 U/mL) in the presence of different ethyl esters (10% v/v) in phosphate buffer (100 mM, pH 8.0) at 25 °C. [a] As determined by gas chromatography. [b] Time refers to maximum conversion observed.



R_1	Initial alcohol (mM)	R_2	Ester main flavor property [1]	Conv. (%)[a]	Time (h)[b]
$(CH_3)_2CHCH_2$-	100	H-	black currant/currant	93	0.5
$(CH_3)_2CHCH_2$-	250	H-		93	0.5
$(CH_3)_2CHCH_2$-	500	H-		86	1
$(CH_3)_2CHCH_2$-	100	CH_3-	banana/pear	94	0.5
$(CH_3)_2CHCH_2$-	250	CH_3-		95	0.5
$(CH_3)_2CHCH_2$-	500	CH_3-		88	0.5
$(CH_3)_2CHCH_2$-	100	CH_2CH_3-	pineapple-apricot/apricot	97	0.5
$(CH_3)_2CHCH_2$-	250	CH_2CH_3-		97	1
$(CH_3)_2CHCH_2$-	500	CH_2CH_3-		95	1
$(CH_3)_2CHCH_2$-	100	$CH_2CH_2CH_3$-	fruity	94	24
$(CH_3)_2CHCH_2$-	250	$CH_2CH_2CH_3$-		93	24
$(CH_3)_2CHCH_2$-	500	$CH_2CH_2CH_3$-		92	24

$CH_3(CH_2)_4$-	100	H-	apple	97	0.5
$CH_3(CH_2)_4$-	250	H-		97	1
$CH_3(CH_2)_4$-	500	H-		88	3
$CH_3(CH_2)_4$-	100	CH_3-	fruity/pear	97	0.5
$CH_3(CH_2)_4$-	250	CH_3-		95	1
$CH_3(CH_2)_4$-	500	CH_3-		90	3
$CH_3(CH_2)_4$-	100	CH_2CH_3-	earthy/metallic-fruity	72	1
$CH_3(CH_2)_4$-	250	CH_2CH_3-		50	1
$CH_3(CH_2)_4$-	250	CH_2CH_3-		35	2
$CH_3(CH_2)_4$-	500	CH_2CH_3-		30	1
$CH_3(CH_2)_4$-	500	CH_2CH_3-		24	2
$CH_3(CH_2)_4$-	100	$CH_2CH_2CH_3$-	apricot/pineapple	94	2
$CH_3(CH_2)_4$-	250	$CH_2CH_2CH_3$-		96	6
$CH_3(CH_2)_4$-	500	$CH_2CH_2CH_3$-		95	24
Ph-	100	H-	floral-fruity/apricot	92	0.5
Ph-	250	H-		84	1
Ph-	500	H-		86	3
Ph-	100	CH_3-	jasmine	92	0.5
Ph-	250	CH_3-		90	1
Ph-	500	CH_3-		87	3
Ph-	100	CH_2CH_3-	floral-fruity/peach	96	0.5
Ph-	250	CH_2CH_3-		95	1
Ph-	500	CH_2CH_3-		89	3
Ph-	100	$CH_2CH_2CH_3$-	plum/pear	95	8
Ph-	250	$CH_2CH_2CH_3$-		92	8
Ph-	500	$CH_2CH_2CH_3$-		95	24
$PhCH_2$-	100	H-	hyacinth	95	0.5
$PhCH_2$-	250	H-		88	2
$PhCH_2$-	500	H-		86	3
$PhCH_2$-	100	CH_3-	rose/raspberry	96	0.5
$PhCH_2$-	250	CH_3-		91	0.5
$PhCH_2$-	500	CH_3-		82	0.5

$PhCH_2$-	100	CH_2CH_3-	red rose/strawberry	97	0.5
$PhCH_2$-	250	CH_2CH_3-		97	0.5
$PhCH_2$-	500	CH_2CH_3-		96	0.5
$PhCH_2$-	100	$CH_2CH_2CH_3$-	rose/honey	97	4
$PhCH_2$-	250	$CH_2CH_2CH_3$-		97	8
$PhCH_2$-	500	$CH_2CH_2CH_3$-		90	24
$PhCH=CHCH_2$-	100	H-	fruity/apple	>98	0.5
$PhCH=CHCH_2$-	250	H-		>98	4
$PhCH=CHCH_2$-	500	H-		82	24
$PhCH=CHCH_2$-	100	CH_3-	floral/pineapple	>98	1
$PhCH=CHCH_2$-	100	CH_3-		>98	4
$PhCH=CHCH_2$-	250	CH_3-		96	24
$PhCH=CHCH_2$-	100	CH_3CH_2-	fruity/woody	>98	1
$PhCH=CHCH_2$-	250	CH_3CH_2-		>98	5
$PhCH=CHCH_2$-	500	CH_3CH_2-		97	24
$PhCH=CHCH_2$-	100	$CH_3CH_2CH_2$-	fruity/honey	>98	16
$PhCH=CHCH_2$-	250	$CH_3CH_2CH_2$-		92	24
$PhCH=CHCH_2$-	500	$CH_3CH_2CH_2$-		80	48

Formylation and acetylation of all the tested primary alcohols allowed for quantitative or almost quantitative conversions with high rates (≥ 90% within 30-60 min) when the initial concentration of alcohols was kept between 100 and 250 mM. When the biotransformation was carried out with higher alcohol concentration (500 mM), still high molar conversions of the flavor-esters were observed (from 80 up to >98%), albeit with longer reaction times. Slower reactions were generally found with acyl donors of increased chain length (propionyl- and butyryl-). The only biotransformation occurring with relatively low yields (30-70% depending on the initial concentration) was the propionylation of *n*-hexanol to give *n*-hexyl propionate; interestingly, in this case hydrolysis of the formed ester was predominant after reaching its maximum accumulation, as noticeable from an increase of the alcohol concentration (data not shown).

In conclusion, a highly efficient preparation of different flavor-esters was achieved using the acyltransferase from *Mycobacterium smegmatis* (MsAcT), which catalyzes the acylation of different primary alcohols in aqueous systems. The results collected so far indicate that the biotransformation can be applied not only to the production of acetate esters, but can be expanded to a range of other acyl donors (e.g., ethyl formate, propionate, butyrate), useful for the formation of different flavor/fragrance esters, making this process strongly versatile. Notably, all the primary alcohols tested were accepted as substrates, even at higher concentrations (up to 500 mM); preparation of geranyl acetate and cinnamyl acetate was carried out on semipreparative scale (10 mL) starting from 250 mM alcohol concentration, allowing for the recovery of 44.2 mg/mL (geranyl acetate) and 41.0 mg/mL (cinnamyl acetate) of the products. Biocatalysis is sometimes perceived to be inefficient compared with conventional chemical processes because of the low productivity, mostly due to substrate/product inhibition of the enzymatic activity which often occurs at somewhat low concentrations. To become an industrially attractive technology. a biocatalytic process must be engineered to improve space time yields:[28] in this work we have described that the use of relatively low amount of MsAcT allowed the conversion of remarkably high substrate concentrations with good yields. The proposed enzymatic method may pave the way for an efficient and environmentally sustainable preparation of natural esters; the biotransformation may be further intensified by immobilizing the enzyme and carry out continuous processes in suited reactors,[29] thus making it appealing at industrial level.

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5.2. Continuous preparation of flavour-active acetate esters by direct biocatalytic esterification

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Continuous preparation of flavour-active acetate esters by direct biocatalytic esterification

Running title: Continuous preparation of flavour esters

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ABSTRACT

Mycelium-bound lipase from *Aspergillus oryzae* catalysed direct esterification of different alcohols and acetic acid in organic solvent, showing high stability towards substrates and products. Water produced during the esterification did not significantly affect the equilibrium of the reaction, allowing for high conversions. These features were exploited for preparing flavour-active acetate esters (isoamyl and cinnamyl acetate) in batch and continuous systems. A continuous stirred tank membrane reactor (CST-MR) was developed securing good reactor productivity and high biocatalyst stability.

KEYWORDS

Flavour esters, enzymatic acetylation, biocatalysis, *Aspergillus oryzae*, continuous reactor

INTRODUCTION

Many acetate esters of primary alcohols are components of natural flavours and are used as flavour and fragrance ingredients.[1] They can be obtained by extraction from natural sources or by enzymatic preparation starting from naturally occurring substrates (e.g., alcohols, carboxylic acids), thus ensuring the definition of "natural". Lipase-catalysed transesterification (or interesterification) in organic solvents or in solvent-free environments is one of the most common enzymatic method for the obtainment of acetate esters.[2.4] Alternatively, interesterification in water can be efficiently catalysed by specific acyltransferase (e.g., the one from *Mycobacterium smegmatis*, MsAcT), furnishing excellent yields of different flavour esters.[5-7]

Direct esterification with acetic acid is a preferred option since acetic acid is largely available as natural molecule, but the biotransformation is often hampered by formation of water, which affects the equilibrium of the reaction, thus limiting the final conversion in the desired esters.[8-10] A large-scale enzymatic production of low molecular weight flavour esters in organic

solvent was developed, where solutions for the elimination of substrate and product inhibitions are presented; water produced during the process was continuously removed by azeotropic distillation or by using molecular sieves, hence enabling high yields of the produced esters.[11] Another major drawback of direct acetylation is the deactivation of lipases in the presence of free acetic acid.[12] Immobilization of lipases has proven an effective method for stabilising these in the presence of acetic acid.[13-20] Novozym 435 (the commercial preparation of lipase B from *Candida antarctica*, CALB, immobilized on a macroporous acrylic resin) has been often used as stable and efficient biocatalyst for acetate esters preparation.[15,18]

Towards the development of a continuous methodology, different type of reactors can be set up for the production of esters by direct esterification catalysed by lipases in organic solvents.[21,22] The final process productivity depends on the activity and stability of the biocatalyst, whereas molar conversion can be modulated by applying the appropriate residence time in the bioreactor. Continuous stirred tank membrane reactor (CST-MR) have been scarcely used for the continuous enzymatic synthesis of esters, albeit they may offer several advantages (enhanced mass transfer, improved liquid mixing, and reduced clogging) over packed bed reactors.[23-26] Moreover, the use of a suited membrane allows for continuous removal of the organic liquid phase containing the product, while retaining the biocatalyst inside the reactor.[27]

Mycelium-bound fungal carboxylesterases have been often used for flavour ester production by direct esterification.[28] Dry mycelium of moulds (e.g. *Aspergillus oryzae*) can be effectively used for different biotransformations,[29,30] including direct esterification of different alcohols with remarkable advantages, such as: easy preparation of the biocatalyst, high stability in organic solvents, and high resistance to the inactivation caused by carboxylic acids (including acetic acid); moreover, this biocatalyst allows direct esterification with high molar conversions, enabled by favourable water partition.[31-33]

In this work, we have investigated the continuous preparation of flavour-active acetate esters (isoamyl and cinnamyl) using mycelium-bound lipase from *Aspergillus oryzae* in a continuous stirred tank membrane reactor (CST-MR).

MATERIALS AND METHODS

Chemicals and biocatalyst

All chemicals were purchased from Sigma-Aldrich and were used without any further purification. *Aspergillus oryzae* MIM[32] (Microbiologia Industriale Milano) was cultured in 500 mL Erlenmeyer flasks containing 125 mL of liquid medium (Tween 80 0.5%v/v, yeast extract 1 g/L, $(NH4)_2SO_4$ 5 g/L, K_2HPO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, pH 5.8) for 48 h at 28 °C on a reciprocal shaker (100 rpm). The mycelium suspension was recovered by vacuum filtration using a Buchner funnel and paper filter and washed with distilled water, and lyophilized.

Batch biotransformations

Different amounts of dry (lyophilized) mycelium of *A. oryzae* were suspended in 2.5 mL of *n*-heptane for 30 minutes and the reactions started by adding different concentrations of isoamyl alcohol and acetic acid; the reaction mixture was magnetically stirred at the desired temperature. Samples (0.25 ml) were taken at different intervals, added to an equal volume of an internal standard solution (*n*-octanol) in *n*-heptane and analysed by GC.

Continuous reaction procedure

The vessel used in this study was a glass-made membrane reactor constructed by the glass workshop of University of Milan. The working volume of the reactor was 200 mL (a picture and a cross section of the membrane reactor are given in Fig. 1). The membrane bioreactor was

composed with a thermostated water-jacketed glass vessel and an ultrafiltration module (0.1. mm) mounted on the bottle cap. The final suspension was maintained under agitation using a magnetic stirrer.

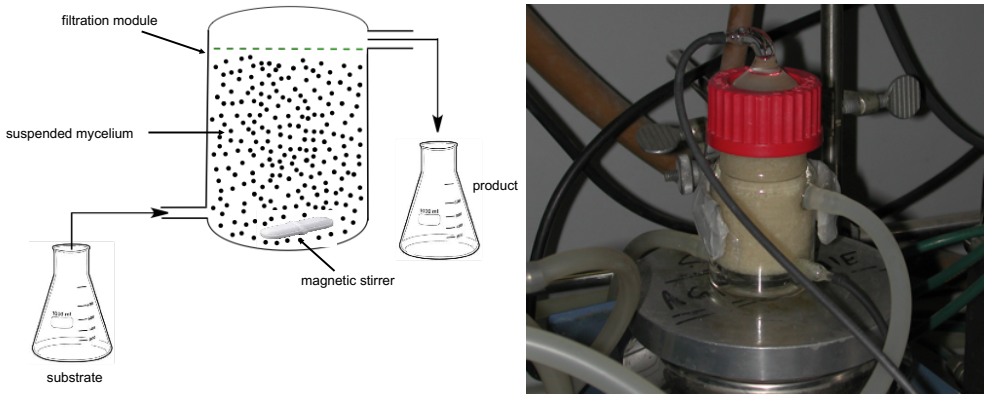

Figure 1. Schematic representation and picture of the membrane reactor

The temperature in the reactor was kept constant by circulating water in the jacket. A Gilson Miniplus 2 peristaltic pump controlled incoming liquid flow.
The molar balance for a continuous stirred tank reactor (CSTR) has the following expression, where F_A stands for the molar flow rate of substrate:

1) $$V = \frac{F_{A,in} - F_{A,out}}{(-r_a)}$$

One can rewrite as:

2) $$\tau = \frac{C_{Ao} - C_A}{(-r_A)} \quad \text{or} \quad \tau = \frac{C_{Ao}\, X_A}{(-r_A)}$$

Finally, rate of the CSTR was calculated using the algebraic equation 3:

3) $$r = \frac{C_{Ao}\, X_A}{\tau}$$

Analysis

Isoamyl alcohol and isoamyl acetate concentrations were determined by gas-chromatographic (GC) analysis on a Carlo Erba Fractovap GC equipped with a fused-silica capillary column MEGA- DEX DMP-Beta (dimethyl pentyl-

β-cyclodextrin, 25 m × 0.25 mm i.d.), with the injector temperature at 200 °C. Oven temperature was a 5 °C/min gradient from 40 to 180 °C. The retention times were: isoamyl alcohol, 6.7 min; isoamyl acetate, 7.1 min. Cinnamyl alcohol and cinnamyl acetate concentrations were determined with a fused-silica capillary column MEGA-SE30 (100% methyl polysiloxane; 25 m × 0.25 mm i.d.), with the injector temperature at 200 °C. Oven temperature was 80 °C (2 minutes) and then from 80 to 180 °C with a 5 °C/min gradient. The retention times were: cinnamyl alcohol, 9.9 min; cinnamyl acetate, 13.2 min.

Isoamyl acetate preparation: optimization of batch reaction

Direct esterification of isoamyl alcohol with acetic acid in *n*-heptane for the preparation of isoamyl acetate was firstly carried out in batch mode using dry mycelium of *Aspergillus oryzae*. Isoamyl acetate is used as a flavouring compound, having a characteristic banana flavour property.[1] Optimisation of the biotransformation was accomplished by sequential experimental trials using a Multisimplex® 2.0 software, previously employed for the optimization of biotransformations.[33] Response variables were the productivity of the biotransformation (defined as amount of product per amount of biocatalyst per unit of time) and the molar conversion of the alcohol, both determined after 24 h; the control variables were alcohol concentration, biocatalyst concentration, molar ratio (acid/alcohol), and temperature. The initial levels considered for the optimisation are listed in Table 1.

	Control variables			
	Alcohol (mM)	Dry mycelium (mg/mL)	Molar ratio	T (°C)
Reference value	25	20	1.5	40
Step size	5	5	0.2	5
Minimum	10	5	1.0	20
Maximum	200	40	3.0	70

Table 1. Control variables and initial levels considered for the optimisation of the esterification between isoamyl alcohol and acetic acid catalysed by mycelium-bound lipase of *Aspergilllus oryzae.*

The results of the sequential experiments aimed at the optimisation of the bioconversion are shown in Fig. 1. Each trial was performed in triplicate and the mean value was introduced as response for further optimisation.

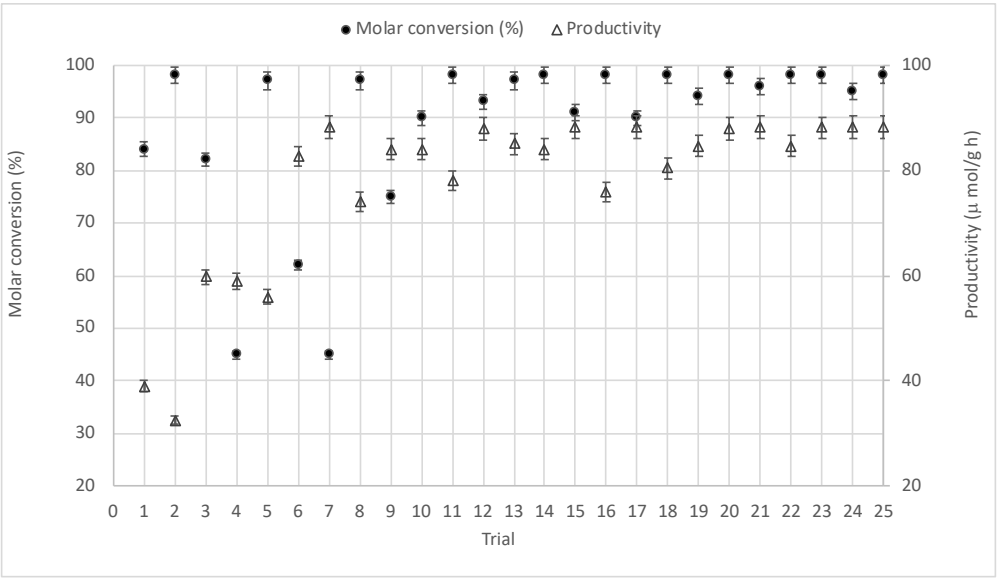

Figure 2. Sequential optimization of the esterification between isoamyl alcohol and acetic acid catalysed by mycelium-bound lipase of *Aspergilllus oryzae*. Experimental

responses (△ productivity; ● molar conversion) are reported as function of sequential trials. Values represent the mean of separate assays in triplicate.

The highest molar conversion (98%) and overall productivity (88.2 mmol g^{-1} h^{-1}) were simultaneously met in trials 20, 23, and 25; conditions corresponding to the trial 25 (isoamyl alcohol = 54 mM, dry mycelium = 25 mg mL^{-1}, molar ratio acid/alcohol 1.2/1 at 50 °C) were chosen for further experiments, since the lowest amount of biocatalyst still gave the highest amount of isoamyl acetate. Figure 3 reports the time-course of the direct esterification of isoamyl alcohol with acetic acid under the optimised conditions.

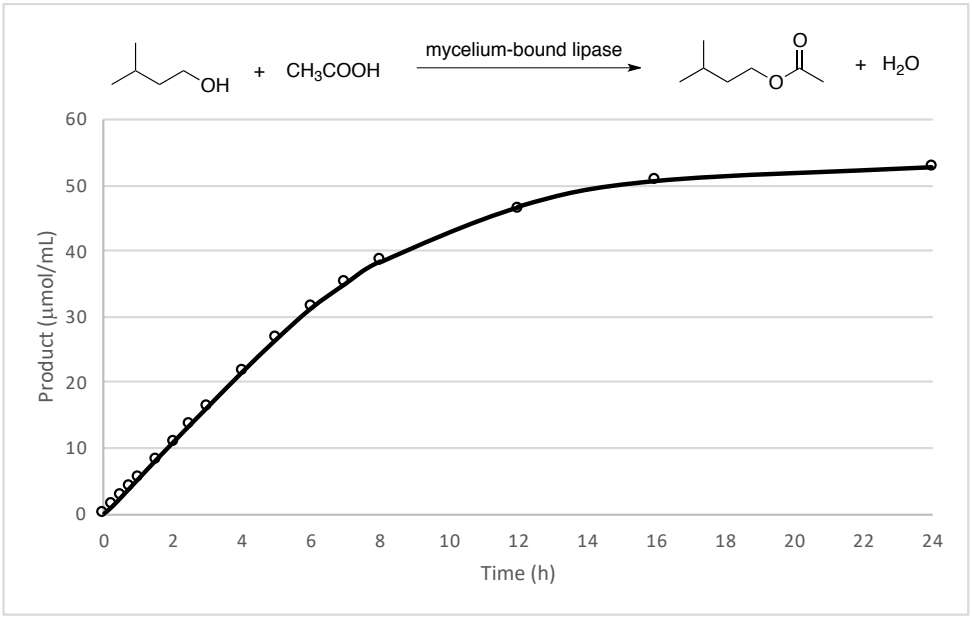

Figure 3. Esterification between isoamyl alcohol and acetic acid catalysed by mycelium-bound lipase from *A. oryzae*. Reaction conditions: initial alcohol concentration 54 mM; initial acetic acid concentration 65 mM in *n*-heptane containing 25 g L^{-1} of biocatalyst at 50°C.

The reaction occurred with high yield and completion of the reaction in 24 h with a reaction rate quite constant in the first 3 hours (0.089 mmol/mL min).

Isoamyl acetate preparation: optimisation of continuous reactor

The encouraging results obtained in batch reactions led us to explore the possibility to carry out continuous biotransformations in a continuous stirred tank membrane reactor (CST-MR), where mixing of the heterogeneous system should be optimal. The continuous stirred tank membrane reactor (CST-MR) described in the Material section (50 mL working volume) was employed for continuous operations; the biocatalyst was retained in the reactor by an ultrafiltration membrane, which allowed for permeation of the outflow solution.

The CST-MR was packed with a suspension of dry mycelium (1.25 g, corresponding to 25 g L^{-1}) in *n*-heptane and the solvent was flown in a recirculation mode for 72 hours at a flow rate (*Q*) of 0.2 mL min^{-1}, corresponding to a hydraulic residence time of 250 min (residence time: t = V/Q, where V is the reactor volume and Q is the flow rate). No filter plugging or release of enzymatic activity was observed during the time of the operation.

Direct esterification of isoamyl alcohol with acetic acid in CST-MR was then studied; substrates were continuously added in a *n*-heptane solution and the composition of the outflow solution monitored by GC. Experiments were initially focused to reach the best compromise between reaction rate and degree of conversion (Table 2).

Q (mL/min)	τ (min)	$C_{alcohol}$ (mM)[a]	$F_{alcohol}$ (μmol/min)	Conversion (%)	Rate (μmol/min)
0.10	500	50	5.0	83	0.083
0.10	500	55	5.5	82	0.090
0.10	500	60	6.0	78	0.094
0.14	350	50	7.0	56	0.090

Table 2. Optimisation of the continuous esterification of isoamyl alcohol with acetic acid catalysed by mycelium-bound lipase from *A. oryzae* carried out in CST-MR. Initial stoichiometric ratio between acid/alcohol was always 1.2. [a]Initial alcohol concentration.

Experiments carried out with a t = 500 min allowed for 78-83% molar conversion, depending on the $F_{alcohol}$ in the inlet flow; the best compromise between conversion and rate was found with a $F_{alcohol}$ of 5.5 mmol/min (corresponding to a $C_{alcohol}$ = 55 mM and $C_{acetic\ acid}$ = 66 mM) corresponding to a rate of 0.092 mmol/min. The rate was slightly better than the one observed in batch reactor, showing the good performance in terms of mixing of the CST-MR. Experiments were also carried out at lower residence time (t = 350 min), leading to a significant decrease in the conversion.

Isoamyl acetate preparation: stability of the biocatalyst in the continuous reactor

The continuous operation aimed at preparing isoamyl acetate was carried out for 10 days under optimised conditions (inflow solution containing 55 mM isoamyl alcohol and 65 mM acetic acid, t = 500 min at 50°C); no decrease of the molar conversion was observed at the end of the process, thus indicating a notable operational stability of the catalyst. Table 3 summarises the results of the continuous biotransformation, which allowed for a remarkable overall reactor productivity (defined here as the amount of isoamyl acetate produced for volume of reactor) of 169 mg mL^{-1}.

Q (mL min^{-1})	τ (min)	Conv.[a] (%)	Space time yield (mmol d^{-1})	Catalyst productivity[b] (mmol g^{-1})	Reactor productivity[b] (mg mL^{-1})
0.10	500	82	6.49	51.95	169

Table 3. Continuous esterification of isoamyl alcohol with acetic acid catalysed by mycelium-bound lipase from *A. oryzae* carried out in CST-MR for 10 days. [a] Conversion detected in the exiting flow after 10 days of operation. [b] Productivity is referred to the amount of ester formed after an operation time of 10 days

A closed-loop reactor was also set up by recirculating the outflowing solution; after 16 h of operation, the mycelium-bound lipase was able to catalyse a conversion of 98% of isoamyl alcohol into the corresponding acetate ester.

Cinnamyl acetate preparation: batch and continuous reactor

Biocatalytic preparation of cinnamyl acetate was also studied; cinnamyl acetate is characterised by a typical pineapple flavor.[1] Direct acetylation of cinnamyl alcohol was accomplished applying the optimised conditions employed for the direct esterification of isoamyl alcohol. Figure 4 shows the time-course of the batch biotransformation.

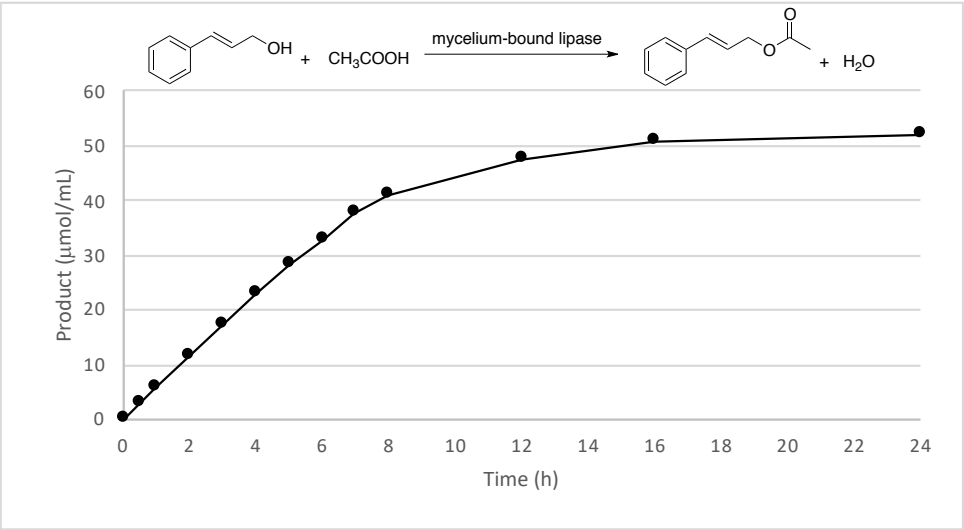

Figure 4. Esterification between cinnamyl alcohol and acetic acid catalysed by mycelium-bound lipase from *A. oryzae*. Reaction conditions: initial alcohol concentration 54 mM; initial acetic acid concentration 65 mM in *n*-heptane containing 25 g L^{-1} of biocatalyst at 50°C.

The acetylation rate of cinnamyl alcohol was slightly higher (steady reaction rate in the first 3 hours of 0.095 mmol/mL min) than what observed with isoamyl alcohol, whereas final conversion after 24 h was 95-96%. Since acetylation of cinnamyl alcohol occurred with a time-course similar to what observed for isoamyl alcohol, continuous operation was carried out under the same conditions ($C_{alcohol}$ = 55 mM, $F_{alcohol}$ =5.5 mmol/min, C_{acid} = 66 mM, Q = 0.1 ml min^{-1}, t = 500 min at 50°C) and in the same CST-MR. Also, in this case, the biocatalyst was stable over 10 days of reaction; Table 4 summarises the data of the continuous bioprocess.

Q (mL min^{-1})	τ (min)	Conv.[a] (%)	Space time yield (mmol d^{-1})	Catalyst productivity[b] (mmol g^{-1})	Reactor productivity[b] (mg mL^{-1})
0.10	500	80	6.34	50.69	223

Table 4. Continuous esterification of cinnamyl alcohol with acetic acid catalysed by mycelium-bound lipase from *A. oryzae* carried out in CST-MR for 10 days. [a] Conversion detected in the exiting flow after 10 days of operation. [b] Productivity is referred to the amount of ester formed after an operation time of 10 days

Conclusion

The mycelium-bound lipase activity of *Aspergillus oryzae* was exploited for the direct esterification of isoamyl and cinnamyl alcohol with acetic acid for the preparation of the corresponding flavour-active esters. Remarkably, similar acetylation rates and molar conversions were observed, although two alcohols with quite different structure were employed. It is worthy to underline that the biotransformation occurred in *n*-heptane without any particular system for the removal of the water produced; it has previously

suggested that the mycelia supply a hydrophobic micro-environment, thus disfavouring water access to the enzymes which catalyses the reaction.[34] A stirred tank membrane reactor (CST-MR) was set up for carrying out continuous biotransformations; reusability of the biocatalyst reduce the cost of fermentation, necessary for its production. Indeed, the CST-MR guaranteed 10 days of operation without any significant loss of the biocatalyst activity; reactor productivity (defined as the amount of ester formed per volume of reactor) was 169 mg/mL for isoamyl acetate and 223 mg/mL for cinnamyl acetate. In conclusion, a highly effective continuous production of isoamyl and cinnamyl acetate was obtained with dry mycelium, without any necessity of costly or laborious enzymatic purification, and without the need of water sorption during the prolonged bioprocess.

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6. GENERAL CONCLUSION

As we have seen, both production systems of these flavour-active molecules are effective. The first alternative, with the enzyme MsAcT, was interesting as it is able to work in aqueous medium, reducing the need to use solvents once in the reaction medium there is only water, the substrates (ester and alcohol). And the enzyme, and further noted that the use of a relatively low amount of MsAcT allowed the conversion of remarkably high substrate concentrations in good yields and in relatively short times. Another feature observed was the versatility as it relates to different substrates, especially those with the shorter chain, which are favoured in the reaction thus obtaining higher rates in a shorter time. The use of the enzyme MsAcT proved to be efficient for the sustainable preparation of natural esters.

The use of the enzyme-bound to the lyophilized *Arpergillus oryzae* mycelium proved to be more accessible, less expensive, since the enzyme does not require purification, and for this reason the enzyme has excellent stability and activity, up to 10 days, without significant loss of stability, in which it was possible to verify the possibility of reuse of the biocatalyst, for this reason it was possible to work in a continuous system. It was observed its direct esterification activity in two alcohols with their very different structure with acetic acid. The enzyme has been shown to work in heptane, and other green solvents (data not shown). The lyophilised mycelium of *Arpergillus oryzae* resulted in a highly effective continuous production of active flavoured acetate esters without the need for any expensive enzymatic purification.

Both production systems are promising, represent two different alternatives and can be further optimized and scaled up for the interests of the industry.

7. IMPLICATIONS AND FUTURE DIRECTIONS

The production of these natural esters through these bioprocesses is viable as consumers increasingly want natural products, and the aromas are present in our daily lives in many ways, be it the food we eat, drinks, perfumes, cosmetics, in even cleaning products, noting that this market is estimated at over US$ 26 billion. But we must also remember that the natural sources of these compounds are limited, since they run in nature at very low concentrations, which makes the natural aromas cost in themselves absurd numbers when compared to those synthesized, because chemically they are the same molecule and there no have difference.

Since the legislation allows and encourages the use of biotechnology for the development of enzymatic molecules, it is highly attractive and since our results have been very efficient, both in terms of production, stability and costs.

Substrates of natural origin do not prove to be a cost problem for the production of esters, as they are abundant and can be derived from agro-industry tailings and the products have a high added value. We can continue testing various other substrates, increasing the possibilities and products produced by these bioprocesses.

Both alternatives for producing natural aroma esters, via the enzyme MsAcT or with *Aspergillus oryzae* mycelium, can be optimized by applying a flow reaction, where this process can be automated and further recovered of the product in line, and later adapted to industrial levels, to finally meet this repressed demand for these products.