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Non-conventional yeast-promoted biotransformation for the production of flavor compounds

Luca Forti[1]*, Maria Rita Cramarossa[1], Sara Filippucci[2], Giorgia Tasselli[2], Benedetta Turchetti[2], Pietro Buzzini[2]*

[1]Department of Life Sciences, University of Modena & Reggio Emilia, via G. Campi 103, Modena 41125, Italy; E-Mail: luca.forti@unimore.it
[2]Department of Agricultural, Environmental and Food Sciences, Industrial Yeasts Collection DBVPG, University of Perugia, Borgo XX Giugno 74, Perugia 06121, Italy; E-Mail: pietro.buzzini@unipg.it

***** Authors to whom correspondence should be addressed; E-Mails: luca.forti@unimore.it (L.F.); pietro.buzzini@unipg.it (P.B.); Tel.: +39-059-2058591 (L.F.); Tel.: +39-075-5856455 (P.B.).

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Abstract

The rising consumer demand for "natural" foodstuffs have encouraged a growing part of both Academic and Industrial scientific community to develop novel biocatalysts for producing flavouring molecules. In this context, non-conventional yeasts (NCYs) have attracted increasing interest due to their biochemical characteristics and potential applications, being able to produce aroma compounds from a variety of carbon sources, including sugars, alkanes, plant oils, starch hydrolysates, ethanol, and glycerol. Apart from classical fermentation processes (*de novo* synthesis), bioconversion of appropriate precursor compounds are also being developed to produce food aromas.

An overview on the potential of NCYs whole cell for producing food flavors by biotransformation is illustrated in this chapter by a discussion of the production of different class of compounds, namely Alcohols, Aldehydes, Ketones Lactones, Terpenes and Terpenoids, Alkenes, Phenols, and Sulphur compounds.

1. Introduction

Flavors play an essential role in the quality perception of food and beverages: whereas the non-volatile compounds contribute mainly to the taste, the volatile ones influence both taste and aroma.

Numerous compounds may be responsible for the aroma of the food products, such as ketones, aldehydes, alcohols, fatty acids, esters, lactones, terpenes, aromatic and sulfur compounds (Table 1).

<Insert table 1 here>

Nowadays, most of the flavoring compounds are produced *via* chemical synthesis or by extraction from natural materials, which still represents the cheaper technology for their production. An alternative route for flavors synthesis is based on *de novo* microbial process (fermentation) or on biotransformation of suitable precursors using microbial cells or enzymes (biocatalysis) (Longo et al. 2006, Janssens et al. 1992, Krings and Berger 1998, Vandamme and Soetaert 2002, Aguedo et al. 2004). Thus, the rising consumer demand for "natural" foodstuffs have boosted a growing part of both Academic and Industrial scientific community to develop novel biocatalysts for producing these flavouring molecules (Krings and Berger 1998, Lomascolo et al. 1999, Brenna et al. 2011). Indeed, according to EU regulations, natural flavours are defined as substances corresponding to substances that are naturally present and have been 'identified in nature': as stated by the European Food Safety Autority (EFSA), a flavor is 'identified in nature' when "it has been identified in materials of plant, animal, microbiological, or mineral origin, and/or it has been identified in food in the raw state or processed or partly processed fo human consumption" (Demyttenaere 2012). Thus, the products obtained through microbial or enzymatic processes and that the precursor and product can be found in nature or are part of traditional foods can be considered as 'natural'.

Although the biocatalytic ability of yeasts is a well-known phenomenon, and some processes using yeast whole cells for selected biotransformation at the industrial level have been reviewed (Johnson and Echavarri-Erasun 2011), the studies aimed at evaluating the potential of the so-called non-conventional yeasts as biocatalysts have never been substantially reviewed so far.

In this chapter, we will review the use of non-conventional yeasts for producing food-grade flavor compounds via biocatalysis.

1.1 Non-conventional yeasts (NCYs) as biocatalysts

Yeasts are unanimously considered as key players of a variety of traditional (i.e. fermented foods and beverages) and innovative (i.e. high-value bulk and fine chemicals) processes. The impact of yeast biotechnology on human activities has been extensively documented (Domínguez et al. 1998, Walker 1998, Flores et al. 2000, Johnson and Echavarri-Erasun 2011). Many of these studies are

closely related with the yeast *Saccharomyces cerevisiae* (otherwise labeled as baker's yeast), which is traditionally used for producing bread, beer, wine, a number of ethnic fermented foods and beverages in Asia, Africa and South America, and some important industrial commodities (e.g. ethanol as additive for biofuels) (Walker 1998).

The wide dissemination of the results from those studies and industrial applications has determined that to most people, including a not negligible number of (micro)biologists, this species was erroneously taken as an example (and model) for studying the whole diversity of yeast world. On the contrary, in recent decades the taxonomic, physiologic and metabolic diversity of the so-called "non-conventional yeasts" (NCYs) have been deeply investigated (Wolf et al. 2003, Sibirny and Scheffers 2002, Buzzini and Vaughan-Martini 2006.). There is no generally accepted definition on NCYs. Even though a number of scientists includes a few additional species (namely, at least *Schizosaccharomyces pombe* and *Kluyveromyces lactis*) into the group of "conventional yeasts" (CYs), many other consider NCYs as synonymous of "non-*Saccharomyces*" yeasts. As early reported by Sibirny and Scheffers (2002) "there is no generally accepted definition on NCYs". A number of scientists consider NCYs simply as synonymous of "non-*Saccharomyces*" yeasts, while many others include the two species *Schizosaccharomyces pombe* and *Kluyveromyces lactis* into the group of "conventional yeasts" (CYs). Because of the above definitions result sometimes incomplete and indeterminate, the same authors (Sibirny and Scheffers 2002) also concluded that, since an increasing number of NCYs is gaining importance in fundamental and applied microbiological sciences, the term NCYs is gradually losing both significance and usefulness. Beyond any definition, because of the current yeast taxonomy accounts for more than 130 ascomycetous and basidiomycetous genera and over 1,600 species, NCYs represent the vast majority of yeast diversity so far described (Kurtzman et al. 2011). Undoubtedly, this huge yeast diversity includes many species possessing useful, and sometimes uncommon, metabolic aptitudes that could be potentially exploited in both food and no-food industry (Johnson et al. 2011, Sibirny and Scheffers 2002, Spencer et al. 2002).

Demands for enhancing both productivity and efficiency of biotechnological processes from wider substrate range, production of novel molecules sometimes due to change of consumer preferences can lead to a great interest in further enhancing the number of NCYs exploited by industry (Steensels et al. 2014).

1.2. Definition of biocatalysis and their impact on the production of flavors

Biocatalysis may be defined as “a process that describes a reaction, or a set of simultaneous reactions, in which a pre-formed precursor molecule is converted using enzymes and/or whole cells, or combinations thereof, either free or immobilized” (Straathof et al. 2002), and sums up both biotrasformations and enzyme catalysis.

The high specificity and selectivity expressed by biocatalysts, and the possibility to use “environmental-friendly” conditions (e.g. solvent-free approaches, low working temperatures and pressures, and waste reduction) together with the possible reduction of the number of steps in a synthetic route actually justify the increasing use of biocatalysts in modern chemical processes. (Matsuda et al. 2009, Bastos Borges et al. 2009, Patel 2011, Faber 2004).

Isolated whole cells are often preferable than purified enzymes, because they are more convenient and stable, with no need for costly purification and coenzyme addition. In fact, although enzymes are considered as powerful tools, particularly for single step biotransformation, the number of free or immobilized enzymes commercially available on the market is still quite limited, particularly for some types of uncommon substrates. Moreover, when the enzymes are kept within their natural environment (i.e. cell cytoplasm) lesser inactivation usually occurs (Liese et al. 2006).

In recent years, biocatalysis has increasingly been investigated as promising manufacturing technique for food-grade flavors production. This is essentially justified by two reasons: firstly, biocatalysis sometimes allow the production of regio- and stereoselective compounds under mild conditions; secondly, the use of cell biocatalysts is unanimously considered a lesser pollutant technology. Accordingly, molecules obtained by such bioprocesses can be labeled as natural, and all volatile organic compounds (VOCs) obtained from living cells, including microorganisms, are labeled as GRAS (Generally Recognized As Safe) (Lomascolo et al. 1999). As a result, the biocatalytic potential of GRAS microorganisms (in particular, the use of whole cells for obtaining specific molecules) has attracted considerable interest.

2. Biocatalytic production of flavours by NCYs

2.1. Aldehydes, ketones and alcohols

Volatile C6- and C9-aldehydes and –alcohols are compounds that are responsible for the green notes characteristic flavors of fruits, vegetables and green leaves (Buchhaupt et al. 2012), and comprise hexanal, (3Z)- and (2E)-hexenal, (3Z)- and (2E)-nonenal, (3Z,6Z)- and (2E,6Z)-nonadienal, and their corresponding alcohols, such as hexanol, (2E)- and (3Z)-hexenol (Buchhaupt et al. 2012). Leaf aldehyde, (2E)-hexenal, and leaf alcohol, (3Z)-hexenol, are considered to be the most valuable ones exhibiting various flavor applications and higher stability (Gigot et al. 2012).

Early studies on the biotransformation of C precursors to aldehydes dated back to 1980s. Armstrong et al. (1984) found that an ethanol-adapted *Candida utilis* (anamorphic state of *Lindnera jadinii*) efficiently converted ethanol to acetaldehyde. A study reported the conversion of (Z)-3-hexenal into the corresponding (Z)-3-hexenol (Fig.1) by *Wickerhamomyces anomalus* (former *Pichia anomala*). Conversion yields higher than 90% were also obtained by directly conducting the reaction in the medium where (Z)-3-hexenal is produced by the action of lipoxygenase and hydroperoxide lyase on linolenic acid (Fauconnier et al., 1999).

<Insert figure 1 here>

More recently, a few ketoreductases isolated from NCYs exhibiting broad substrate range and high selectivity on ketone reduction chemistry have been studied (Faber 2004, Moore et al. 2007). They exhibited broad substrate range and high selectivity on ketone reduction chemistry, thus making biocatalysis the general method of choice for ketone reductions (Moore et al., 2007). Nicaud et al. (2002) studied the introduction of multiple fatty acid hydroperoxide (HPO) lyase genes into *Yarrowia lipolytica* genome for increasing its aptitude towards fed-batch cultivation and inducing highest production of HPO lyase activity and, consequently, the production of high quantities of C6-aldehydes via biocatalysis (Nicaud et al. 2002, Bourel et al. 2004).

Furaneol® (2,5-dimethyl-4-hydroxy-3(2H)-furanone - DMHF) is an volatile ketone, exhibiting strawberry flavour in dilute solutions and caramel-like flavour in concentrates. Furanones occur in many fruits (e.g. pineapple, strawberries, mangoes, raspberries), but also in certain microbial cultures and in soy sauce. It is also formed by the chemical reaction of sugars with amines during the Maillard reaction. A study reported that *Zygosaccharomyces rouxii* is able to form DMHF via D-fructose-1,6-diphosphate metabolism (Dahlen et al. 2001).

The flavoring compounds 2-phenylethanol (and its derivative 2-phenylethylacetate) are widely applied in food industries (Hua and Xu 2011), and can be produced from L-phenylalanine via batch and fed-batch biocatalysis by whole cells of *Kluyveromyces marxianus* (Etschmann et al. 2005, Etschmann and Schrader 2006, Gao and Daugulis 2009). The use of an organic phase to make easy downstream processing steps made fed-batch approach an attractive and cheap alternative to current industrial processes (Etschmann et al. 2005, Etschmann and Schrader 2006). More recently, Gao and Daugulis (2009) used a solid-liquid two-phase partition bioreactor system to reduce the aqueous 2-phenylethanol concentration to non-inhibitory levels and to allow an *in situ* product removal technique. A final 2-phenylethanol concentration of 20.4 g/L was achieved (with 1.4 g/L in the aqueous and 97 g/L in the polymer phase) (Gao and Daugulis 2009). An alternative process for

producing 2-phenylethanol from L-phenylalanine via biocatalysis was the Ehrlich pathway (Hua and Xu 2011).

In the last fifteen years, a number of studies described the optimal conditions for producing 2-phenylethanol via biotransformation processes: yields from 0.5 to 5.6 g/L were reported (Fabre et al. 1998, Huang et al. 2000, Huang et al. 2001, Etschmann et al. 2004, Garavaglia et al. 2007). The production of other food-grade alcohols via biocatalysis was recently studied. Andreu and Del Olmo (2014) described the use of whole cells of *K. marxianus* and *L. jadinii* (former *Pichia jardinii*) as biocatalysts for producing the chiral products derived from the aldol reaction between acetone and some aromatic aldehydes, as well as the chiral 1,3-diols deriving from their reduction. The resolution of the racemic starting material and the recovery of the aldol with the *S* configuration was sometime possible using *K. marxianus* whole cells. Both species showed complementary enantioselectivity.

2.2. Lactones

Lactones are cyclic esters of primary γ- and δ-hydroxyacids: they are currently found in foods and beverage, and contribute to change their taste and flavor. Accordingly, the industry uses some of them, in particular γ-lactones as flavoring ingredients in many fruity aromatic foods (Romero-Guido et al. 2011). Although many synthetic γ-lactones are at present still utilized as flavoring additives, the consumer request is nowadays orienting to the use of natural flavors. this tendency increased the interest (and consequently the studies) for the production of γ-lactones via biocatalysis. Hence, γ-decalactone and γ-dodecalactone have been produced using free fatty acids, hydroxyl fatty acids, or oils as precursors through several enzymatic steps that catalyze their γ-oxidation (Krings and Berger 1998, An et al. 2013). Some high yield biotransformation used efficient NCYs for producing γ-lactones were developed (Kondo et al. 2000, Lee et al. 2004, de Carvalho 2011).

γ-Dodecalactone (responsible for a peach-like aroma) is a food-grade compound presently used as an aroma or taste ingredient of foods, chewing gums, toothpastes, cosmetics, pharmaceutics, cigarettes, soaps, and perfumes (Farbood et al. 1998, Karagül-Yüceer et al. 2001, Pino and Marbot 2001, Herianus et al. 2003, Elss et al. 2005, Nunes et al. 2008). An et al. (2013) developed a process for biotransforming oleic acid into γ-dodecalactone catalyzed by permeabilized cells of *Waltomyces lipofer* (now *Lipomyces lipofer*) with a conversion yield of 76%. The of ricinoleic acid to γ-decalactone was also studied. The species *Sporobolomyces odorus* (now *Sporobolomyces salmonicolor*) was found to be able to convert castor oil (or its derivatives) to γ-decalactone (Dufossé et al. 1998).

The species *Y. lipolytica* was broadly studied for its capability to produce lactones via biocatalysis. Groguenin et al. (2004) reported that this ascomycetous yeast synthesizes five acyl-CoA oxidases (Aox1p to 5), that catalyze the first of β-oxidation step. the same Authors ey constructed an overproducing that gave 10 times more γ-decalactone than the wild type. In contrast, Escamilla-Garcia et al. (2007) optimized the production of γ-decalactone by *Y. lipolytica* using an experimental design (variables: pH and aeration conditions): 496 mg/L of γ-decalactone were obtained at pH around 5 and increased at low aeration. On the contrary, 660 mg/L of 3-hydroxy-γ-decalactone were accumulated at low pH and high aeration, thus postulating that hydroxylation of γ-decalactone was essentially oxygen-dependent (Escamilla-Garcia et al., 2009). More recently, Braga and Belo (2015) studied the production of γ-decalactone from ricinoleic acid by whole cells of *Y. lipolytica* in batch and fed-batch: a high γ-decalactone productivity (215 mg/L·h) was found (Fig.2). These interesting results suggest that these two strategies could be good alternatives for industrial production processes.

<Insert figure 2 here>

Likewise, Kang et al., (2016) produced δ-decalactone from linoleic acid by one-pot reaction of enzymatic hydroxylation and whole cell biotransformation: using the unsaturated fatty linoleic acid supplementation with whole cells of *Y. lipolytica*, 1.9 g/L δ-decalactone were obtained (Fig. 3). This study represents the first production of δ -decalactone using unsaturated fatty acid as precursor, with the highest yield reported so far.

<Insert figure 3 here>

Other studies carried out in the last fifteen years on the use of NCYs for producing lactones via biotransformation of fatty acids are reported in Table 2.

<Insert table 2 here>

2.3. Terpenes and terpenoids

Terpenes are widespread in nature, mainly as constituents of essential oils, and are also obtained in large scale as industrial residues. They are composed of isoprene units, and can be cyclic, open-chained, saturated, unsaturated, and oxidized. Monoterpenes represent a valuable resource for the

flavor industry, and include over 400 different naturally occurring structures constituents of essential oils. Because of low odor activity, high hydrophobicity and their high tendency to auto-oxidize and polymerize, some monoterpenes are usually separated from oils by rectification. Thus, a few abundant monoterpenes, namely α-pinene and limonene, may be considered industrial wastes and inexpensive starting materials for chemical and biochemical transformations. In this framework, the rising consumer demand for natural flavors have determined the development of novel prokaryotic and eukaryotic biocatalysts to biotransform monoterpenes into their more valuable food-grade oxygenated derivatives (van Rensburg 1997, de Carvalho 2006).

α-Pinene is a low-price bicycle monoterpene largely employed in flavor industries as raw material for the synthesis of high-value products (Gomes 2001, da Silva 2003), and is commonly used as substrates for biotransformation. Different NCYs, isolated from orange juice industry residues, soils of citric fruits, and leaves of citric fruits, were able to convert the substrate (-)-α-pinene to verbenol (Fig. 4) (Rottava 2010).

<Insert figure 4 here>

The biotransformations of α- and β-pinene by seven different microorganisms, including whole cells of *Candida albicans* have been studied (Javidnia 2009). The results showed that this species was unable to biotransform α-pinene, while traces of sabinol, myrtenol and myrtenal were observed. (+)-Limonene is one of the most studied precursor for the production of food-grade flavors *via* biotrasformation. Different NCYs were screened to biotransform this molecule to flavoring compounds (van Rensburg 1997): *Blastobotrys adeninivorans* (former *Arxula adeninivorans*) and *Y. lipolytica* exhibited a superior ability to convert (+)-limonene to perillic acid by hydroxylation on the *exo*-cyclic methyl group (Fig. 5). More recently, Ferrara et al. (2013) reported a study on the bioconversion of R-(+)-limonene to perillic acid by whole cells of *Y. lipolytica*. Interestingly, the stepwise addition of limonene increased the perillic acid concentration by over 50%.

<Insert figure 5 here>

The biotransformation of (-)-(*R*)-α-phellandrene with 16 different microorganisms, including some NCYs, has been recently reported (Iscan 2012): the major product (yields 16%) obtained by *Y. lipolytica*-promoted biotransformation was identified as 5-*p*-menthene-1,2-diol (Fig. 6).

<Insert figure 6 here>

The microbial and enzymatic biotransformation of some monoterpenoids (i.e. carvone, myrtenal and geraniol) into highly valuable flavoring derivatives is becoming of increasing interest because of their economic potential for the food, and beverage industry (Brenna et al. 2011, Lemos et al. 2009). Carvone is a monoterpenoid isolated from the essential oil of spearmint, and can be synthesized from d-limonene. It can occur in the dextro, levo, and racemic form. d-Carvone exhibits odor reminiscent of caraway, and is used as food-grade flavoring ingredient, exhibiting odor of spearmint. It is usually prepared by fractional distillation of oil caraway or also from dillseed and dillweed oils, but this type differs in odor and flavor. The reduced derivatives of carvones, such as cis-dihydrocarvone and dihydrocarveol also found application as flavoring ingredients. cis-Dihydrocarvone has a warm, powerful, herb-like odor and a spearmint-like flavor, and may be synthesized by isomerization of limonene oxide, by oxidation of dihydrocarveol or reduction of carvone. Dihydrocarveol has a floral, woody odor and a sweet, somewhat spicy flavor (peppery).

Different NCYs were found to be able to catalyze the reduction of C=C and C=O double bonds in carvones competitively, affording a mixture of saturated ketones, saturated alcohol and, more rarely, the allylic alcohol. Since 1990s, a number of ascomycetous and basidiomycetous NCYs, belonging to species of the genera *Dekkera, Eremothecium, Geotrichum, Hanseniaspora, Kloeckera, Kluyveromyces, Lipomyces, Metschnikowia, Pichia, Rhodotorula, Schwanniomyces, Sporodiobolus, Torulaspora, Trichosporon,* and *Yarrowia* were screened for the their ability to catalyze the biotransformation of monoterpenoid ketones. Although with different enzyme activity, almost all NCYs tested gave reduction of carvone (Fig. 7 and 8). (4*R*)-carvone was often reduced much faster than (4*S*)-carvone, and yields of up to 90% were obtained within a few hours. Some NCYs also exhibited the ability to reduce the C=C bond to yield dihydrocarvone isomers with the stereochemistry at C-1 always *R*, while with other yeasts the C-O double bond was also reduced to give the dihydrocarveols with stereochemistry at C-2 always *S* for (4*R*)-carvone, but sometimes *S* and sometimes *R* for (4*S*)-carvone (van Dyk 1998).

The reduction of (4*R*)- or (4*S*)-carvones catalyzed by *Schizosaccharomyces octosporus* whole cells showed that the stereochemistry of the ketones determined the reduction pathway reported in Fig. 7 (Carballeira 2004 et al.), where (4*S*)-carvone yields (2*R*,4*S*)-carveol (50% yield) and the (4*R*)-carvone carries to (1*R*,2*S*,4*R*)-dihydrocarveol (80% yield) as the major product. In another study, (Goretti et al. 2009) a set of environmental NCYs lyophilized whole cells were screened for their aptitude to reduce (4*S*)-carvone. Although the species *Lindnera amylophila* and *Kazachstania naganishii* (formerly *Pichia amylophila* and *Saccharomyces naganishii*, respectively) exhibited a bioconversion of this compound close to 100%, a quite different biocatalytic route was found (Fig. 7). In the first case, a mixture of (1*R*,2*S*,4*S*)- and (1*S*,2*S*,4*S*)-dihydrocarveol are produced as the

major compounds (25.3 and 63% respectively), by the consecutive reduction of C-C and C-O double bonds respectively; on the contrary, the cells of *L. naganishii* catalyzed almost exclusively the reduction of the carbon-carbon double bond of (4*S*)-carvone affording (1*S*,4*S*)- and (1*R*,4*S*)-dihydrocarvones (44.4 and 39.7% respectively) as major products.

<Insert figure 7 here>

The prevalent catalytic activity was the ene-reductase(ER)-catalyzed reduction of the substrate into a mixture of (1*R*,4*R*)- and (1*S*,4*R*)-dihydrocarvone, with a clear-cut preference towards the production of (1*R*,4*R*)-diastereomer. Variable amounts of dihydrocarveols, derived from the subsequent carbonyl reductase-catalyzed reduction of the carbonyl group were sometimes produced. *Hanseniaspora guilliermondii* exhibited a good bioconversion yield (about 63%), coupled with an excellent selectivity (diastereomeric excess, d.e. = 98%), compatible with its application as potential source of dihydrocarvone (Goretti et al. 2009). Response surface methodology was also recently applied for simultaneously maximize the bioreduction yield of (4*S*)-(+)-carvone (>95%) by whole cells of *Cryptococcus gastricus* and to minimize the rate of side reactions below 1% (Goretti et al. 2012). Bioreduction of (4*R*)-carvone promoted by NCYs whole cells of the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Kazachstania*, *Kluyveromyces*, *Lindnera*, *Nakaseomyces*, *Vanderwaltozyma* and *Wickerhamomyces* has also been reported [Goretti et al. 2013]. The ene-reductase (ER)-catalyzed reduction of the substrate into a mixture of (1R,4R)- and (1S,4R)-dihydrocarvone was showed to be the prevalent catalytic activity, with a clear-cut preference towards the production of (1*R*,4*R*)-diastereomer. The dihydrocarveols, derived from the subsequent carbonyl reductase-catalyzed reduction of the carbonyl group, were found only in traces. *Hanseniaspora guilliermondii* exhibited a good bioconversion yield (about 63%), coupled with an excellent selectivity (diastereomeric excess, d.e. = 98%), compatible with its application as potential source of dihydrocarvone.

Goretti et al. (2011) recently reported. the biotransformation of some terpene aldehydes [(S)-perillaldehyde and the aromatic terpene aldehyde α-methyl-cinnamaldehyde] by a set of NCYs. NCYs reduce the monocyclic (*S*)-perillaldehyde with moderate yields: whole cells of *K. naganishii* gave bioconversion yields higher than 60% (Fig. 8). (*S*)-Perillaldehyde was initially reduced to dihydro-perillaldehyde and successively to dihydroperillic alcohol. Furthermore, it is noteworthy that the same species catalyzed the direct reduction of the carbonyl group of (*S*)-perillaldehyde, which led to the formation of perillic alcohol.

<Insert figure 8 here>

On the contrary, NCYs poorly reduced α-methyl-cinnamaldehyde: only *Kazachstania spencerorum* exhibited bioconversion yields higher than 60%. α-Methyl-cinnamaldehyde was initially reduced to α-methyl-dihydro-cinnamaldehyde, while the subsequent reduction of the carbonyl group led to the formation of α-methyldihydrocinnamyl alcohol (Fig. 9).

<Insert figure 9 here>

The biotransformation of the α,β-unsaturated aldehyde (1*R*)-myrtenal catalyzed by NCYs was also investigated (Goretti et al. 2013). Overall, NCYs showed good (sometimes even excellent) aptitudes to biotransform (1*R*)-myrtenal into derivative compounds (Fig. 10): about one third of strains gave percentage of conversion ≥ 95%. Among them, *Candida freyschussii* and *K. spencerorum* converted 100% of the precursor. Interestingly, in almost all cases, biocatalytic ability was prevalently driven towards the reduction of C=O catalysed by carbonyl reductases (CRs) associated to whole cells, affording myrtenol as the main product of the bioconversions. On the contrary, the results obtained apparently suggest that (1*R*)-myrtenal is not a good substrate for the ER activity.

<Insert figure 10 here>

The ability of NCYs to convert acyclic monoterpenes (e.g., geraniol and nerol) has received little attention. NCYs can produce linalool and α-terpineol from both geraniol and nerol (King and Dickinson 2000). *Torulaspora delbrueckii* also exhibited the ability to form geraniol from nerol (King and Dickinson 2000). More recently, a set of NCYs have been screened for their ability to biotransform the acyclic monoterpenes geraniol and nerol (Fig. 11) (Ponzoni et al. 2008). The aptitude to convert both compounds was apparently frequent in NCYs. The production of linalool, α-terpineol, β-myrcene, d-limonene, (E)-β-ocimene, (Z)-β-ocimene, or carene was observed, depending upon the substrate used: thus, linalool was the main product obtained from geraniol, whereas linalool and α-terpineol were the main products obtained through the conversion of nerol. Yet, differently from nerol, the aptitude to exhibit high bioconversion yields of geraniol to linalool was an apparently genus-related character, whereas the ability to produce other monoterpenes was a both genus- and habitat-related character (Ponzoni et al. 2008).

<Insert figure 11 here>

2.4. Alkenes

The reduction of C-C double bonds represents a powerful tool in asymmetric synthesis, and different methods to accomplish this reaction are available on industrial scale (Winkler et al. 2012). Bioreduction of carbonyl-activated alkenes to flavoring food-grade compounds has been reported. The ability of NCYs to bioreduce the α,β-unsaturated ketones ketoisophorone, 2-methyl- and 3-methyl-cyclopentenone has been checked by Goretti et al. (2011). Generally, NCYs exhibited an extremely high capability (currently 90 or even 100% yields) to reduce, via ER activity, the conjugated C=C bond of ketoisophorone and 2-methyl-ciclopentenone (Fig. 12).

<Insert figure 12 here>

Due to low competing CRs activities, high chemoselectivity was sometimes observed, together with a moderate stereoselectivity for *R*-isomers: an enantiomeric excess varying from 10 to 44 % was found for 6*R*-dihydro-oxoisophorone, 6*R*-DOIP, (Fig. 12). 6*R*-DOIP is a key food-grade aroma constituent of tobacco and saffron (Sode et al. 1987), and, interestingly, *K. spencerorum* converted more than 80% of KIP in 6*R*-DOIP, which represented, after 24 h., the almost exclusive product of bioreduction.

NCYs whole cells also showed a moderate ability to bioreduce 2-methyl-cyclopentenone (2-MCPO): whole cells of *Debaryomyces coudertii*, *Debaryomyces nepalensis* and *K. spencerorum* exhibited bioconversion yields higher than 90% following the scheme reported in Fig. 13.

<Insert figure 13 here>

2.5 Sulphur compounds

A number of sulphur compounds such as methanethiol, dimethyl disulphide, dimethyl trisulphide, S-methylthioacetate and S-methylthiobutyrate have been identified as normal components of aroma of cheeses and truffles (Molimard and Spinnler 1996; López del Castillo-Lozano et al., 2007). Bondar et al. (2005) early reported the biotrasformation of L-methionine to 4-methylthio-2-oxobutyric acid, via transamination catalyzed by *Y. lipolytica*.

In recent years, some studies explored the ability of NCYs to biotransform L-methionine into a set of volatile sulphur compounds (VSC). A set of strains belonging to both Ascomycota (genera *Candida*, *Debaryomyces*, *Geotrichum*, *Kluyveromyces*, *Lindnera* and *Yarrowia*) and Basidiomycota (*Cryptococcus*, *Rhodotorula* and *Trichosporon*) exhibited the aptitude to biotransform L-methionine or L-methionine/L-cysteine mixtures into 2-methyl butanol, 3-methyl

butanol, methanethiol, S-methyl thioacetate, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, dihydro-2-methyl-3(2H)-thiophenone, 3-(methylthio)-1-propene, 3-(methylthio)-1-propanal, 3-(methylthio)-1-propanol, 3-(methylthio)-1-propyl acetate, 3-(methylthio)-1-propanoic acid and ethyl 3-(methylthio)-1-propanoate (Buzzini et al. 2005, López del Castillo-Lozano et al. 2007, Tan et al. 2012, Koh et al. 2013).

3-(Methylthio)-1-propanol (methionol), which has cauliflower- and cabbage-like aroma, is of great importance in the overall aroma of soy sauce and cheese (Yvon and Rijnen 2001). Different yeasts (*Candida kefyr*, *Candida utilis*, *Kluyveromyces lactis*, *Saccharomyces bayanus*, *Saccharomyces chevalieri*, *Candida famata* (previously *Torulopsis candida*) and *Williopsis saturnus* were recently screened for their ability to produce flavour-active methionol in coconut cream supplemented with L-methionine (Seow et el. 2010). The yeasts showed different ability to produce methionol from methionine (Fig. 14), with *Kluyveromyces lactis* producing the most. More recently, the bioproduction of methionol from *K. lactis* was optimized be the application of response surface methodology (RSM) and fractional factorial design methods: under the optimum conditions the production of methionol reached 990.1 ± 49.7 μg/mL (Koh et al. 2013).

<Insert figure 14 here>

Tan et al. (2010) reported the first study on the production of VSCs in relation to L-methionine catabolism by yeasts from the genus of *Williopsis*. Five strains were screened for VSC production in a synthetic medium supplemented with L-methionine. A diverse range of VSCs were produced, including methional, methionic acid, 3-(methylthio)propyl acetate, and 3-(methylthio)propionic acid ethyl ester.

An investigation on how VSC production by yeasts could be affected through the supplementation of methionine-cysteine mixtures in comparison with methionine alone has been reported (López del Castillo-Lozano et al. 2007). In this study four cheese-ripening NCYs yeasts were screened, i.e. *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Geotrichum candidum*, and *Yarrowia lipolytica*: all of them produced VSC with L-methionine or L-methionine/L-cysteine, although with different VSC profiles, confirming the hypothesis of the authors that L-methionine must be present in culture medium for the production of VSC by the yeast. Furthermore, this study showed that L-methionine is catabolized to a lower extent when L-cysteine is also added in the cultures.

2.6. Phenols

The ability of yeasts to utilize monomeric phenolic compounds is a well-know phenomenon (Middelhoven 1992). Here, a typical example is the biosynthesis of vanillin. This compound (4-hydroxy-3-methoxy-benzaldehyde) is the second highest produced aroma component in the world (Zheng et al. 2007) and plays an important role in foods and beverages, as well as in other industries (Priefert et al. 2001). It is usually produced *via* extraction from vanilla beans (natural vanillin content 2%) or *via* chemical synthesis from guaiacol and lignin, leading to a cheaper product but of lower quality (Clark 1990).

Like to other flavoring compounds, the trends towards natural flavors and the high price of natural vanillin has driven the search of microbial processes as alternative sources of natural vanillin via biocatalytic transformation of ferulic acid. Though the biotransformation of this precursor to vanillin has been extensively studied (Priefert 2001), there are other value-added minor metabolites produced during this biodegradation, including 4-vinyl guaiacol, vanillic acid, acetovanillone, vanillyl alcohol, dihydroferulic acid, coniferyl alcohol, dihydroconiferyl alcohol or homovanillic acid (Shanker 2007). However, only few studies regarding production of vanillin and associated metabolites by yeast strains are reported.

Whole cells of *D. hansenii* metabolized ferulic acid to 4-vinyl guaiacol by the non-oxidative decarboxylation of its side chain (Mathew 2007). This biotransformation is a highly value added process as 4-vinyl guaiacol is nearly 40 times costlier than ferulic acid. Whole cells of *D. hansenii* produced 1470 mg/L of vinyl guaiacol in ten hour, corresponding to a molar yield of 95%. However, the production of vanillin from 4-vinyl guaiacol through this biotechnological route is considered not very economical as the vanillin levels were 169 mg/L at the fifth hour.

Recently Max et al. [2012] used a factorial design to optimize the biotransformation of ferulic acid into higher value added products such as 4-vinyl guaiacol, vanillic acid and acetovanillone by whole cells of *D. hansenii* (Fig. 15). The major degradation products of ferulic acid were 4-vinyl guaiacol after 72 h (molar yield of 86.0 %), vanillic acid after 360 h (molar yield of 91.1 %) or acetovanillone after 408 h (molar yield of 98.8%). Traces of vanillin, vanillyl alcohol or 4-ethylguaiacol were also found. Previous studies have also reported that *Rhodotorula mucilaginosa* (former *Rhodotorula rubra*), *Rhodotorula minuta* and *Pichia fermentans* (former *Candida lambica*) (Donaghy 1999) were able to transform ferulic acid into 4-vinylguaiacol.

<Insert figure 15 here>

A recent study showed the evidence for biotransformation of isoeugenol to vanillin and vanillic acid by a strain of *Candida galli* (Ashengroph et al. 2011). This yeast strain, isolated from oil-

contaminated water, was able to transform isoeugenol to vanillin and vanillic acid, yielding a vanillin concentration of 1.12 gl^{-1} (Fig. 16)

<Insert figure 16 here>

3. Conclusions

Flavors are key players for modifying and improving organoleptic quality of a number of foods and beverages. There is a general trend pushing towards the progressive replacement of flavoring additives produced via chemical synthesis with those obtained from natural sources, including plants, food-grade microorganisms and enzymes in order to meet the rising consumer requests for natural flavors and fragrances.

In this way, the use of microbial whole cells for catalyzing alternative and attractive routes for the production of these compounds via biotransformation have been extensively studied. However, despite the high number of studies so far published, only a few processes have been successfully scaled-up to the industrial scale, while most of other possible biotransformations are still confined to the laboratory scale.

NCYs whole cells could be considered as excellent biocatalysts for promoting and scaling-up cheap system for producing food-grade flavors. The array of flavors potentially produced by whole cells of NCYs via biotransformation of suitable precursors may match with ever increasing biological and metabolic diversity these organisms. So, the study of NCYs, together with the interaction among yeast biodiversity, synthetic biology, DNA recombinant technology and yeast biotechnology may improve in the future the biological (e.g. bioactivity, olfactory properties, etc.) and the physico-chemical properties (e.g. solubility, lipophilic aptitude, etc.) of flavors, available in the market (Antoniotti et al. 2014).

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Table 1. Representative food aroma compounds grouped by chemical classes

Table 2. Production of lactones via biotransformation of fatty acids catalyzed by NCYs

Figure 1. Bioreduction of (Z)-3-hexenal to (Z)-3-hexenol by *Wickerhamomyces anomalus*

Figure 2. Biotransformation of ricinoleic acid to γ-decalactone promoted by whole cells of *Yarrowia lipolytica*

Figure 3. Biotransformation of linoleic acid to δ-decalactone promoted by whole cells of *Yarrowia lipolytica*

Figure 4. Biotransformation of (-)-α-pinene to verbenol by NCYs

Figure 5. Biotransformation of (+)-limonene to perillic acid promoted by whole cells of *Yarrowia lipolytica*

Figure 6. Biotransformation of (-)-(*R*)-α-phellandrene to 5-*p*-menthene-1,2-diol promoted by whole cells of *Yarrowia lipolytica*

Figure 7. Biotransformation pathway of (4*S*)-carvone by whole cells of NCYs

Figure 8. Biotransformation of (*S*)-perillaldehyde by whole cells of *Kazachstania naganishii*

Figure 9. Biotransformation of α-methyl-cinnamaldehyde by whole cells of *Kazachstania spencerorum*

Figure 10. Biotransformation of (1*R*)-myrtenal by whole cells of NCYs

Figure 11. Biotransformation of geraniol and nerol by whole cells of NCYs

Figure 12. Biotransformation of ketoisophorone by whole cells of NCYs

Figure 13. Biotransformation of 2-methyl-cyclopentenone by whole cells of *Kazachstania spencerorum*

Figure 14. Biotransformation of L-methionine to methionol

Figure 15. Biotransformation of ferulic acid by whole cells of *Debaryomyces hansenii*

Figure 16. Biotransformation of eugenol to vanillin and vanillic acid by whole cells of *Candida galli*

Ketones	acetone, 2-butanone, 2-pentanone, 3-pentanone, 2-hexanone, 2-heptanone, 2-octanone, 2-nonanone, 2-undecanone, 2-tridecanone, acetophenone, 2,3-butanedione, 2,3-pentandione, 3-hydroxy-2-butanone, 3-methyl-2-butanone, 4-methyl-2-pentanone, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, ketoisophorone
Aldehydes	acetaldehyde, propanal, butanal, pentanal, hexanal, isohexanal, heptanal, octanal, nonanal, decanal, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, propenal, 2-hexenal, (Z)-4-heptenal, (Z)-2-nonenal, (E)-2-nonenal, (E,E)-2,4-nonadienal
Esters	methyl acetate, ethyl acetate, 3-octyl acetate, pentyl acetate, phenethyl acetate, 2-methyl-1-butyl acetate, 3-methyl-1-butyl acetate, 2-hydroxyethyl propionate, 2-methyl-2-ethyl-3-hydroxyhexyl propionate, ethyl butanoate, ethyl isobutanoate, isobutyl butanoate, propyl butanoate, ethyl 2-methyl butanoate, ethyl 3-methyl butanoate, ethyl hexanoate, ethyl octanoate
Lactones	δ-decalactone, γ-decalactone, γ-butyrolactone, δ-dodecalactone,d-octalactone, (Z)-6-dodecen-δ-lactone
Acids	acetic, phenylacetic, propanoic, , butanoic, isobutanoic, 2-methylbutanoic, 3-methylbutanoic, valeric, caproic, octanoic, decanoic,
Alcohols	ethanol, phenyl ethanol, 2-phenyl ethanol, 2-methyl propanol, 2-butanol, isobutanol, 2-methyl butanol, 3-methyl butanol, 2-ethyl butanol, 1,2-butanediol, 2,3-butanediol, 1-pentanol, 1-hexanol, 2-heptanol, 2-octanol, 1-nonanol, 2-nonanol, 1-octen-3-ol, (Z)-1,5-octadien-3-ol
Terpenes	α-pinene, β-pinene, limonene, carvones, carveols, dihydrocarvones, dihydrocarveols, perillaldehyde, perillic alcohol, α-methyl-cinnamaldehyde, myrtenal, geraniol, nerol
Phenols	vanillin, vanillic acid, benzaldehyde
Sulphur compounds	methanethiol, dimethyl disulphide, dimethyl trisulphide, S-methylthioacetate and S-methylthiobutyrate, methionol

Tab. 1

Species	Substrate	Product(s)	References
Lindnera saturnus (formerly *Hansenula saturnus*)	Ricinoleic acid	3-hydroxylactone, decen-4-olides	Waché et al. 2001
Yarrowia lipolytica	Ricinoleic acid	3-hydroxylactone, decen-4-olides	Waché et al. 2001
Yarrowia lipolytica	Ricinoleic acid	γ-decalactone	Waché et al. 2003
Candida albicans	Linoleic acid	γ-nonalactone	Feron and Waché 2005
Candida tropicalis	Linoleic acid	γ-nonalactone	Feron and Waché 2005
Yarrowia lipolytica (formerly *Candida lipolytica*)	Linoleic acid	γ-nonalactone	Feron and Waché 2005

Tab. 2